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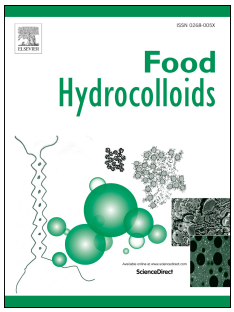

Structural mechanism and kinetics of *in vitro* gastric digestion are affected by process-induced changes in bovine milk

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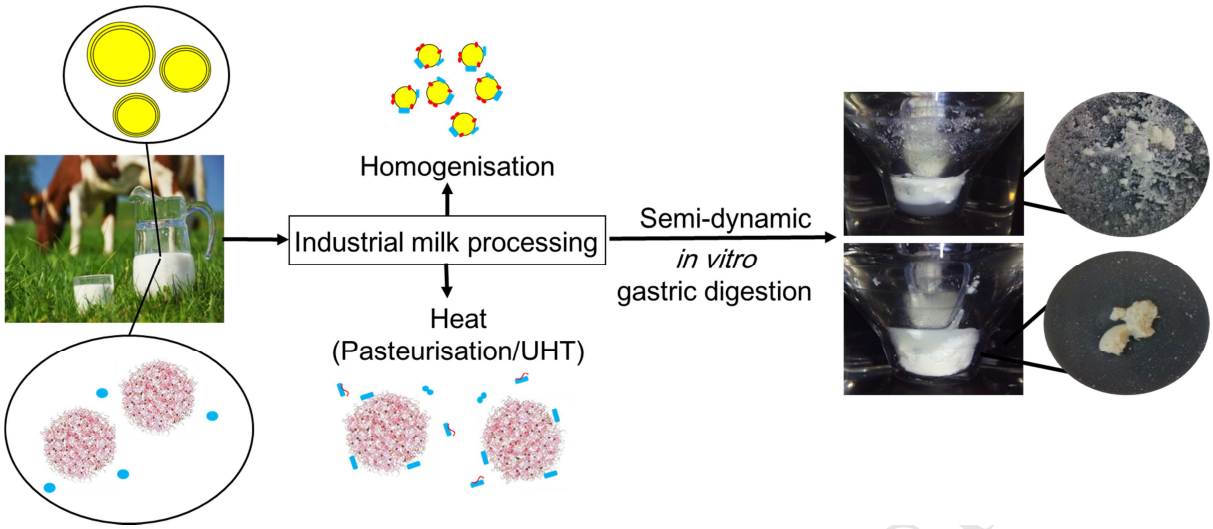

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Title: Structural mechanism and kinetics of *in vitro* gastric digestion are affected by process-induced changes in bovine milk

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Abstract

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

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

Keywords

Milk; Homogenisation; Heat treatment; Gastric behaviour; Nutrient delivery; Protein digestion

1. Introduction

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

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

Studies of the *in vivo* digestion of processed milk are very rare. Lacroix *et al.* (2008) found, in healthy humans, that UHT-treated milk consumption induced a significantly higher and faster transfer of dietary nitrogen into serum amino acids and proteins but also to body urea compared to pasteurised and microfiltrated milk. It was suggested that this modulation of the digestive kinetics was due to the possible formation of a softer coagulum in the stomach and a higher enzyme accessibility in the case of UHT-treated milk. These results have been supported by Bach *et al.* (2017), who showed that urinary nitrogen secretion was greater for UHT-milk compared to raw and pasteurised milk using young dairy calves as a model. In addition, Miranda and Pelissier (1987) found that heat treated milk (UHT and autoclaving) increased gastric emptying rate and casein hydrolysis in rats. This contrasts to other work where a higher mean retention time in the stomach of heated skim milk (90 ^{o}C, 10 min) was observed compared to a non-heated system observed in mini-pigs (Barbé *et al.*, 2013). It is broadly reported that heat treatment, using temperatures above 90 ^{o}C, facilitates protein digestion, which has been observed for β-Lg (Wada & Lönnerdal, 2014). However, opposing observations have been made for caseins. Heated skim milk (90 ^{o}C, 10 min) promoted hydrolysis resistance of casein fraction compared to unheated skim milk during gastric digestion using an *in vitro* adult dynamic model (Sánchez-Rivera *et al.*, 2015) and *in vitro* infant static model (Dupont *et al.*, 2010), which could affect the kinetics of protein digestion in a mini-pig model (Barbé *et al.*, 2013). This was reportedly related to chemical modifications of the protein during heating, i.e. lactosylation, glycosylation as well as casein-whey interactions, resulting in different peptides generated during digestion. In contrast, using a static digestion model, Tunick *et al.* (2016) found a rapid digestion of caseins in the gastric phase of both processed (heated at pasteurisation and UHT conditions and homogenised) and non-processed samples. Moreover, homogenisation was observed to increase β-Lg hydrolysis compared to pasteurised milk (Islam *et al.*, 2017). Despite the

differences in enzymatic digestion of the major milk proteins, Wada and Lönnerdal (2014) reported no significant differences in the overall *in vitro* digestion kinetics among the heat treatments (pasteurisation, UHT and in-can sterilisation). A sophisticated *in vitro* model, the Human Gastric Simulator (Kong & Singh, 2010), was used to investigate the effect of milk treatment on the gastric behaviour (Ye *et al.*, 2016). They showed the formation of coagula of different structures led to different protein digestion behaviour. The homogenisation and heat treatment resulted in the formation of a crumbly structure compared to the tight clot obtained in raw milk. This was similar to what was proposed to occur *in vivo* and highlights the limitations of the static *in vitro* digestion models. However, the heating conditions used, 90 ^{o}C for 20 min, are less representative of the typical conditions of industrial milk processing.

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

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

2. Materials and methods

119 2.1. Materials

120 Fresh whole bovine milk was collected from a bulk tank of the Moorepark Dairy Unit,
121 Teagasc Animal and Grassland Research and Innovation Center, Moorepark, Fermoy, Co.
122 Cork, Ireland. The milk was from Friesian cows that were fed a total mixed ration diet
123 consisted of grass silage, maize silage and concentrates. Bulk milk samples were collected
124 post-morning milking. The sampling was conducted between November 2016 and February
125 2017.

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

141 Milk fat, protein, lactose and total solids values were obtained using a Milkoscan FT 6000
142 (FOSS, Denmark) with a tolerance of ± 0.06 %. The nutrient composition of milk was

143 measured before each sample (see Table S.1 Supplementary Material) and the caloric content
144 was calculated using the Atwater factors. This ranged from 0.78 to 0.68 kcal/mL.

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

149

150 2.2. Methods

151 2.2.1. Semi-dynamic gastric digestion model

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

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

159 The oral phase was applied before the gastric digestion, in which 20 g of milk sample was
160 mixed with oral mixture using a rotator (SB3 Model, Stuart, Bibby Scientific, UK) at 30 rpm
161 for 2 min. The total oral mixture consists of Simulated Salivary Fluid (SSF), prepared
162 accordingly to Minekus et al. (2014). The volume of the added SSF corresponded to the total
163 solid content of the milk sample, which was measured for each individual milk. For example,
164 a SSF volume of 2.8 mL is required for the digestion of a 20 mL (containing 2.8 g total
165 solids). The SSF addition varied slightly between samples, ranging from 2.52 to 2.82 mL due

166 to the difference of the total solid concentration in the analysed milk samples during the
167 period of study. The resulting mixture was then put through the gastric digestion.

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

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

IKA®, Germany) at approximately 30,000 rpm for 30 s to obtain a homogenous sample for the remaining analysis. The pH of each GE samples was measured using a pH meter and a sufficient volume of 2 M NaOH was added to the samples to increase the pH above 7, inhibiting pepsin activity. Finally, samples were snap-frozen in liquid nitrogen and stored at -80 °C until subsequent treatment.

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

2.2.2. Confocal Laser Scanning Microscopy (CLSM)

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

2.2.3. Particle size distribution

The particle size distribution and average lipid droplet size of initial and digested samples were determined using a laser-light diffraction unit (Mastersizer, Malvern Instruments Ltd, Worcestershire, UK) equipped with a 300 RF lens. The optical parameters chosen were a particle and dispersant (water) refractive index of 1.456 and 1.330, respectively. The absorbance value of the fat globules was 0.001. A volume of initial and digested samples was added in order to reach a laser obscuration range of 5-10 %. A volume of the initial and GE5 samples (0.2 mL) was dispersed in 10 mL of 0.02 M sodium dodecyl sulphate (SDS) to dissociate clusters of proteins (as described in van Aken *et al.* (2011)). The size distribution was obtained using polydisperse analysis, while droplet size measurements were recorded as surface area weighted ($d_{3,2}$) and volume weighted ($d_{4,3}$) means, where $d_{3,2}$ is defined as $\sum n_i d_i^3 / n_i d_i^2$ 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. Each measurement was carried out in triplicate.

2.2.4. Protein content analysis

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

2.2.5. Lipid content analysis

The lipid content of the initial milk and emptied digesta was measured using a CEM Smart Trac System-5 and a CEM Smart Trac Rapid Fat Analyzer (CEM Corp., Matthews, N.C., U.S.A.). Approximately 2 g of sample (previously warmed up to 40 ^{o}C to disperse the lipid)

was placed on a glass fiber sample pad and dried in the Smart Trac System by microwave drying. Immediately after drying, samples were placed in the Smart Trac Rapid Fat Analyzer to determine total lipid content by nuclear magnetic resonance. The lipid content was reported as a percentage of g lipid per g meal. Each measurement was carried out in duplicate.

2.2.6. Protein analysis

2.2.6.1. Quantification of protein hydrolysis

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

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

Different concentrations of standard L-leucine solution (made with phosphate buffer solution) ranged from 0 to 10 mM were used to obtain a calibration curve. 10 µl of standard/sample was placed into each well and mixed with 200 µl of OPA reagent. The reaction was allowed to proceed for 15 min, then the absorbance was measured at 340 nm using a multi-mode

microplate reader (Synergy HT, BioTek Instruments, Inc.). Each measurement was carried out in duplicate.

2.2.6.2. Identification of proteins during digestion

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed on the initial and digested samples normalised to a total protein concentration of 0.1 %. NuPAGE Novex bis-Tris 12-well precast gels (Invitrogen, Life Technologies Corp., CA, USA), 4-12 % polyacrylamide, were used according to the manufacturer's instructions. The amount of protein loaded in each well was 6.5 µg. A fixing solution (50% methanol and 10% acetic acid in v/v) was applied to the gels for 2 hrs before staining with Coomassie Blue. Mark 12™ Unstained Standard (Invitrogen, Life Technologies Corp., CA, USA) was used as a molecular weight marker.

2.2.7. Rheological analysis

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

2.2.8. Statistical Analysis

The results were expressed as means ± standard deviation of two replicates. For each replicate, raw milk was collected, analysed (composition) and processed independently, i.e. one milk per day. To identify differences in normally distributed results of the repeated measure within groups during gastric digestion, one-way ANOVA was applied. Where overall significant interaction was observed ($P < 0.05$), the means of individual milk treatments were compared using Tukey's post hoc test. Statistical analyses were performed using GraphPad Prism software (Prism 5 for Windows, Version 5.04).

3. Results

3.1. Gastric pH profile

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

3.2. Gastric behaviour

Using the semi-dynamic model, a range of different structures and behaviours during gastric digestion were obtained (Fig. 2). Protein coagulation was visible for all the samples within the first 10 min of digestion and the formation of larger aggregates was observed a few minutes later, at which time the pH ranged from 5.5 to 6. Subsequently, there was the formation of a more compact coagulum with clear serum within the following 15 min. Differences in coagulum consistency were observed throughout the gastric phase as illustrated in Fig. 2. There were remarkable differences, in particular, between the firm coagulum of Raw (Fig. 2A) and the fragmented structure of UHT+Homo (Fig. 2I). In the absence of pepsin, we observed later aggregation and coagulum formation. Protein coagulation was visually observed after 75 min at which time the pH was around 5, with the exception of the UHT-treated samples in which the aggregation was first seen at 35min.

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

The consistency of the milk coagulum was further studied by small deformation rheology analysing the coagulum remaining in GE5. Table 1 shows the values of the complex modulus (G*) obtained after 15 min of measurement. The non-heated samples, Raw and Homo, generated the highest levels of G* accounting for 4,555 and 4,113 Pa, respectively. The pasteurised samples (Past and Past+Homo) presented an intermediate situation accounting for 2,934 and 1,569 Pa. The lowest G* values were found in UHT and UHT+Homo representing

for 501 and 206 Pa, respectively. The same behaviour was observed during the rheological analysis, which was performed for 30 min.

It is important to note that some alteration of the structure could have been induced while transferring the sample to the rheometer in order to perform the analysis.

3.3. Microstructure of the emptied samples

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

The changes in the droplet size were followed during digestion (Table 1). Initially, the volume mean particle diameter, $d_{4,3,}$ of non-homogenised samples was about 2.5 µm whereas that of homogenised samples was about 0.4 µm, showing the significant size reduction due to homogenisation treatment. The particle size of the milk samples, with the addition of SDS,

356 increased to a different extent at the end of digestion. The digestion of the raw milk resulted
357 in an increase from the initial size of 2.96 µm to 8.26 µm after 182 min of digestion but the
358 particle size of UHT+Homo increased from 0.41 to 0.97 µm.

359

360 3.4. Nutrient delivery

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

369

370 3.5. Protein digestion

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

respectively. Conversely, in GE5, UHT+Homo showed the highest level of proteolysis (1,736 mM NH_2/g) being statistically different from Raw and Homo (897 and 1,065 mM NH_2/g, respectively).

The protein composition during the gastric phase was also studied by SDS-PAGE and shown in Fig. 7. The bands corresponding to the samples before digestion (I) did not differ due to processing. Moreover, there were no differences between homogenised and non-homogenised samples. The non-heated samples, Raw and Homo, had similar patterns than those of pasteurised samples (Past and Past+Homo). The caseins were detectable in the first emptying points, in particular GE1 and GE2 points, but they were almost not observed in GE3 and GE4 points. In the last emptying point (GE5) intact caseins could again be observed together with a wide range of peptides. β-Lg, in contrast, was present during gastric digestion even though the band weakened in the last GE points. Also, α-La was present in the three first GE points, after which it was not detected anymore. Many small molecular weight peptides were present during digestion and could be seen from GE1 onwards. This behaviour differed from that observed in the UHT-treated samples (UHT and UHT+Homo). In those samples, both caseins and whey proteins could only be observed in the two first GE points.

4. Discussion

4.1.Influence of the milk processing on gastric behaviour.

By using a physiologically relevant gastric model (Mulet-Cabero *et al.*, 2017), we have been able to show that homogenised samples showed significantly more creaming compared to non-homogenised samples where sedimentation was observed, regardless the heat treatment (Fig. 3). Homogenisation caused the disruption of the native MFGM, reduced the droplet size and promoted adsorption of milk proteins onto the droplet surface (Lopez, 2005; Sharma &

Dalgleish, 1993). This change of the droplet interfacial composition might be one of the main reasons for the distinct gastric behaviour. The milk proteins on the droplet surface, especially the denatured and aggregated proteins in the heated UHT+Homo sample, may be more susceptible to be hydrolysed by pepsin leading to the destabilisation of the droplets by flocculation and some coalescence, and ultimately leading to the phase separation observed. The non-homogenised samples, in contrast, still possessed the native MFGM, which could provide more stability during gastric digestion. These structural changes were certainly due to the proteolytic action of pepsin since there was no phase separation in the homogenised samples when pepsin was absent. Further investigation was undertaken in order to gain insight into the mechanism of the different gastric behaviour observed. The lipid/protein ratio in both coagulum and serum in the first GE point was determined (Fig. S.1 Supplementary Material). The non-homogenised samples had significantly higher lipid/protein ratio in the serum compared to the homogenised samples. Moreover, the microstructure imaging showed that most of the droplets in the non-homogenised samples tended to be in the serum (Fig. 4D-F) compared to those of the homogenised samples (Fig. 4M-O). This might be due to easier incorporation of the smaller droplets into the coagulum and also the possible interactions of the droplet surface coated by milk protein with the protein network. Therefore, a higher inclusion of droplets into the protein matrix could lead to a lower density of the coagulum resulting in the phase separation whereas the higher lipid content in the serum seen in the non-homogenised samples could lead to a dense coagulum that sedimented. Hence, the different colloidal behaviour of the samples was driven by both droplet destabilisation and aggregate density.

Heat treatment was shown to be the main driver for the differences in coagulum consistency. Both pasteurisation (72 ^{o}C for 15 s) and UHT (140 ^{o}C for 3 s) treatments were used, and compared to the non-heated raw milk. It is well established that heating above 70 ^{o}C induces

the denaturation of whey proteins, in particular β-Lg. The extent of whey protein denaturation in UHT milk is much higher than that in pasteurised milk (Douglas *et al.*, 1981). The denatured whey proteins have been reported to interact with κ-casein, forming complexes both at casein micelle surface and in serum phase, the prevalence of which depends on the pH of heated milk (Anema *et al.*, 2011). Therefore, the level of protein association is higher in UHT-treated compared to that of pasteurised milk. This could have impaired casein coagulation and led to the more fragmented structures obtained in heated milk samples, in particular UHT (Fig. 2). This different consistency persisted throughout digestion and the rheological analysis (Table 1) confirmed that the heat treatment was the main cause of the consistency of coagulum.

The initial protein aggregation to form the coagulum and the gastric behaviour was induced by pepsin action. The protein aggregation was visually observed within the first 10 min, at which time the pH was above 5.5. In contrast, when pepsin was not included, the protein aggregation was observed after 75 min at which the pH was around 5. It has been reported that the pH for coagulation of unheated and heated milk is about 5 and 5.3 respectively (Donato *et al.*, 2007). There was a more rapid decrease of pH when pepsin was present in raw milk digestion caused by the rapid formation of the coagulum whereas the pH profile of the heated sample was similar in the absence of pepsin (data not shown), which is in accordance to Ye *et al.* (2016). Pepsin has been reported to favour the hydrolysis of κ-caseins among the other caseins at pH 6.0 (Tam & Whitaker, 1972). The coagulation is caused by the destabilisation of casein micelles since pepsin cleavages the Phe-105-Met-106 bond in κ-casein, which is the same than that for chymosin (Drøhse & Foltmann, 1989) that is used for cheese making. Hence, it seems possible to draw parallels to the effects of heat-induced changes on the functional properties, which has been widely reported for the rennet coagulation. Kethireddipalli *et al.* (2010) showed that the poor rennet clotting of heat-treated

454 milk resulting in weak curds was due to the interactive effect of the following: (i)
455 modification of the surface of casein micelles with bound denatured whey proteins; (ii)
456 formation of soluble complexes between denatured whey proteins and κ-casein; (iii)
457 reduction of calcium concentration in the serum. In the present study, milk was heated at its
458 natural pH (6.67). It was shown that about 30 % of whey proteins can bind to the micelle
459 surface when milk, at the mentioned pH, was heated at 90 ^{o}C (Kethireddipalli *et al.*, 2010).
460 This impairs the micelle aggregation by steric effects, which in combination with the protein
461 complexation and alteration of the ionic equilibrium in the serum might explain the different
462 consistency of the coagulum obtained in the present study.

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

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

473

474 4.2. Effect of gastric behaviour on nutrient delivery and protein digestion

475 The gastric behaviour caused by the milk processing affected the nutrients emptied and
476 protein digestion kinetics. The sampling simulating the emptying was influenced by the
477 consistency of the coagulum. Mostly serum liquid was emptied in the first GE points for the

478 samples having a firmer coagulum, in particular Raw (Fig. 2A) accounting for the lowest
479 content of nutrients delivered in the GE1 (Fig. 5). In contrast, the very soft coagulum
480 obtained from UHT+Homo (Fig. 2F) allowed more of the coagulum to be emptied. Hence,
481 the delivery of both lipid and protein in GE1 was the highest for UHT+Homo (Fig. 5). It was
482 found that the release of lipid (Fig. 5A) was influenced by the phase separation obtained in
483 the homogenised samples. The lipid content in GE5 point was generally higher in the
484 homogenised samples, as the cream layer remained in the *in vitro* stomach until the last GE
485 point. One exception for that was UHT+Homo due to the high nutrient content at early stage.
486 Similar results could be seen in the protein profile (Fig. 5B) even though the differences were
487 more subtle. This might be due to the more constant delivery of protein throughout digestion,
488 which might be attributed to the emptying of serum containing mainly whey proteins.

489 The proteolysis levels might be linked to the consistency of the coagulum, which was mainly
490 affected by heat treatment. The softness of the coagulum (Table 1) and the greater number of
491 smaller particles (Fig.2) from the heat treated samples, in particular in the UHT+Homo could
492 facilitate pepsin diffusion within the structure leading to the higher proteolysis obtained at the
493 end of digestion (Fig. 6). In contrast the lowest level of proteolysis was found in raw milk, in
494 which the hardness of the coagulum and larger particles hampered the pepsin accessibility.
495 The UHT treatment resulted in an enhancement of both caseins and whey protein digestion
496 (Fig. 7). For the UHT samples, almost no detectable intact caseins or whey proteins were
497 found after 73 min, corresponding to the GE2 point. This finding is in agreement with the
498 protein composition of the heated homogenised milk shown in Ye *et al.* (2016). The UHT
499 treatment has been reported to greatly denature β-Lg, which exposes the peptide bonds to
500 pepsin. The temperature of the pasteurisation process was not sufficient to induce any
501 important changes in the protein digestion; the SDS-PAGE profile did not differ from that
502 obtained of the non-heated samples similarly to the observations of Wada and Lönnerdal

503 (2014) during *in vitro* gastric digestion. Also, β-lg remained largely intact during gastric
504 digestion, which was already reported in humans with the ingestion of purified caseins and β-
505 Lg (Mahe *et al.*, 1996). The degradation of α-La was observed after about 109 min (GE3) at
506 which the pH was under 4, which is in agreement with its pepsin hydrolysis susceptibility by
507 the change of protein conformation at that pH.

508

509 4.3. Physiological relevance

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

514 The gastric behaviour found in the stomach has been seen to influence satiety responses,
515 which are linked partly to the release of gut hormones such as cholecystokinin (CCK). The
516 clinical study performed by Mackie *et al.* (2013) showed the sedimentation of a semi-solid
517 matrix (cheese and yogurt) caused a lower GE rate and prolonged fullness response, in
518 contrast to the isocaloric comparison in a liquid matrix that creamed and increased hunger.
519 This was explained by the patterns of digestion obtained *in vitro* (Mulet-Cabero et al., 2017).
520 The liquid system showed a delayed nutrient release due to the formation of the cream layer
521 during gastric digestion whereas the sedimentation in semi-solid system led to the early
522 emptying of high nutrient content. In the present study, we found creaming and sedimentation
523 in the homogenised and non-homogenised samples respectively. Therefore, one might expect
524 that non-homogenised samples may induce more fullness compared to the homogenised
525 samples. However, according to the nutrient delivery results obtained in this study,

526 UHT+Homo showed early release of both protein and lipid, which may promote the release
527 of CCK and thus increase satiety.

528 The heat treatment of milk has been reported to affect protein postprandial kinetics. Lacroix
529 *et al.* (2008) showed that the UHT treatment enhanced the rate of digestion of milk protein
530 causing a higher transfer of dietary nitrogen into serum amino acids and protein, but
531 pasteurisation treatment did not alter the outcome. In the present study, in agreement with the
532 *in vivo* data, the UHT treated samples had a higher protein release in the early stages of
533 digestion, in particular UHT+Homo. Also, these samples showed higher digestion of both
534 caseins and whey proteins. This may lead to a different postprandial release of peptides
535 (Boutrou *et al.*, 2013), which may favour certain population groups, for instance elderly and
536 athletes may benefit form a higher postprandial nitrogen absorption rate.

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

545

546 ## 5. Conclusions

547 In this study, it was shown that processed-induced changes in milk affect gastric digestion *in*
548 *vitro*, which may impact nutrient metabolism *in vivo*. This study showed for the first time
549 clear evidence of different milk behaviour, sedimentation vs. creaming. Homogenisation was

the main driver for the gastric phase separation, which was caused by the different droplet surface and coagulum density. The different consistency of the coagulum was a consequence of the heat treatment. The non-heated samples, especially Raw, formed a firm coagulum whereas the heated samples had a fragmented coagulum particularly observed in UHT+Homo. This stems from the formation of complexes between milk proteins, which weakens the protein network. These structural changes occurring during the gastric phase resulted in different nutrient emptying, with significant differences between Raw and UHT+Homo, and higher digestion of milk proteins in the UHT-treated samples due to the drastic heat treatment. This study provides valuable information for understanding the gastric emptying of milk in relation to its processing and can be applied to manipulate the nutrient release rate of the dairy matrices addressed to specific population groups.

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Abbreviations

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

573 Figure Captions

574

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

582

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

587

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

592

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

596 milk (K, N, Q), UHT+homogenised milk (L, O, R). Red shows the lipid and green shows the
597 protein. The scale bar corresponds to 75 µm.

598

599 Fig. 5. The nutrient content (w/w, %) in terms of protein (A) and lipid (B) of initial (before
600 digestion) and the gastric emptying points (GE1-GE5). Each data point is the average and
601 error bars represent standard deviation of two independent replicates. The values were
602 corrected by the different gastric dilution in each point. Mean values within a column with
603 different superscript letters (a, b, c) were significantly different ($p < 0.05$).

604

605 Fig. 6. Concentration of free amine groups per mass of total protein in sample; initial (before
606 digestion) and gastric emptying points (GE1-GE5). Each data point is the average and error
607 bars represent standard deviation of two independent replicates. The values were corrected by
608 the different gastric dilution in each point. Mean values within a column with different
609 superscript letters (a, b, c) were significantly different ($p < 0.05$).

610

611 Fig. 7. SDS-PAGE (under reducing conditions) of the milk samples, initial (I) referred to
612 before digestion and the gastric emptying points (GE1-GE5), and a molecular weight (MW)
613 marker. The samples are labelled in the figure accordingly. The protein content in each
614 sample was 0.1%.

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Table 1. Volume-weighted mean diameter ($d_{4,3}$) of the initial samples (before digestion), with and without SDS addition, and the last gastric emptying (GE) point, GE5, including SDS. The values represent the mean and standard deviation of two independent replicates. Values of the complex module, G*, at 15 min of shear of the milk coagulum collected at GE5 time (after about 182 min). Means within the same column and having the same superscript lower case letter and means within the same superscript uppercase letter are not significantly different by 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}

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641 Supplementary Material

642

643 Table S.1. Nutritional composition of the milk samples. Values are the mean of two
644 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

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

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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

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Table S.3. Time (min) at which gastric emptying was applied in the milk samples. Five 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

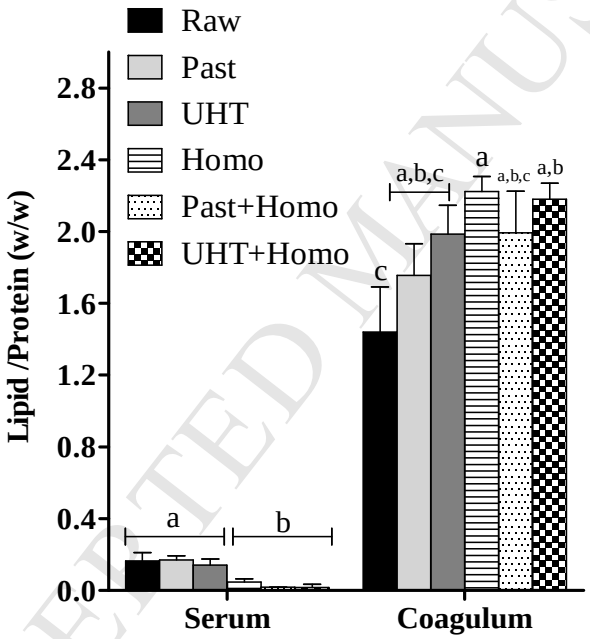

Fig S.1. Lipid/protein ratio (w/w) of both serum and coagulum the digesta at approximately 36 min of digestion (time referred to GE1 point). Mean values within a column with different superscript letters (a, b, c) were significantly different ($p < 0.05$).

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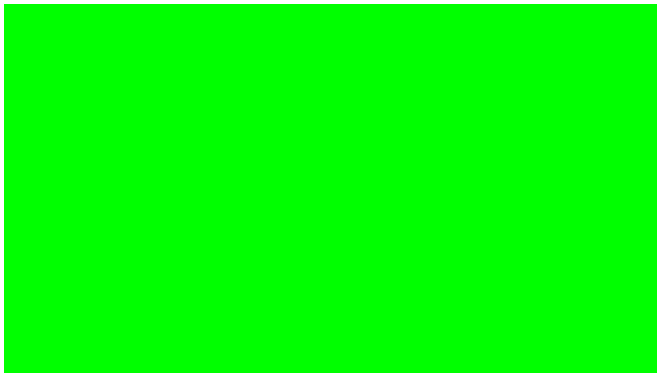

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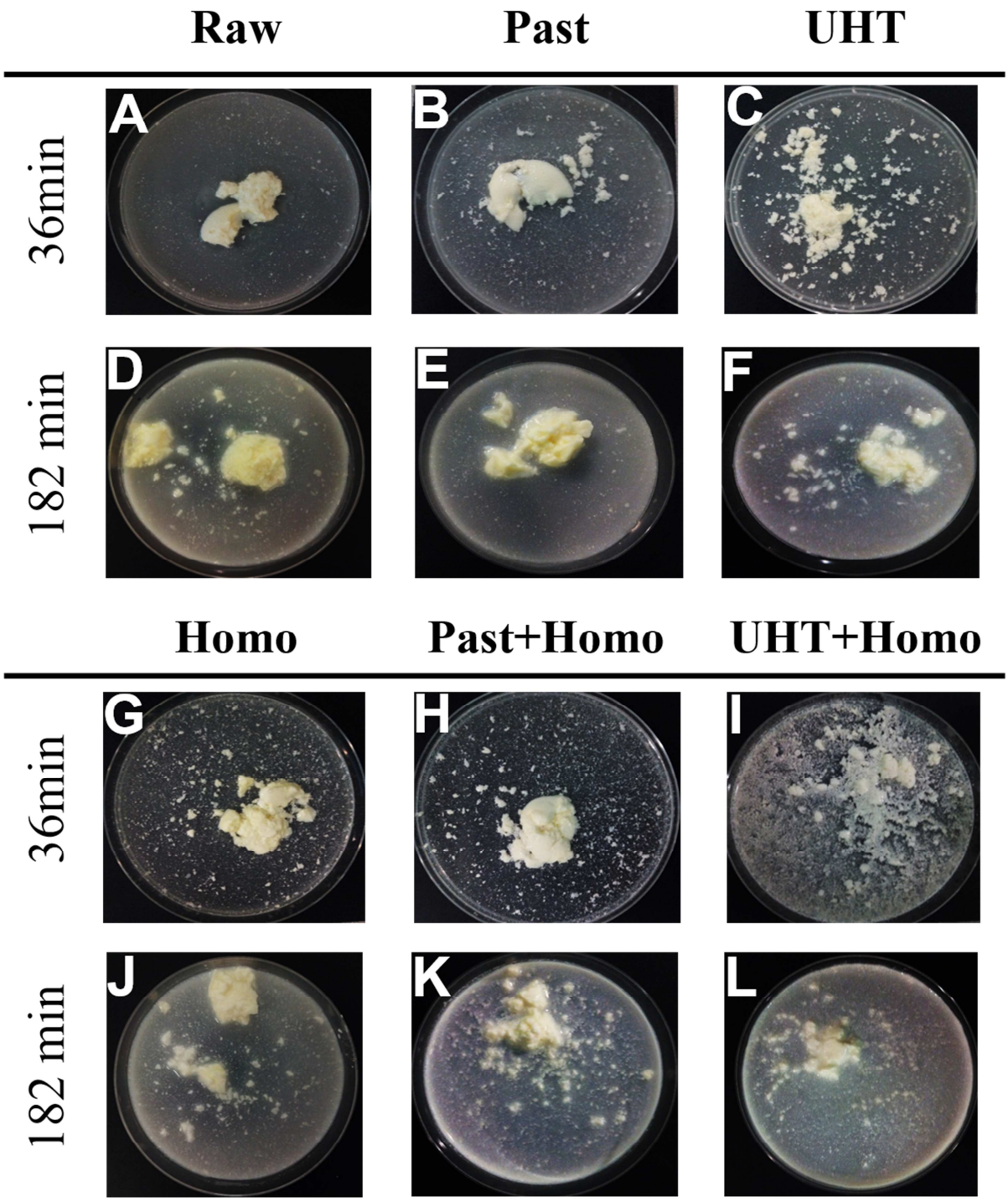
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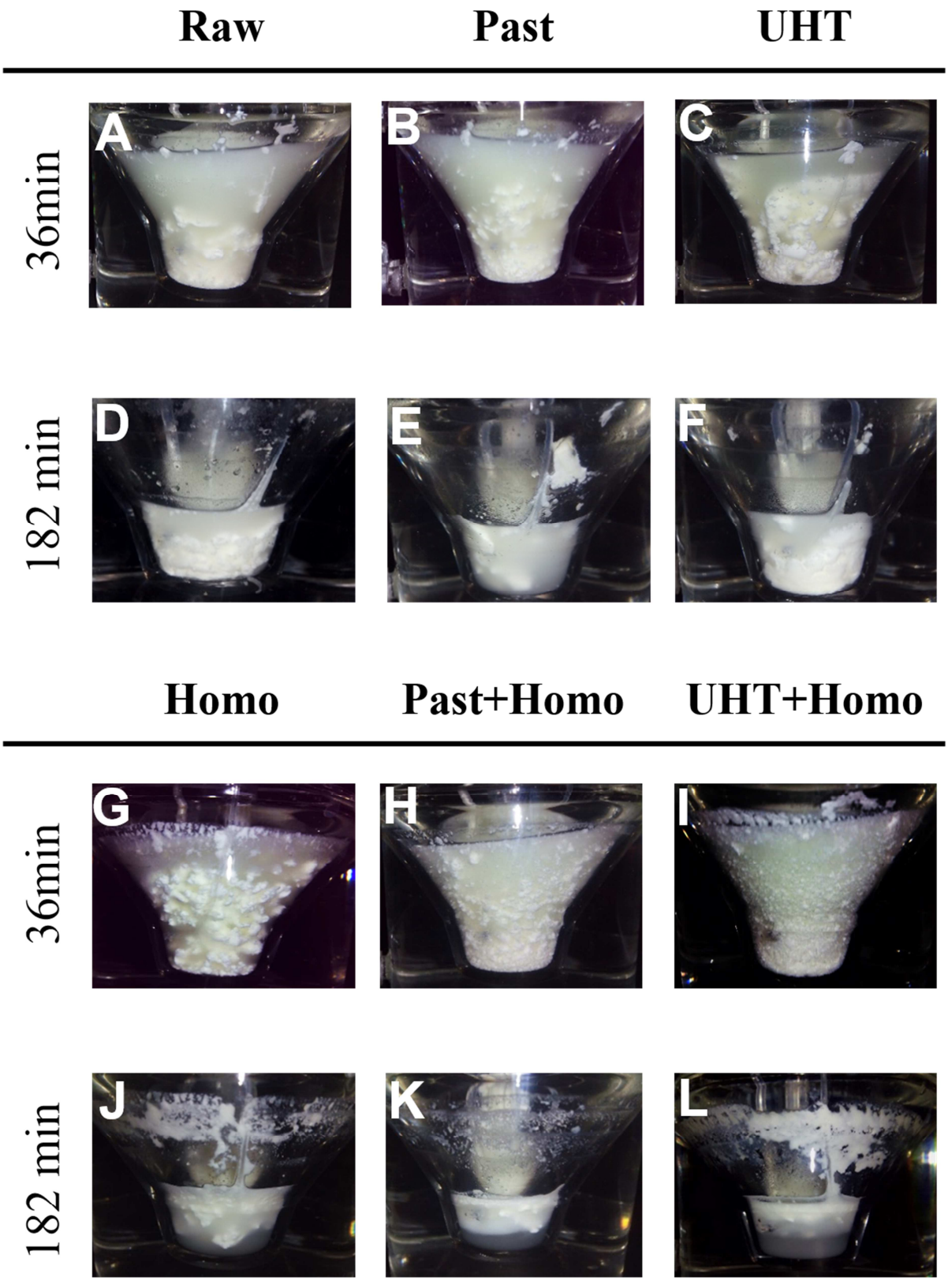
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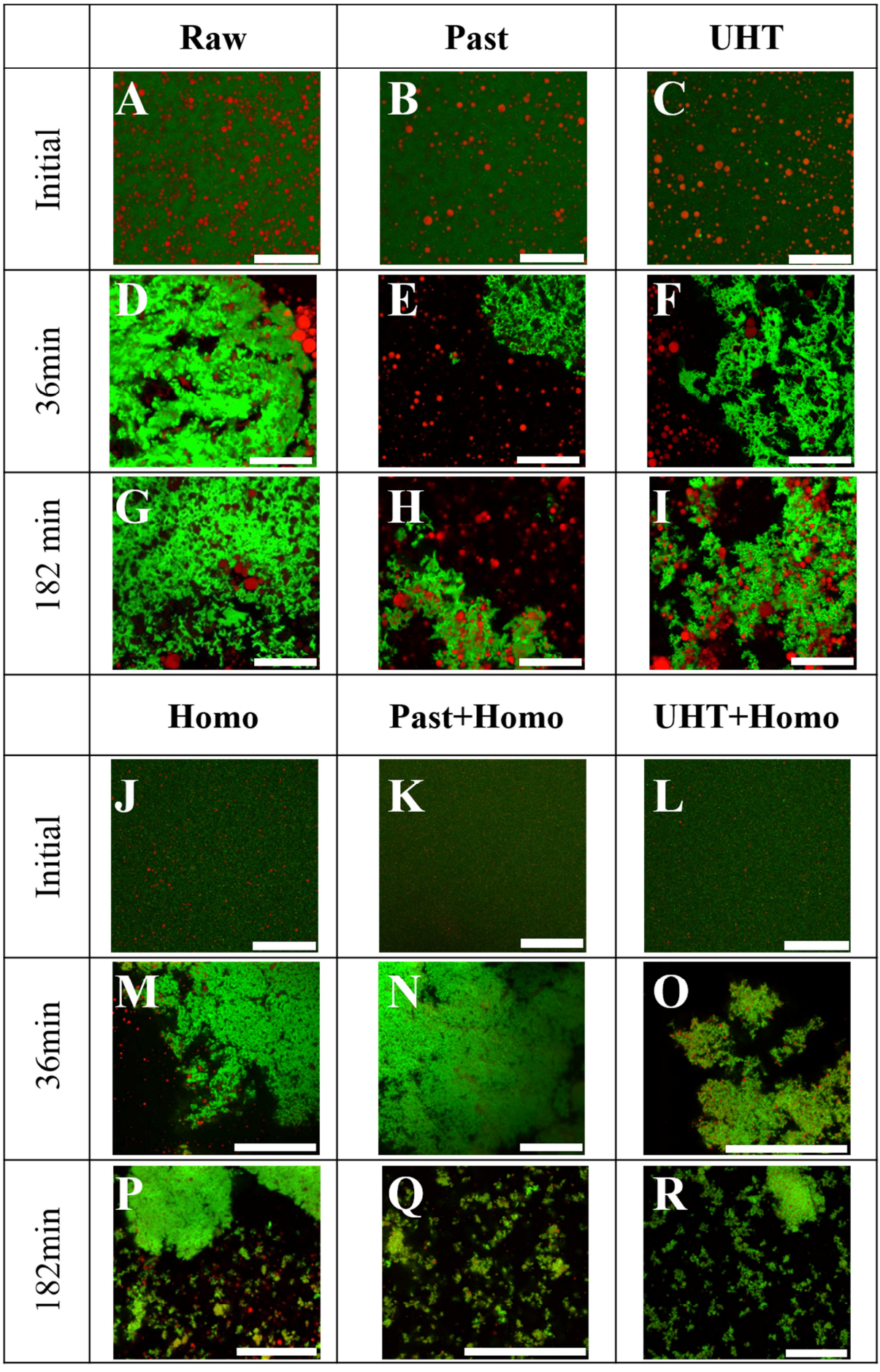
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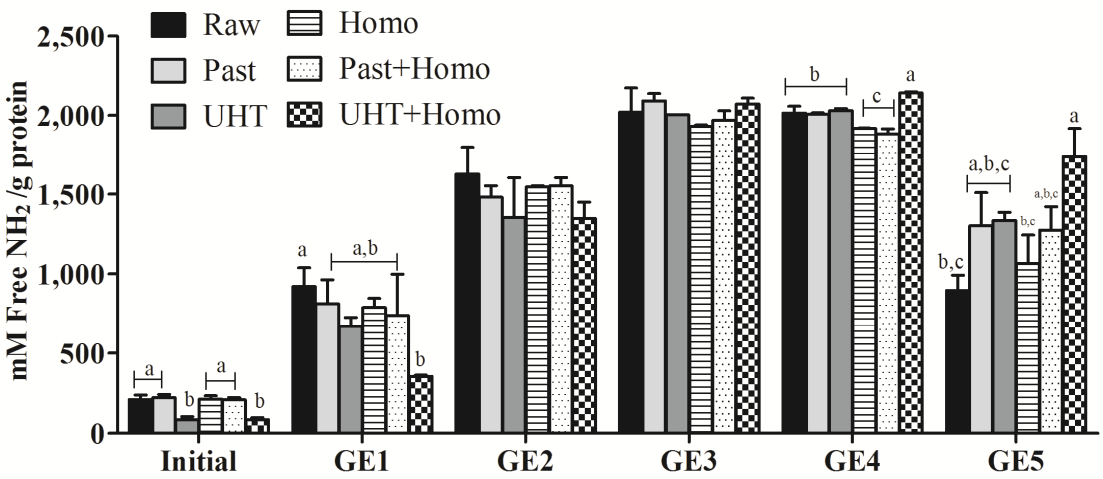

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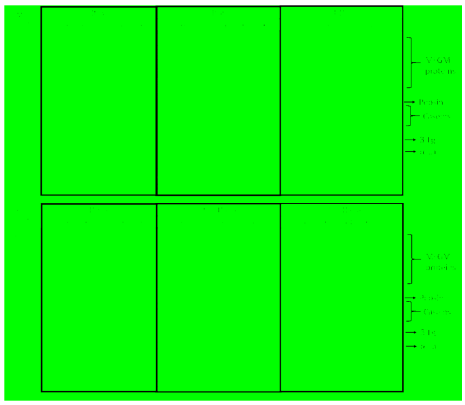

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- Gastric digestion of milk was studied by a semi-dynamic model including emptying
- Milk homogenisation caused phase separation during in vitro gastric digestion
- Coagulation during in vitro gastric digestion was influenced by heat treatment
- The rate of nutrient emptying was affected by the gastric structural changes
- UHT treatment but not pasteurisation accelerated protein digestion in gastric phase

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