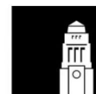


UNIVERSITY OF LEEDS

This is a repository copy of *Structural mechanism and kinetics of in vitro gastric digestion are affected by process-induced changes in bovine milk*.

White Rose Research Online URL for this paper:
http://eprints.whiterose.ac.uk/153229/

Version: Accepted Version

Article:

Mulet-Cabero, A-I, Mackie, AR orcid.org/0000-0002-5681-0593, Wilde, PJ et al. (2 more authors) (2019) Structural mechanism and kinetics of in vitro gastric digestion are affected by process-induced changes in bovine milk. Food Hydrocolloids, 86. pp. 172-183. ISSN 0268-005X

https://doi.org/10.1016/j.foodhyd.2018.03.035

© 2019, Elsevier. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/.

Reuse
This article is distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND) licence. This licence only allows you to download this work and share it with others as long as you credit the authors, but you can't change the article in any way or use it commercially. More information and the full terms of the licence here: https://creativecommons.org/licenses/

Takedown
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

eprints@whiterose.ac.uk
https://eprints.whiterose.ac.uk/

1 Title: Structural mechanism and kinetics of in vitro gastric digestion are affected by process-
2 induced changes in bovine milk

3

4 Author names and affiliations: Ana-Isabel Mulet-Cabero[a,b], Alan R. Mackie[c] , Peter J. Wilde[a]
5 , Mark Fenelon[b] , André Brodkorb[b*]

6 [a] Quadram Institute Bioscience, Norwich Research Park, Norwich, Norfolk, NR4 7UA, UK

7 [b] Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

8 [c] School of Food Science and Nutrition, University of Leeds, LS2 9JT, UK

9

10 *Corresponding author: André Brodkorb
11 Tel.: +353-25-42-431
12 E-mail address: Andre.Brodkorb@teagasc.ie

13
14

15

16

17

18

19

20

21

22 Abstract

23 Bovine milk is commonly exposed to industrial processing, which can alter the structure,
24 biochemical composition, physico-chemical properties and sensory quality. While many of
25 these changes have been studied extensively, little is known about their effect on digestive
26 behaviour. In this study, heat treatments of pasteurisation at 72 °C for 15 s or Ultra-High-
27 Temperature (UHT) treatment at 140 °C for 3 s and homogenisation at pilot-plant scale were
28 applied to whole milk. The gastric behaviour was investigated using a recently developed semi-
29 dynamic adult in vitro model. The emptied digesta were analysed to assess the nutrient delivery
30 kinetics, changes in microstructure and protein digestion.

31 All samples showed protein aggregation and coagulum formation within the first 15 min of
32 gastric digestion at which time the pH ranged from 5.5 to 6. Homogenised samples creamed
33 regardless of heat treatment, whereas all non-homogenised samples exhibited sedimentation.
34 The consistency of the coagulum of the heated samples was more fragmented compared to
35 those of the non-heated samples. Rheological analysis showed that the higher the temperature
36 of the heat treatment, the softer the obtained coagulum and the higher the protein hydrolysis at
37 the end of digestion. The study also confirmed that gastric emptying of caseins from milk is
38 delayed due to coagulation in the stomach, while β-lactoglobulin was emptied throughout the
39 gastric phase, except for UHT-treated milk. The gastric behaviour also had an impact on the
40 lipid and protein content of the emptied chyme. The homogenised samples seemed to release
41 more nutrients at the end of gastric digestion.

42

43 Keywords

44 Milk; Homogenisation; Heat treatment; Gastric behaviour; Nutrient delivery; Protein
45 hydrolysis

46 1. Introduction

47 Bovine milk is conventionally heat treated and homogenised to improve consumer acceptance
48 and ensure microbial stability, and the shelf life. These dairy processes cause changes in the
49 physical structure, which has been widely characterised. Homogenisation results in size
50 reduction of the native fat globule, initially surrounded by the milk fat globule membrane
51 (MFGM), from an average size of 3-5 μm to below 1 μm (Keenan et al., 1983; Michalski &
52 Januel, 2006). Moreover, homogenisation disrupts the MFGM drastically changing the
53 interface composition, which mainly consists of adsorbed milk protein, and organisation of the
54 droplet (Lopez, 2005; Sharma & Dalgleish, 1993). The most common heat treatments applied
55 to milk are pasteurisation that consists of heating to a minimum of 72 ºC for ≥ 15 s and ultra-
56 high temperature (UHT) sterilization involving heating at 135-150 ºC during a few seconds.
57 These heat processes cause the denaturation of whey protein, in particular β-Lg (Douglas et al.,
58 1981), which can be bound to κ-casein on the new formed droplet surface (Sharma & Dalgleish,
59 1993).

60 The structure of food at different length scales has been shown to impact nutrient digestion and
61 absorption. However, there has been little research performed on the impact of these process-
62 induced changes on milk digestion. In some cases conflicting results have been obtained mainly
63 due to the different digestion models applied. The gastric compartment is a key site to regulate
64 nutrient digestion and differences in intestinal absorption kinetics of dairy products have been
65 associated with gastric emptying (Gaudichon et al., 1994). The first steps of hydrolysis and
66 breakdown of food are in the gastric compartment mainly due to the presence of pepsin and
67 gastric lipase and acid. Digested products are progressively emptied through the pylorus and
68 released into the small intestine, which has important implications for postprandial responses.

69 Studies of the in vivo digestion of processed milk are very rare. Lacroix et al. (2008) found, in
70 healthy humans, UHT-treated milk consumption induced a lower postprandial retention of
71 dietary nitrogen leading to a faster appearance in plasma compared to pasteurised milk. It was
72 suggested that this modulation of the digestive kinetics was due to the possible formation of a
73 softer coagulum in the stomach and a higher enzyme accessibility in the case of UHT-treated
74 milk. These results have been supported by Bach et al. (2017), who showed that urinary
75 nitrogen secretion was greater for UHT-milk compared to raw and pasteurised milk using
76 young dairy calves as a model. In addition, Miranda and Pelissier (1987) found that heat treated
77 milk (UHT and autoclaving) increased gastric emptying rate and casein hydrolysis in rats. This
78 contrasts to the higher mean retention time in the stomach of heated skim milk (90 ºC, 10 min)
79 compared to a non-heated system observed in mini-pigs (Barbé et al., 2013). It is broadly
80 reported that heat treatment, using temperatures above 90 ºC, facilitates protein digestion,
81 which has been observed for β-Lg (Wada & Lönnerdal, 2014). However, opposing
82 observations have been made for caseins. Heated skim milk (90 ºC, 10 min) promoted
83 hydrolysis resistance of casein fraction compared to unheated skim milk during gastric
84 digestion using an in vitro adult dynamic model (Sánchez-Rivera et al., 2015) and in vitro
85 infant static model (Dupont et al., 2010), which could affect the kinetics of protein digestion
86 in a mini-pig model (Barbé et al., 2013). This was reportedly related to chemical modifications
87 of the protein during heating, i.e. lactosylation, glycosylation as well as casein-whey
88 interactions, resulting in different peptides generated during digestion. In contrast, using a static
89 digestion model, Tunick et al. (2016) found a rapid digestion of caseins in the gastric phase of
90 both processed (heated at pasteurisation and UHT conditions and homogenised) and non-
91 processed samples. Moreover, homogenisation was observed to increase β-Lg hydrolysis
92 compared to pasteurised milk (Islam et al., 2017). Despite the differences in enzymatic
93 digestion of the major milk proteins, Wada and Lönnerdal (2014) reported no significant

94 differences in the overall in vitro digestion kinetics among the heat treatments (pasteurisation,
95 UHT and in-can sterilisation). A sophisticated in vitro model, the Human Gastric Simulator
96 (Kong & Singh, 2010), was used to investigated the effect of milk treatment on the gastric
97 behaviour (Ye et al., 2016). They showed the formation of coagulum of different structure was
98 leading to different protein digestion behaviour. The homogenisation and heat treatment
99 resulted in the formation of a crumbly structure compared to the tight clot obtained in raw milk.
100 This was similar to what proposed to occur in vivo and highlights the limitations of the static
101 in vitro digestion models. However, the conditions of heating used, 90 ºC for 20 min, are less
102 representative of the typical conditions of industrial milk processing.

103 In addition, gastric conditions may lead to different gastric colloidal behaviours, which could
104 affect postprandial responses by different nutrient delivery. Mackie et al. (2013) showed that
105 the homogenised droplets stabilised by milk proteins caused creaming in the human stomach,
106 as monitored by MRI, and decreased fullness due to the delayed of lipid emptying, in contrast
107 to the early delivery of nutrients from a mixture of cheese and yogurt, which sustained fullness.

108 In this study, the recently developed semi-dynamic gastric model was used, which replicates
109 some gastric behaviour seen in the human stomach (Mulet-Cabero et al., 2017). The model
110 can simulate the main dynamics of the stomach including gradual acidification, gastric fluid
111 and enzyme secretion and emptying. The most commonly used milk processes,
112 homogenisation and the heat treatments of pasteurisation and UHT, were used and compared
113 to raw milk in order to assess the influence in gastric behaviour, protein coagulation, nutrient
114 delivery and protein digestion.

115

116 ## 2. Material and methods

117 ### 2.1. Material

118 Fresh whole bovine milk was collected from a bulk tank of the Moorepark Dairy Unit, Teagasc
119 Animal and Grassland Research and Innovation Center, Moorepark, Fermoy, Co. Cork,
120 Ireland. The milk was from Friesian cows that were fed a total mixed ration diet consisted of
121 grass silage, maize silage and concentrates. Bulk milk samples were collected post-morning
122 milking. The sampling was conducted between November 2016 and February 2017.

123 The raw milk was collected on different days for each process performed. The processes were
124 conducted at pilot-plant scale using industrially relevant conditions. Homogenisation was
125 applied at 40 ºC using a 2-stage valve-type homogeniser (Gaulin Labor Homogenizer, type Lab
126 60; APV Gaulin GmbH, Lubeck, Germany). The pressures used were 15 and 5 MPa for first
127 and second stage, respectively. The sample is referred as Homo in the text. Pasteursation and
128 ultra-high temperature (UHT) treatments were carried out using a MicroThermics tubular heat
129 exchanger (MicroThermics, NC, USA). The conditions were a final heat temperature at 72 ºC
130 with a holding time of 15 s for pasteurisation and 140 ºC with a holding time of 3 s for UHT
131 treatment (pre-heating temperature of 91 ºC). The samples are referred as Past and UHT
132 respectively in the text. These heat treatments were also carried out with a subsequently
133 homogenisation using an in-line-two stage valve homogeniser, Model NS 2006IT (Niro Soavi,
134 Parma, Italy) employing first-stage pressure of 15 MPa and a second-stage pressure of 5 MPa.
135 The samples are referred as Past+Homo and UHT+Homo respectively in the text. The samples
136 were stored at 4 ºC after preparation. The Raw, Homo, Past and UHT were studied within 1
137 day and Past/UHT+Homo were used within 2 days.

138 Milk fat, protein, lactose and total solids values were obtained using a Milkoscan FT 6000
139 (FOSS, Denmark) with a tolerance of ± 0.06 %. The nutrient composition of milk was measured
140 before each sample (see Table S.1 Supplementary Material) and the caloric content was
141 calculated using the Atwater factors. This ranged from 0.78 to 0.68 kcal/mL.

142 Pepsin from porcine gastric mucosa (Sigma Chemical Co., USA) had an enzymatic activity of
143 3,875 units/mg protein, calculated by measuring the TCA-soluble products using haemoglobin
144 as substrate as described by Minekus et al. (2014). All other chemicals were purchased from
145 Sigma-Aldrich unless specified otherwise.

146

147 2.2. Methods

148 2.2.1. Semi-dynamic gastric digestion model

149 After collection of the raw milk and the respective milk processes, the samples went through a
150 simulated digestion. This was performed using two independent samples on different days.
151 Therefore, the simulated digestion experiments were conducted independently, and subsequent
152 analyses were performed from these independent samples

153 The simulation of the oral and gastric phase was done using a semi-dynamic adult digestion
154 model previously described in Mulet-Cabero et al. (2017) with some modifications. An
155 example of the parameters used is shown in Table S.2 Supplementary Material.

156 The oral phase was applied before the gastric digestion, in which 20 g of milk sample was
157 mixed with oral mixture using a rotator (SB3 Model, Stuart, Bibby Scientific, UK) at 30 rpm
158 for 2 min. The total oral mixture consists of Simulated Salivary Fluid (SSF), prepared
159 accordingly to Minekus et al. (2014). The volume of the added SSF corresponded to the total
160 solid content of the milk sample, which was measured for each individual milk. For example,
161 for 20 mL sample with a total solid content of 14 %, the added SSF 2.8 mL was corresponded
162 to be added. It slighType equation here.tly varied among samples ranging from 2.52 to 2.82 mL
163 due to the difference of the total solid concentration in the analysed milk samples during the
164 period of study. The resulting mixture was then put through the gastric digestion.

165 The sample was placed into a 70 mL glass v-form vessel thermoregulated at 37 °C after the
166 addition of 10 % of the total volume of gastric mixture, simulating the residue in the stomach
167 during the fasted state. The gastric mixture contained 80 % simulated gastric fluid (SGF,
168 prepared according the protocol described in Minekus et al. (2014) at pH 7), 7.7 % Milli-Q®
169 water, 8.8 % 1.5 M HCl and 0.05 % 0.3 M $CaCl_2(H_2O)_2$. Two solutions were added at a constant
170 rate: (1) the remaining gastric mixture was added using a pH-stat dosing device (800 Dosino,
171 Metrohm, Switzerland) and (2) 0.8 mL pepsin solution (made with Milli-Q® water) was added
172 using a syringe pump (New Era Pump Systems, Inc., NY, USA). A 3D action shaker (Mini-
173 gyro rocker, SSM3 Model, Stuart, Barloworld Scientific limited, UK) at 35 rpm was used for
174 agitation.

175 After 25 min of gastric digestion, the sample was mixed using a 50 mL plastic syringe (BD
176 Plastipak, Ireland), the aperture of which had an inner diameter of 6.80 mm with a plastic tube
177 attached (6 mm inner diameter). This mixing was required to make the sampling more accurate.
178 Nevertheless, the colloidal behaviour during digestion seemed not to be impaired by the initial
179 mixing. Gastric emptying (GE) was simulated by taking 5 samples, referred to as GE points in
180 the text. The average time of those were 36 min (GE1), 72 min (GE2), 109 min (GE3), 145
181 min (GE4) and 182 min (GE5). Samples were taken from the bottom of the vessel using a
182 serological pipette with a tip internal diameter of 2 mm because it approximates the upper limit
183 of particle size that has been seen to pass through the pyloric opening into the duodenum
184 (Thomas, 2006). It is important to note that there was some residue left in the last GE point
185 that could not be taken using a pipette; this was taken using a spatula and included in the last
186 point. An aliquot of these GE samples was used for microscopic and particle size analysis.
187 Otherwise, the sample was mixed using a homogeniser (T10 basic Ultra-Turrax®, IKA®,
188 Germany) at approximately 30,000 rpm for 30 s to obtain a homogenous sample for the
189 remaining analysis. The pH of each GE samples was measured using a pH meter and a

190 sufficient volume of 2 M NaOH was added to the samples to increase the pH above 7, inhibiting
191 pepsin activity. Finally, samples were snap-frozen in liquid nitrogen and stored at -80 °C until
192 subsequent treatment.

193 The simulation of the emptying was based on caloric density. A linear GE rate of 2 kcal/min,
194 which is considered the average caloric content that is emptied in vivo in a regulated manner
195 by the antrum for an average food volume of 500 mL (Hunt & Stubbs, 1975) was used and
196 scaled it down for this reduced-volume system. This implied that the volume and time of each
197 emptying point (Table S.3 Supplementary Material) differed due to the slight variations in the
198 caloric content of the milk samples during the period of the study.

199

200 ### 2.2.2. Confocal Laser Scanning Microscopy (CLSM)

201 The microstructure of the initial and digested samples were observed using a Leica TCS SP5
202 microscope (Leica Microsystems, Baden-Württemberg, Germany). All the images were taken
203 using a 63 x oil-immersion objective and simultaneous dual-channel imaging, He–Ne laser
204 (excitation wavelength at 633 nm) and an Argon laser (excitation wavelength at 488 nm). A
205 mixture of two dyes was used, which consisted of 1:1 0.1 % Fast green FCF solution (in water)
206 to detect protein and 0.1 % Nile red solution (in propanediol) to detect the lipid phase. 500 µl
207 of initial/digested sample was gently mixed with 50 µl of mixed dye.

208

209 ### 2.2.3. Particle size distribution

210 The particle size distribution and average lipid droplet size of initial and digested samples were
211 determined using a laser-light diffraction unit (Mastersizer, Malvern Instruments Ltd,
212 Worcestershire, UK) equipped with a 300 RF lens. The optical parameters chosen were a

213 particle and dispersant (water) refractive index of 1.456 and 1.330, respectively. The
214 absorbance value of the fat globules was 0.001. A volume of initial and digested samples was
215 added in order to reach a laser obscuration range of 5-10 %. A volume of the initial and GE5
216 samples (0.2 mL) was dispersed in 10 mL of 0.02 M sodium dodecyl sulphate (SDS) to
217 dissociate clusters of proteins (as described in van Aken et al. (2011)). The size distribution
218 was obtained using polydisperse analysis, while droplet size measurements were recorded as
219 surface area weighted ($d_{3,2}$) and volume weighted ($d_{4,3}$) means, where $d_{3,2}$ is defined as $\sum n_i d_i^3 /$
220 $n_i d_i^2$ and $d_{4,3}$ is defined as $\sum n_i d_i^4 / n_i d_i^3$, where n_i is the number of particles with diameter d_i.
221 Each measurement was carried out in triplicate.

222

223 ### 2.2.4. Protein content analysis

224 The protein content of the initial milk and emptied digesta was determined by the Dumas
225 method using a LECO FP628 Protein analyser (LECO Corp., St. Joseph, MI, USA). A
226 conversion factor of 6.38 was used to obtain the protein content from the nitrogen content. The
227 protein content was reported as a percentage of g protein per g meal. Each measurement was
228 carried out in duplicate.

229

230 ### 2.2.5. Lipid content analysis

231 The lipid content of the initial milk and emptied digesta was measured using a CEM Smart
232 Trac System-5 and a CEM Smart Trac Rapid Fat Analyzer (CEM Corp., Matthews, N.C.,
233 U.S.A.). Approximately 2 g of sample (previously warmed up to 40 ^{o}C to dispersed lipid) was
234 placed on a glass fiber sample pad and dried in the Smart Trac System by microwave drying.
235 Immediately after drying, samples were placed in the Smart Trac Rapid Fat Analyzer to

236 determine total lipid content by nuclear magnetic resonance. The lipid content was reported as
237 a percentage of g lipid per g meal. Each measurement was carried out in duplicate.

238

239 2.2.6. Protein analysis

240 2.2.6.1. Quantification of protein hydrolysis

241 The samples were treated before protein hydrolysis analysis. This involved the addition of
242 trichloroacetic acid (3.12 % final concentration) to digested sample to cause the precipitation
243 of insoluble protein that could interfere in the further analysis. Then, the samples were
244 centrifuge at 10,000 g for 30 min at room temperature and the supernatant was filtered using a
245 syringe filter of PVDF 0.22 µm membrane (Millex-GV, Millipore, Cork, Ireland)

246 The levels of free NH_2 groups were determined using the standardised o-phthaldialdehyde
247 (OPA) spectrophotometric assay in micro-titre plates. OPA reagent consisted of 3.81 g sodium
248 tetraborate dissolved in approximately 80 mL water. Once dissolved, 0.088 g dithiothreitol and
249 0.1 g sodium dodecyl sulphate were added. Then, 0.080 g OPA dissolved in 2-4 mL ethanol
250 was placed in the solution that was finally made up to 100 mL with Milli-Q® water.

251 Different concentrations of standard L-leucine solution (made with phosphate buffer solution)
252 ranged from 0 to 10 mM were used to obtain a calibration curve. 10 µl of standard/sample was
253 placed into each well and mixed with 200 µl of OPA reagent. The reaction was allowed to
254 proceed for 15 min, then the absorbance was measured at 340 nm using a multi-mode
255 microplate reader (Synergy HT, BioTek Instruments, Inc.). Each measurement was carried out
256 in duplicate.

257

258 2.2.6.2. Identification of proteins during digestion

259 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed
260 on the initial and digested samples normalised to a total protein concentration of 0.1 %.
261 NuPAGE Novex bis-Tris 12-well precast gels (Invitrogen, Life Technologies Corp., CA,
262 USA), 4-12 % polyacrylamide, were used according to the manufacturer's instructions. A
263 fixing solution (50% methanol and 10% acetic acid in v/v) was applied to the gels for 2 hrs
264 before staining with Coomassie Blue. Mark 12™ Unstained Standard (Invitrogen, Life
265 Technologies Corp., CA, USA) was used as a molecular weight marker.

266

267 2.2.7. Rheology analysis

268 The consistency of the coagulum that persisted at the end of digestion, after about 182 min
269 (GE5 point) was analysed by small deformation rheology. The coagulum was separated from
270 the serum using a 70 µm Nylon strainer (BD Falcon). The mass of the sample and, the separated
271 coagulum and serum was recorded. The coagulum was gently placed in a rheometer (AR 2000
272 EX Rheometer, TA Instruments, Crawley, UK). The rheometer geometry consisted of a 40 mm
273 parallel steel plate cylindrical using a shear strain of 0.5 and a frequency of 1 Hz for 30 min at
274 37 ^{o}C. The complex modulus (G*) was calculated as follows G*=stress*/strain.

275

276 2.2.8. Statistical Analysis

277 The results were expressed as means ± standard deviation of two replicates. For each replicate,
278 raw milk was collected, analysed (composition) and processed independently, i.e. one milk per
279 day. To identify differences in normally distributed results within groups during gastric
280 digestion, one-way ANOVA was applied. Where overall significant interaction was observed
281 ($P < 0.05$), the means of individual milk treatments were compared using Tukey's post hoc

282 test. Statistical analyses were performed using GraphPad Prism software (Prism 5 for
283 Windows, Version 5.04).

284 3. Results

285 3.1. Gastric pH profile

286 The simulation of the gastric phase was performed by a semi-dynamic model that can simulate
287 the main biochemical dynamics of the human stomach. These are gradual enzyme and acid
288 secretion and progressive gastric emptying. The changes in pH during gastric digestion are
289 shown in the Fig. 1. The gastric model had a low initial pH of about 1 simulating the fasting
290 conditions. The pH increased rapidly, up to values of about 6, after the addition of sample from
291 the oral phase. Subsequently, there was a progressive decrease reaching pH values below 1.4
292 after 3 hours due to the continuous gastric fluid secretion containing acid as well as the
293 reduction of buffering capacity of the digested food by gastric emptying. All samples showed
294 a similar pH behaviour of the predefined profile seen in in vivo studies (Malagelada et al.,
295 1976). The mean pH of the samples did not show any statistically difference except in the initial
296 ($p = 0.034$) and GE1 ($p = 0.041$) points. The mean pH between Raw and UHT+Homo in GE1
297 were significanly different using the Tukey's multiple comparison post-hoc test.

298

299 3.2. Gastric behaviour

300 Using the semi-dynamic model, a range of different structures and behaviours during gastric
301 digestion were obtained (Fig. 2). Protein coagulation was visible for all the samples within the
302 first 10 min of digestion and the formation of larger aggregates was observed a few minutes
303 later, at which time the pH ranged from 5.5 to 6. Subsequently, there was the formation of a
304 more compact coagulum with clear serum within the first 15 min. Differences in coagulum
305 consistency were observed throughout the gastric phase as illustrated in Fig. 2. There were

306 remarkable differences, in particular, between the firm coagulum of Raw (Fig. 2A) and the
307 fragmented structure of UHT+Homo (Fig. 2I). In the absence of pepsin, we observed later
308 aggregation and coagulum formation. Protein coagulation was visually observed after 75 min
309 at which time the pH was around 5, with the exception of the UHT-treated samples in which
310 the aggregation was first seen at 35min.

311 Fig. 3 shows the gastric behaviour of the milk samples in the model stomach at about 36 min
312 (Fig. 3A, B, C, G, H, I) and 182 min (Fig. 3D, E, F, J, K, L) of gastric digestion. The
313 homogenised samples showed creaming, having an opaque layer on the top, (Fig. 3J, K, L)
314 whereas the non-homogenised samples resulted in sedimentation (Fig. 3D, E, F). In the
315 homogenised samples, phase separation was initially observed when aggregates could form a
316 layer at the top, with a cloudy layer in the middle part and clearer layer in the bottom at about
317 109 min. This was different in the absence of pepsin since there was no phase separation and
318 the coagulum of all the samples remained of the bottom of the vessel.

319 The consistency of the milk coagulum was further studied by small deformation rheology
320 analysing the coagulum remaining in GE5. Table 1 shows the values of the complex modulus
321 (G*) obtained after 15 min of measurement. The non-heated samples, Raw and Homo,
322 generated the highest levels of G* accounting for 4,555 and 4,113 Pa, respectively. The
323 pasteurised samples (Past and Past+Homo) presented an intermediate situation accounting for
324 2,934 and 1,569 Pa. The lowest G* values were found in UHT and UHT+Homo representing
325 for 501 and 206 Pa, respectively. The same behaviour was observed during the rheology
326 analysis, which was performed for 30 min.

327 It is important to note that some alteration of the structure could have been induced while
328 placing the sample on the plate in order to perform the analysis.

329

330 3.3. Microstructure of the emptied samples

331 The coagulation, observed within the first 15 min of digestion, was reflected in the
332 microstructures of the emptied samples (Fig. 4). There were differences in the structure of the
333 protein matrix in the first stages of gastric digestion. The non-heated samples, in particular
334 Raw, seemed to form a more compact and dense network (Fig. 4D) in accordance with the
335 visual observation. This differs from the heated samples, in particular UHT (Fig. 4F), in which
336 the structure of the protein coagulum was open with more pores. This can be linked with the
337 particulate and soft macrostructure observed. Moreover, in the GE1 point of the non-
338 homogenised samples (Fig. 4D, E, F), the native fat droplets appeared to be in the aqueous
339 phase showing some coalescence. In contrast, the fat droplets seemed to be easily entrapped in
340 protein network of the homogenised samples (Fig. 4M, N, O), in which fine particles could be
341 seen distributed within the coagulum particles, in particular UHT+Homo (Fig. 4O). The effect
342 of homogenisation on the structure at the end of gastric digestion (182 min) was significant.
343 All the homogenised samples presented a great number of small aggregates (Fig. 4P, Q, R)
344 compared to the large particles of non-homogenised samples (Fig. 4G, H, I).

345 The changes in the droplet size were followed during digestion (Table 1). Initially, the volume
346 mean particle diameter, $d_{4,3}$, of non-homogenised samples was about 2.5 µm whereas that of
347 homogenised samples was about 0.4 µm, showing the significant size reduction due to
348 homogenisation treatment. The particle size of the milk samples, with the addition of SDS,
349 increased to a different extent at the end of digestion. The digestion of the raw milk resulted in
350 an increase from the initial size of 2.96 µm to 8.26 µm after 182 min of digestion but the particle
351 size of UHT+Homo increased from 0.41 to 0.97 µm.

352

353 3.4. Nutrient delivery

354 The protein (Fig. 5A) and lipid (Fig. 5B) delivery was low in the first GE points and then there
355 was an increase in the last point, GE5. The content in GE5 ranged from 3.42 to 9.45 % and
356 from 7.21 to 16.14 % for protein and lipid, respectively. The means of protein and lipid content
357 were significantly different in both GE1 and GE5 due to differences between Raw and
358 UHT+Homo. The profile of the protein content showed a more constant and higher levels in
359 the first GE points in comparison to those in lipid profile. In the case of lipid content profile,
360 in GE5, the homogenised samples seemed to have higher levels with exception of UHT+Homo.

361

362 3.5. Protein digestion

363 Fig. 6 shows the levels of free NH_2 groups of the milk samples before digestion and in the
364 different GE points. The means of the initial samples were significantly different (p=0.0008)
365 due to the samples in which UHT treatment was applied. The low values obtained in these
366 samples may be due to the Maillard reaction products, which might be favoured by the high
367 heating of UHT treatment (Morgan et al., 1999). The proteolysis showed a similar profile in
368 all samples. There was an increase in the three first GE points, after which it levelled off
369 showing no increase in the GE4 point. After that, the level of proteolysis decreased in GE5.
370 Levels of proteolysis among samples differed greatly in GE1 and GE5. In GE1, Raw and
371 UHT+Homo were statistically different accounting for 921.07 and 354.31 mM NH_2/g,
372 respectively. Conversely, in GE5, UHT+Homo showed the highest level of proteolysis (1,736
373 mM NH_2/g) being statistically different from Raw and Homo (897 and 1,065 mM NH_2/g,
374 respectively).

375 The protein composition during the gastric phase was also studied by SDS-PAGE and shown
376 in Fig. 7. The bands corresponding to the samples before digestion (I) did not differ due to
377 processing. Moreover, there were no differences between homogenised and non-homogenised

378 samples. The non-heated samples, Raw and Homo, had similar patterns than those of
379 pasteurised samples (Past and Past+Homo). The caseins were detectable in the first emptying
380 points, in particular GE1 and GE2 points, but they were almost not observed in GE3 and GE4
381 points. In the last emptying point (GE5) intact caseins could again be observed together with a
382 wide range of peptides. β-Lg, in contrast, was present during gastric digestion even though the
383 band weakened in the last GE points. Also, α-La was present in the three first GE points, after
384 which it was not detected anymore. Many small molecular weight peptides were present during
385 digestion and could be seen from GE1 onwards. This behaviour differed from that observed in
386 the UHT-treated samples (UHT and UHT+Homo). In those samples, both caseins and whey
387 proteins could only be observed in the two first GE points.

388

389 ## 4. Discussion

390 ### 4.1. Influence of the milk processing on gastric behaviour.

391 By using a physiologically relevant gastric model (Mulet-Cabero et al., 2017), we have been
392 able to show that homogenised samples creamed whereas sedimentation was observed in non-
393 homogenised samples, regardless the heat treatment (Fig. 3). Homogenisation causes the
394 disruption of the native MFGM and promotes adsorption of milk proteins onto the droplet
395 surface (Lopez, 2005; Sharma & Dalgleish, 1993). This change of the droplet interfacial
396 composition might be one of the main reasons for the distinct gastric behaviour. The milk
397 proteins on the droplet surface, especially the denatured and aggregated proteins in the heated
398 UHT+Homo sample, may be more susceptible to be hydrolysed by pepsin leading to the
399 destabilisation of the droplets by flocculation and some coalescence, and ultimately leading to
400 the phase separation observed. The non-homogenised samples, in contrast, still possessed the
401 native MFGM, which could provide more stability during gastric digestion. These structural

402 changes were certainly due to the proteolytic action of pepsin since there was no phase
403 separation in the homogenised samples when pepsin was absent. Further investigation was
404 undertaken in order to gain insight into the mechanism of the different gastric behaviour
405 observed. The lipid/protein ratio in both coagulum and serum in the first GE point was
406 determined (Fig. S.1 Supplementary Material). The non-homogenised samples had
407 significantly higher lipid/protein ratio in the serum compared to the homogenised samples.
408 Moreover, the microstructure imaging showed that most of the droplets in the non-
409 homogenised samples tended to be in the serum (Fig. 4D-F) compared to those of the
410 homogenised samples (Fig. 4M-O). This might be due to easier incorporation of the smaller
411 droplets into the coagulum and also the possible interactions of the droplet surface coated by
412 milk protein with the protein network. Therefore, a higher inclusion of droplets into the protein
413 matrix could lead to a lower density of the coagulum resulting in the phase separation whereas
414 the higher lipid content in the serum seen in the non-homogenised samples could lead to a
415 dense coagulum that sedimented. Hence, the different colloidal behaviour of the samples was
416 driven by both droplet destabilisation and aggregate density.

417 Heat treatment was shown to be the main driver for the differences in coagulum consistency.
418 Both pasteurisation (72 °C for 15 s) and UHT (140 °C for 3 s) treatments were used, and
419 compared to the non-heated raw milk. It is well established that heating above 70 °C induces
420 the denaturation of whey proteins, in particular β-Lg. The extent of whey protein denaturation
421 in UHT milk is much higher than that in pasteurised milk (Douglas et al., 1981). The denatured
422 whey proteins have been reported to interact with κ-casein, forming complexes both at casein
423 micelle surface and in serum phase, the prevalence of which depends on the pH of heated milk
424 (Anema et al., 2011). Therefore, the level of protein association is higher in UHT-treated
425 compared to that of pasteurised milk. This could have impaired casein coagulation and led to
426 the more fragmented structures obtained in heated milk samples, in particular UHT (Fig. 2).

427 This different consistency persisted throughout digestion and the rheological analysis (Table
428 1) confirmed that the heat treatment was the main cause of the consistency of coagulum.

429 The initial protein aggregation to form the coagulum and the gastric behaviour was induced by
430 pepsin action. The protein aggregation was visually observed within the first 10 min, at which
431 time the pH was above 5.5. In contrast, when pepsin was not included, the protein aggregation
432 was observed after 75 min at which the pH was around 5. It has been reported that the pH for
433 coagulation of unheated and heated milk is about 5 and 5.3 respectively (Donato et al., 2007).
434 There was a more rapid decrease of pH when pepsin was present in raw milk digestion caused
435 by the rapid formation of the coagulum whereas the pH profile of the heated sample was similar
436 in the absence of pepsin (data not shown), which is in accordance to Ye et al. (2016). Pepsin
437 has been reported to favour the hydrolysis of κ-caseins among the other caseins at pH 6.0 (Tam
438 & Whitaker, 1972). The coagulation is caused by the destabilisation of casein micelles since
439 pepsin cleavages the Phe-105-Met-106 bond in κ-casein, which is the same than that for
440 chymosin (Drøhse & Foltmann, 1989) that is used for cheese making. Hence, it seems possible
441 to draw parallels to the effects of heat-induced changes on the functional properties, which has
442 been widely reported for the rennet coagulation. Kethireddipalli et al. (2010) showed that the
443 poor rennet clotting of heat-treated milk resulting in weak curds was due to the interactive
444 effect of the following: (i) modification of the surface of casein micelles with bound denatured
445 whey proteins; (ii) formation of soluble complexes between denatured whey proteins and κ-
446 casein; (iii) reduction of calcium concentration in the serum. In the present study, milk was
447 heated at its natural pH (6.67). It was shown that about 30 % of whey proteins can bind to the
448 micelle surface when milk, at the mentioned pH, was heated at 90 ºC (Kethireddipalli et al.,
449 2010). This impairs the micelle aggregation by steric effects, which in combination with the
450 protein complexation and alteration of the ionic equilibrium in the serum might explain the
451 different consistency of the coagulum obtained in the present study.

452 It is important to note that in this study the heat treatment was followed by the homogenisation.
453 The impact of the order of these processes is still subject of past and current research projects
454 (Michalski & Januel, 2006).

455 The comparison of the obtained gastric behaviour with other studies is difficult because the in
456 vivo studies using similar samples did not show the structural changes in the stomach even
457 though they suggested similar behaviours in terms of the consistency of coagulum. Moreover,
458 most in vitro studies use a static model, which does not allow to assess the structural changes.
459 Nevertheless, the results in terms of coagulation behaviour, timing and consistency, were in
460 agreement with the findings reported by Ye et al. (2016) using a dynamic model, the Human
461 Gastric Simulator.

462

463 ### 4.2. Effect of gastric behaviour on nutrient delivery and protein digestion

464 The gastric behaviour caused by the milk processing affected the nutrients emptied and protein
465 digestion kinetics. The sampling simulating the emptying was influenced by the consistency of
466 the coagulum. Mostly serum liquid was emptied in the first GE points for the samples having
467 a firmer coagulum, in particular Raw (Fig. 2A) accounting for the lowest content of nutrients
468 delivered in the GE1 (Fig. 5). In contrast, the very soft coagulum obtained from UHT+Homo
469 (Fig. 2F) allowed more of the coagulum to be emptied. Hence, the delivery of both lipid and
470 protein in GE1 was the highest for UHT+Homo (Fig. 5). It was found that the release of lipid
471 (Fig. 5A) was influenced by the phase separation obtained in the homogenised samples. The
472 lipid content in GE5 point was generally higher in the homogenised samples, as the cream layer
473 remained in the in vitro stomach until the last GE point. One exception for that was
474 UHT+Homo due to the high nutrient content at early stage. Similar results could be seen in the
475 protein profile (Fig. 5B) even though the differences were more subtle. This might be due to

476 the more constant delivery of protein throughout digestion, which might be attributed to the
477 emptying of serum containing mainly whey proteins.

478 The proteolysis levels might be linked to the consistency of the coagulum, which was mainly
479 affected by heat treatment. The softness of the coagulum (Table 1) and the greater number of
480 smaller particles (Fig.2) from the heat treated samples, in particular in the UHT+Homo could
481 facilitate pepsin diffusion within the structure leading to that higher proteolysis obtained at the
482 end of digestion (Fig. 6). In contrast to the lowest level of proteolysis found in raw milk, in
483 which the hardness of the coagulum and larger particles hampered the pepsin accessibility. The
484 UHT treatment resulted in an enhancement of both caseins and whey protein digestion (Fig.
485 7). Almost no detectable intact caseins and whey proteins were found after 73 min,
486 corresponding to the GE2 point. This finding is in agreement with the protein composition of
487 the heated homogenised milk shown in Ye et al. (2016). The UHT treatment has been reported
488 to greatly denature β-Lg, which exposures the peptides bonds to pepsin. The temperature of the
489 pasteurisation process was not sufficient to induce any important changes in the protein
490 digestion; the SDS-PAGE profile did not differ from that obtained of the non-heated samples
491 similarly to the observations of Wada and Lönnerdal (2014) during in vitro gastric digestion.
492 Also, β-lg remained largely intact during gastric digestion, which was already reported in
493 humans with the ingestion of purified caseins and β-Lg (Mahe et al., 1996). The degradation
494 of α-La was observed after about 109 min (GE3) at which the pH was under 4, which is in
495 agreement with its pepsin hydrolysis susceptibility by the change of protein conformation at
496 that pH.

497

498 4.3. Physiological relevance

499 The study has shown that the processing of milk resulted in different coagulation and colloidal
500 behaviour in gastric conditions influencing the nutrient digestion kinetics. This may influence
501 nutrient bioavailability and absorption in the intestine, and subsequently the metabolic
502 responses.

503 The gastric behaviour found in the stomach has been seen to influence satiety responses, which
504 are linked partly to the release of gut hormones such as cholecystokinin (CCK). The clinical
505 study performed by Mackie et al. (2013) showed the sedimentation of a semi-solid matrix
506 (cheese and yogurt) caused a lower GE rate and prolonged fullness response, in contrast to the
507 isocaloric comparison in a liquid matrix that creamed and increased hunger. This was explained
508 by the patterns of digestion obtained in vitro (Mulet-Cabero et al., 2017). The liquid system
509 showed a delayed nutrient release due to the formation of the cream layer during gastric
510 digestion whereas the sedimentation in semi-solid system led to the early emptying of high
511 nutrient content. In the present study, we found creaming and sedimentation in the
512 homogenised and non-homogenised samples respectively. Therefore, one might expect that
513 non-homogenised samples may induce more fullness compared to the homogenised samples.
514 However, according to the nutrient delivery results obtained in this study, UHT+Homo showed
515 early release of both protein and lipid, which may promote the release of CCK and thus increase
516 satiety.

517 The heat treatment of milk has been reported to affect protein postprandial kinetics. Lacroix et
518 al. (2008) showed that the UHT treatment enhanced the rate of digestion of milk protein
519 causing a higher transfer of dietary nitrogen into plasma, but pasteurisation treatment did not
520 alter the outcome. In the present study, in agreement with the in vivo data, the UHT treated
521 samples had a higher protein release in the early stages of digestion, in particular UHT+Homo.
522 Also, these samples showed higher digestion of both caseins and whey proteins. This may lead
523 to a different postprandial release of peptides (Boutrou et al., 2013), which may favour certain

524 population groups, for instance elderly and athletes may benefit form a higher postprandial
525 nitrogen absorption rate.

526 The metabolic responses relate to the nutrients delivered as a result of gastric emptying, which
527 is linked with the different structural changes occurring in the stomach. In the present study,
528 we used a convenient linear GE rate of 2 kcal/min, which is considered the average caloric
529 content that is emptied in a regulated manner by the antrum (Hunt & Stubbs, 1975). However,
530 this is a simplistic approach since the GE rate differs in response to the behaviour developed
531 during gastric conditions as was shown by Mackie et al. (2013). According to the structural
532 changes observed in the differently processed milk presented in this study, we expect that the
533 GE rate in humans could differ between the samples.

534

535 ## 5. Conclusions

536 In this study, it was shown that processed-induced changes in milk affect gastric digestion in
537 vitro, which may impact nutrient metabolism in vivo. This study showed for the first time clear
538 evidence of different milk behaviour, sedimentation vs. creaming. Homogenisation was the
539 main driver for the gastric phase separation, which was caused by the different droplet surface
540 and coagulum density. The different consistency of the coagulum was a consequence of the
541 heat treatment. The non-heated samples, especially Raw, formed a firm coagulum whereas the
542 heated samples had a fragmented coagulum particularly observed in UHT+Homo. This stems
543 from the formation of complexes between milk proteins, which weakens the protein network.
544 These structural changes occurring during the gastric phase resulted in different nutrient
545 emptying, with significant differences between Raw and UHT+Homo, and higher digestion of
546 milk proteins in the UHT-treated samples due to the drastic heat treatment. This study provides
547 value information for understanding the gastric emptying of milk in relation to its processing

548 and can be applied to manipulate the nutrient release rate of the dairy matrices addressed to
549 specific population groups.

550

551 Acknowledgements

552 This work has funded by the Irish Dairy Levy Research Trust (project number MDDT6261).
553 Ana-Isabel Mulet-Cabero was funded under Teagasc Walsh Fellowship scheme and BBSRC
554 in the UK (grant BB/J004545/1).

555

556 Abbreviations

557 GE, gastric emptying; UHT, Ultra High Temperature; SSF, simulated salivary fluid; SGF,
558 simulated gastric fluid; OPA, o-phthaldialdehyde; β-Lg, β-Lactoglobulin; MW, molecular
559 weight; TCA, trichloroacetic acid; SDS-PAGE, Sodium Dodecyl Sulphate-Polyacrylamide Gel
560 Electrophoresis

561

562 Figure Captions

563

564 Fig. 1. Change in pH of milk samples during gastric digestion in semi-dynamic model
565 corresponding to each gastric emptying (GE) point. The time represents an approximation of
566 the actual values displayed in Table S.1 Supplementary Material. The pH values are referred
567 to the basal stage (before gastric digestion), initial (milk sample including oral phase and basal
568 volumes) and the different GE samples (GE1-GE5). Each data point is the average of 2
569 independent determinations. Significance difference in pH between milk samples in each GE
570 point was determined by one-way ANOVA, $p<0.05$ (*).

571

572 Fig. 2. Images of the milk samples at approximately 36 and 182 min of gastric digestion,
573 corresponding to the first and last gastric emptying points (displayed in a petri dish for a better
574 visualisation). Raw milk (A, D), pasteurised milk (B, E), UHT milk (C, F), homogenised milk
575 (G, J), pasteurised+homogenised milk (H, K) and UHT+homogenised milk (I, L).

576

577 Fig. 3. Images of the milk samples at approximately 36 and 182 min, corresponding to the first
578 and last gastric emptying points (displayed in the gastric model). The times. Raw milk (A, D),
579 pasteurised milk (B, E), UHT milk (C, F), homogenised milk (G, J), pasteurised+homogenised
580 milk (H, K) and UHT+homogenised milk (I, L).

581

582 Fig. 4. Examples of confocal microscopy images of the milk samples before digestion and, at
583 about 36 min (GE1) and 182 min (GE5) of gastric digestion. Raw milk (A, D, G), pasteurised
584 milk (B, E, H), UHT milk (C, F, I), homogenised milk (J, M, P), pasteurised+homogenised

585 milk (K, N, Q), UHT+homogenised milk (L, O, R). Red shows the lipid and green shows the

586 protein. The scale bar corresponds to 75 µm.

587

588 Fig. 5. The nutrient content (w/w, %) in terms of protein (A) and lipid (B) of initial (before

589 digestion) and the gastric emptying points (GE1-GE5). Each data point is the average and error

590 bars represent standard deviation of two independent replicates. The values were corrected by

591 the different gastric dilution in each point. Mean values within a column with different

592 superscript letters (a, b, c) were significantly different ($p < 0.05$).

593

594 Fig. 6. Concentration of free amine groups per mass of total protein in sample; initial (before

595 digestion) and gastric emptying points (GE1-GE5). Each data point is the average and error

596 bars represent standard deviation of two independent replicates. The values were corrected by

597 the different gastric dilution in each point. Mean values within a column with different

598 superscript letters (a, b, c) were significantly different ($p < 0.05$).

599

600 Fig. 7. SDS-PAGE (under reducing conditions) of the milk samples, initial (I) referred to before

601 digestion and the gastric emptying points (GE1-GE5), and a molecular weight (MW) marker.

602 The samples are labelled in the figure accordingly. The protein content in each sample was

603 0.1%.

604

605

606

607

608 Table 1. Volume-weighted mean diameter ($d_{4,3}$) of the initial samples (before digestion), with
609 and without SDS addition, and the last gastric emptying (GE) point, GE5, including SDS. The

610 values represent the mean and standard deviation of two independent replicates. Values of the
611 complex module, G*, at 15 min of shear of the milk coagulum collected at GE5 time (after
612 about 182 min). Means within the same column and having the same superscript lower case
613 letter and means within the same superscript uppercase letter are not significantly different by
614 Tukey's t-test at $p < 0.05$.

	$d_{4,3}$ (µm)			
	Initial	**Initial+SDS**	**GE5+SDS**	**G* (Pa)**
Raw	$2.48±0.48^{aA}$	$2.96±0.08^{aA}$	$8.26±5.44^{aA}$	$4,555±236^{a}$
Past	$2.49±0.61^{aA}$	$3.62±0.65^{aA}$	$6.92±2.26^{aA}$	$2,934±1426^{a}$
UHT	$2.49±0.15^{aA}$	$3.82±0.02^{aA,B}$	$4.28±0.57^{aB}$	$501±186^{b}$
Homo	$0.42±0.02^{bA}$	$0.37±0.01^{bA}$	$0.42±0.03^{aA}$	$4,113±501^{a}$
Past+Homo	$0.34±0.01^{bA}$	$0.87±0.77^{bA}$	$2.99±2.23^{aA}$	$1,569±730^{b}$
UHT+Homo	$0.35±0.06^{bA}$	$0.41±0.08^{bA}$	$0.97±0.70^{aA}$	$206±45^{b}$

615
616
617
618
619
620
621
622
623
624
625
626
627
628

629 Supplementary Material

630

631 Table S.1. Nutritional composition of the milk samples. Values are the mean of two
632 independent replicates.

	% Lipid	% Protein	% Lactose	% Total solids
Raw	4.67±0.26	3.44±0.41	4.72±0.09	13.53±0.67
Past	4.55±0.43	3.32±0.25	4.71±0.10	13.24±0.59
UHT	4.49±0.53	3.43±0.42	4.71±0.05	13.35±1.03
Homo	4.74±0.28	3.76±0.09	4.66±0.02	13.82±0.25
Past+Homo	4.55±0.43	3.32±0.25	4.71±0.10	13.24±0.59
UHT+Homo	4.49±0.53	3.43±0.42	4.71±0.05	13.35±1.03

633

634

635

636

637 Table S.2. Example of the parameters used in the semi-dynamic gastric model. In this example,
638 the nutrient composition was the following 4.94 % fat, 3.82 % protein, 4.64 % lactose. The
639 sample had 14 % of total solids. The energy content was 0.78 kcal/mL calculated using the
640 Atwater factors of 9 kcal/g for fat and 4 kcal/g for protein and carbohydrates. The gastric
641 emptying was scaled down from the considered in vivo emptying average of 2 kcal/min in a
642 500 mL meal (Hunt & Stubbs, 1975) . Then, the gastric half time ($t_{1/2}$) was considered to be
643 the same. The density was set at 1 g/cm^3.

A. Milk sample (example)							
Food volume (g)	20						
Energy content (kcal/mL)	0.78						
Total solids (g)	2.8						
B. Gastric emptying and total digestion time							
	in vitro	in vivo					
Food volume (mL)	20.00	500.00					
Gastric volume (Oral +basal) at t=0 (mL)	25.08	550.00					
Energy content of food (kcal)	15.66	391.43					
Energy emptying rate (kcal/min)	0.08	2.00					
Volume emptying rate (mL/min) (Emptied in 5 steps of 9.12 mL every 39.1 min)	0.13	2.81					
$t_{1/2}$ (min)	97.86	97.86					
Total digestion time (min)	195.71						
C. Digestion							
	Oral Phase		Gastric phase				
Compound	Volume (mL)	%	Total gastric (mL)	Total gastric (%)	Basal (mL)	Gastric mixture (mL). Rate 0.1 mL/min	Pepsin solution (mL). Rate 0.004 mL/min
SSF electrolyte	2.24	79.89	0	0	0	0	0
0.3M M $CaCl_2(H_2O)_2$	0.014	0.50	0.0114	0.05	0.00114	0.01026	0
Milli-Q® Water	0.55	19.61	1.75	7.68	0.18	1.58	0
SGF electrolyte	0	0	18.24	80.00	1.82	16.42	0
1.5M HCl	0	0	2	8.77	0.20	1.80	0
Pepsin solution (2,000 U/mL final)	0	0	0.8	3.51	0	0	0.8
Total	2.80	100	22.8	100	2.20	19.80	0.8

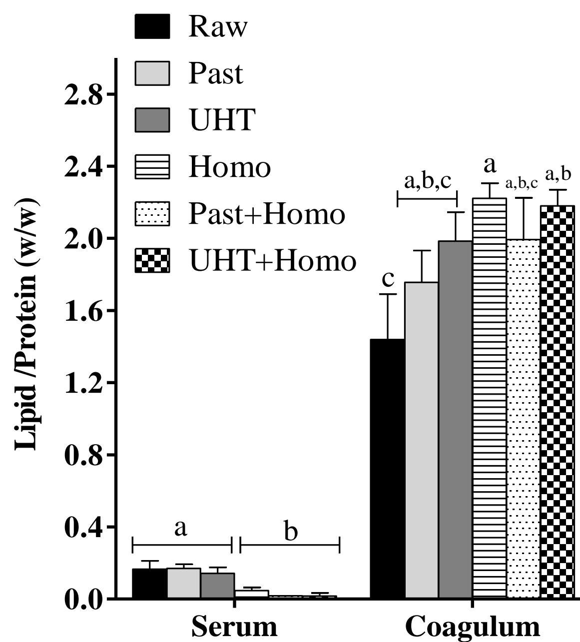
644

645 Table S.3. Time (min) at which gastric emptying was applied in the milk samples. Five
646 emptying points were used. Values are the mean of two independent replicates.

	Gastric emptying time (min)					
	Raw	**Past**	**UHT**	**Homo**	**Past+Homo**	**UHT+Homo**
Initial	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
GE1	36.2 ± 0.2	36.2 ± 2.7	36.4 ± 3.2	36.7 ± 0.6	36.2 ± 2.7	36.4 ± 3.2
GE2	72.4 ± 0.5	72.4 ± 5.3	72.9 ± 6.3	73.3 ± 1.4	72.4 ± 5.3	72.9 ± 6.3
GE3	108.6 ± 0.7	108.6 ± 8.0	109.4 ± 9.5	110.0 ± 2.1	108.6 ± 8.0	109.4 ± 9.5
GE4	144.8 ± 0.9	144.8 ± 10.6	145.8 ± 12.6	146.6 ± 2.8	144.8 ± 10.6	145.8 ± 12.6
GE5	180.9 ± 1.2	181.0 ± 13.3	182.3 ± 15.8	183.3 ± 3.5	181.0 ± 13.3	182.3 ± 15.8

647

648



649

650 Fig S.1. Lipid/protein ratio (w/w) of both serum and coagulum the digesta at approximately 36
651 min of digestion (time referred to GE1 point). Mean values within a column with different
652 superscript letters (a, b, c) were significantly different ($p < 0.05$).

653

654

655

656 References
657
658 Anema, S. G., Lee, S. K., & Klostermeyer, H. (2011). Rennet-Induced Aggregation of Heated
659 pH-Adjusted Skim Milk. Journal of Agricultural and Food Chemistry, 59(15), 8413-
660 8422.
661 Bach, A., Aris, A., Vidal, M., Fàbregas, F., & Terré, M. (2017). Influence of milk processing
662 temperature on growth performance, nitrogen retention, and hindgut's inflammatory
663 status and bacterial populations in a calf model. Journal of Dairy Research, 84(3),
664 355-359.
665 Barbé, F., Ménard, O., Le Gouar, Y., Buffière, C., Famelart, M.-H., Laroche, B., Le
666 Feunteun, S., Dupont, D., & Rémond, D. (2013). The heat treatment and the gelation
667 are strong determinants of the kinetics of milk proteins digestion and of the peripheral
668 availability of amino acids. Food Chemistry, 136(3), 1203-1212.
669 Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A.,
670 Benamouzig, R., Tomé, D., & Leonil, J. (2013). Sequential release of milk protein–
671 derived bioactive peptides in the jejunum in healthy humans. The American journal of
672 clinical nutrition, 97(6), 1314-1323.
673 Donato, L., Alexander, M., & Dalgleish, D. G. (2007). Acid Gelation in Heated and Unheated
674 Milks: Interactions between Serum Protein Complexes and the Surfaces of Casein
675 Micelles. Journal of Agricultural and Food Chemistry, 55(10), 4160-4168.
676 Douglas, F., Greenberg, R., Farrell, H., & Edmondson, L. (1981). Effects of ultra-high-
677 temperature pasteurization on milk proteins. Journal of Agricultural and Food
678 Chemistry, 29(1), 11-15.
679 Drøhse, H. B., & Foltmann, B. (1989). Specificity of milk-clotting enzymes towards bovine
680 κ-casein. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular
681 Enzymology, 995(3), 221-224.
682 Dupont, D., Mandalari, G., Mollé, D., Jardin, J., Rolet-Répécaud, O., Duboz, G., Léonil, J.,
683 Mills, C. E., & Mackie, A. R. (2010). Food processing increases casein resistance to
684 simulated infant digestion. Molecular Nutrition & Food Research, 54(11), 1677-1689.
685 Gaudichon, C., Roos, N., Mahé, S., Sick, H., Bouley, C., & Tomé, D. (1994). Gastric
686 emptying regulates the kinetics of nitrogen absorpti. The Journal of nutrition,
687 124(10), 1970.
688 Hunt, J., & Stubbs, D. (1975). The volume and energy content of meals as determinants of
689 gastric emptying. The Journal of physiology, 245(1), 209.
690 Islam, M. A., Devle, H., Comi, I., Ulleberg, E. K., Rukke, E.-O., Vegarud, G. E., & Ekeberg,
691 D. (2017). Ex vivo digestion of raw, pasteurised and homogenised milk - Effects on
692 lipolysis and proteolysis. International Dairy Journal.
693 Keenan, T. W., Moon, T.-W., & Dylewski, D. P. (1983). Lipid Globules Retain Globule
694 Membrane Material After Homogenization. Journal of Dairy Science, 66(2), 196-203.
695 Kethireddipalli, P., Hill, A. R., & Dalgleish, D. G. (2010). Protein interactions in heat-treated
696 milk and effect on rennet coagulation. International Dairy Journal, 20(12), 838-843.
697 Kong, F., & Singh, R. P. (2010). A Human Gastric Simulator (HGS) to Study Food Digestion
698 in Human Stomach. Journal of Food Science, 75(9), E627-E635.
699 Lacroix, M., Bon, C., Bos, C., Léonil, J., Benamouzig, R., Luengo, C., Fauquant, J., Tomé,
700 D., & Gaudichon, C. (2008). Ultra High Temperature Treatment, but Not
701 Pasteurization, Affects the Postprandial Kinetics of Milk Proteins in Humans. The
702 Journal of nutrition, 138(12), 2342-2347.
703 Lopez, C. (2005). Focus on the supramolecular structure of milk fat in dairy products.
704 Reproduction Nutrition Development, 45(4), 497-511.

705 Mackie, A., Rafiee, H., Malcolm, P., Salt, L., & van Aken, G. (2013). Specific food
706 structures supress appetite through reduced gastric emptying rate. Am J Physiol
707 Gastrointest Liver Physiol, 304(11), G1038-1043.
708 Mahe, S., Roos, N., Benamouzig, R., Davin, L., Luengo, C., Gagnon, L., Gausserges, N.,
709 Rautureau, J., & Tome, D. (1996). Gastrojejunal kinetics and the digestion of N-15
710 beta-lactoglobulin and casein in humans: The influence of the nature and quantity of
711 the protein. American Journal of Clinical Nutrition, 63(4), 546-552.
712 Malagelada, J.-R., Longstreth, G. F., Summerskill, W. H. J., & Go, V. L. W. (1976).
713 Measurement of Gastric Functions During Digestion of Ordinary Solid Meals in Man.
714 Gastroenterology, 70(2), 203-210.
715 Michalski, M.-C., & Januel, C. (2006). Does homogenization affect the human health
716 properties of cow's milk? Trends in Food Science & Technology, 17(8), 423-437.
717 Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carriere, F.,
718 Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya,
719 S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S.,
720 McClements, D. J., Menard, O., Recio, I., Santos, C. N., Singh, R. P., Vegarud, G. E.,
721 Wickham, M. S. J., Weitschies, W., & Brodkorb, A. (2014). A standardised static in
722 vitro digestion method suitable for food - an international consensus. Food &
723 function, 5(6), 1113-1124.
724 Miranda, G., & Pelissier, J.-P. (1987). Influence of heat treatment of bovine skim-milk on in
725 vivo digestion in rat stomach. Lait, 67(3), 365-377.
726 Morgan, F., Léonil, J., Mollé, D., & Bouhallab, S. (1999). Modification of Bovine β-
727 Lactoglobulin by Glycation in a Powdered State or in an Aqueous Solution: Effect on
728 Association Behavior and Protein Conformation. Journal of Agricultural and Food
729 Chemistry, 47(1), 83-91.
730 Mulet-Cabero, A.-I., Rigby, N. M., Brodkorb, A., & Mackie, A. R. (2017). Dairy food
731 structures influence the rates of nutrient digestion through different in vitro gastric
732 behaviour. Food Hydrocolloids, 67, 63-73.
733 Sánchez-Rivera, L., Ménard, O., Recio, I., & Dupont, D. (2015). Peptide mapping during
734 dynamic gastric digestion of heated and unheated skimmed milk powder. Food
735 Research International, 77, Part 2, 132-139.
736 Sharma, S. K., & Dalgleish, D. G. (1993). Interactions between milk serum proteins and
737 synthetic fat globule membrane during heating of homogenized whole milk. Journal
738 of Agricultural and Food Chemistry, 41(9), 1407-1412.
739 Tam, J. J., & Whitaker, J. R. (1972). Rates and Extents of Hydrolysis of Several Caseins by
740 Pepsin, Rennin, Endothia parasitica Protease and Mucor pusillus Protease1. Journal
741 of Dairy Science, 55(11), 1523-1531.
742 Thomas, A. (2006). GastroGut motility, sphincters and reflex control. Anaesthesia &
743 Intensive Care Medicine, 7(2), 57-58.
744 Tunick, M. H., Ren, D. X., Van Hekken, D. L., Bonnaillie, L., Paul, M., Kwoczak, R., &
745 Tomasula, P. M. (2016). Effect of heat and homogenization on in vitro digestion of
746 milk. Journal of Dairy Science, 99(6), 4124-4139.
747 van Aken, G. A., Bomhof, E., Zoet, F. D., Verbeek, M., & Oosterveld, A. (2011). Differences
748 in in vitro gastric behaviour between homogenized milk and emulsions stabilised by
749 Tween 80, whey protein, or whey protein and caseinate. Food Hydrocolloids, 25(4),
750 781-788.
751 Wada, Y., & Lönnerdal, B. (2014). Effects of Different Industrial Heating Processes of Milk
752 on Site-Specific Protein Modifications and Their Relationship to in Vitro and in Vivo
753 Digestibility. Journal of Agricultural and Food Chemistry, 62(18), 4175-4185.

754 Ye, A., Cui, J., Dalgleish, D., & Singh, H. (2016). Effect of homogenization and heat
755 treatment on the behavior of protein and fat globules during gastric digestion of milk.
756 Journal of Dairy Science.
757 Ye, A., Cui, J., Dalgleish, D., & Singh, H. (2016). Formation of a structured clot during the
758 gastric digestion of milk: Impact on the rate of protein hydrolysis. Food
759 Hydrocolloids, 52, 478-486.

760