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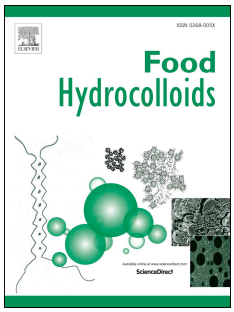

Dairy food structures influence the rates of nutrient digestion through different *in vitro* gastric behaviour

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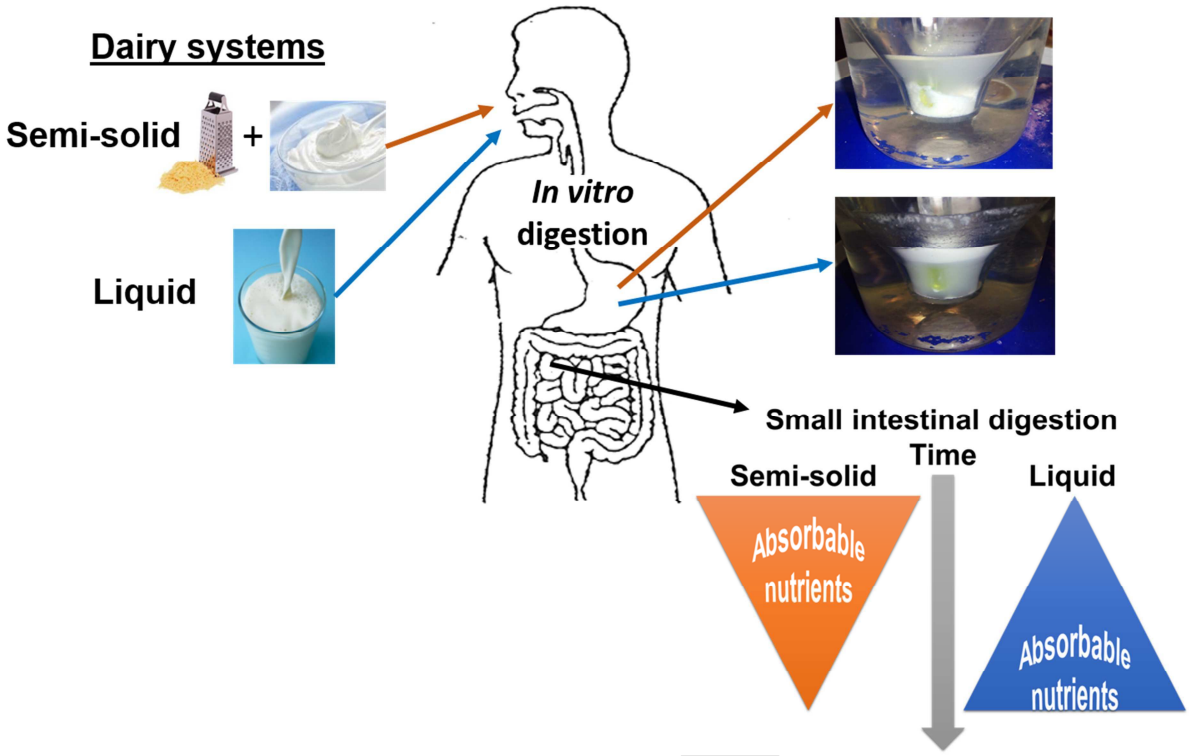

Title: Dairy Food Structures Influence the Rates of Nutrient Digestion through Different *in vitro* Gastric Behaviour

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Keywords

Dairy structure; gastric behaviour; lipid digestion; protein digestion; satiety

Abstract

The purpose of this study was to investigate *in vitro* the extent to which specific food structures alter gastric behaviour and could therefore impact on nutrient delivery and digestion in the small intestine. Results obtained from a specifically developed gastric digestion model, were compared to results from a previous human study on the same foods. The semi-dynamic model could simulate the main gastric dynamics including gradual acidification, lipolysis, proteolysis and emptying. Two dairy-based foods with the same caloric content but different structure were studied. The semi-solid meal comprised a mixture of cheese and yogurt and the liquid meal was an oil in water emulsion stabilised by milk proteins. Our findings showed similar gastric behaviour to that seen previously *in vivo*. Gastric behaviour was affected by the initial structure with creaming and sedimentation observed in the case of liquid and semi-solid samples, respectively. Lipid and protein digestion profiles showed clear differences in the amount of nutrients reaching the simulated small intestine and, consequently, the likely bioaccessibility after digestion. The semi-solid sample generated higher nutrient released into the small intestine at an early stage of digestion whereas nutrient accessibility from liquid sample was delayed due to the formation of a cream layer in the gastric phase. This shows the strong effect of the matrix on gastric behaviour, proteolysis and lipolysis, which explains the differences in physiological responses seen previously with these systems in terms of fullness and satiety.

1. Introduction

The worldwide prevalence of diet-related diseases such as obesity is one of the main food related health concerns. This is projected to lead to health-care cost of about £1.9-2 billion a year in the UK (Wang, McPherson, Marsh, Gortmaker, & Brown, 2011). Several strategies have been developed to address this problem, mainly by reducing the caloric content of the diet focussing on fat and/or sugar (Fiszman & Varela, 2013). However, this strategy does not seem to be working, given the ongoing increase of obesity and this is, at least in part, due to the decrease in palatability of foods. Therefore, approaches looking beyond caloric content have to be investigated. Enhancing satiation and satiety could provide a method to control energy intake (Halford & Harrold, 2012). This could lead to the design of foods inducing feelings of fullness for a longer time.

The satiety cascade is a complex phenomenon involving different pathways (Benelam, 2009). The main factors affecting satiation are gastric distension (Barber & Burks, 1983) and nutrient sensing in the duodenum, which releases gut hormones such as glucagon-like peptide 1 (GLP-1), peptide YY (PYY) and cholecystokinin (CCK), particularly after fat- or protein-rich meals (Feinle, et al., 2002). The release of CCK has important consequences for gastrointestinal (GI) flow including the delay of gastric emptying (GE) (Wren & Bloom, 2007). Rapid emptying leads to a reduction of negative feedback satiety signals and then promotes overconsumption of calories (Delzenne, et al., 2010). Therefore, GE can be modulated by controlling the rate of nutrient digestion. However, the delivery of nutrients in the duodenum is affected by their behaviour in the stomach.

In this context, the structure in which nutrients are presented in food can be designed to exert specific biophysical behaviour in the stomach modulating postprandial physiological

responses to enhance satiation for longer time. This approach has already been highlighted as a potential route to aid weight management (Wilde, 2009) and it comprised the core of this piece of work.

The physical state of food influences the satiety sensation through different physicochemical changes in the GI tract in *in vivo*. For example Marciani, et al. (2012) studied two meals with different consistency, solid/liquid and homogenised soup. They showed that the homogenised meal delayed GE and enhanced satiation compared to the same meal consumed in solid state. This was mainly attributed to the steady release of nutrients into the duodenum of the soup meal which maintained a homogenous appearance throughout gastric digestion. In contrast, using similar food structures but dairy-based systems, Mackie, Rafiee, Malcolm, Salt, and van Aken (2013) found that a semi-solid meal increased the feeling of fullness by a slower rate of GE compared to the same isocaloric meal in a liquid form. However, in this case, different gastric behaviours of sedimentation and creaming were observed for semi-solid and liquid sample, respectively. The authors linked the satiety responses observed to differences in composition of the chyme being emptied from the stomach.

In an *in vitro* study using dairy proteins, casein and whey, susceptibility to hydrolysis by pepsin and trypsin was studied (Guo, Fox, Flynn, & Kindstedt, 1995). They found casein proteins were more susceptible to proteolysis than β-lactoglobulin due to the different structure. The globular structure of β-lactoglobulin hinders the access of proteases to the cleavage sites in contrast to the open structure of casein proteins. However, gastric conditions such as pH and ionic strength can affect the physiochemical properties of proteins. Caseins lose their micellar structure in the stomach at around pH 4.6, their iso-electric point, and precipitate forming aggregates whereas whey proteins remain soluble which has led to differences in digestion. This has been reported to result in more rapid gastric emptying of

whey proteins and a delayed gastric emptying of caseins introducing the concept of 'fast' and 'slow' protein, respectively (Boirie, et al., 1997).

Lipid is another important nutrient playing a key role in satiety. There are several *in vivo* studies looking at the impact of emulsion structure on lipid digestion rate (Keogh, et al., 2011; Marciani, et al., 2009a; Marciani, et al., 2007). They have shown that lipid droplets can be designed to exert specific behaviours in the stomach taking into account different physical processes (i.e. flocculation, coalescence and creaming) that they might undergo under the gastric conditions due to changes in the interfacial properties (Dickinson, 1997). Marciani, et al. (2009a) compared two emulsions with different acid stabilities. They showed that the acid-stable emulsion, homogenous in the stomach, provided a slower and more consistent gastric emptying. In contrast, the acid-unstable emulsion that broke into two phases upon gastric acidification presented a more rapid initial gastric emptying of the aqueous layer followed by the emptying of the upper fat layer in a slower rate.

These studies have highlighted the implications of food structure for gastric emptying and post-prandial responses. However, the underlying mechanisms in terms of nutrient digestion rates are not well understood. Most of these studies have been performed *in vivo,* nevertheless, the influence of food structure on digestion can be studied using *in vitro* systems providing ease of access to samples and minimal variation. Dynamic gastric *in vitro* models such as Human Gastric Simulator (HGS) developed at Riddet Institute or Dynamic Gastric Model (DGM) set up in the Institute of Food Research are sophisticated models that can closely mimic human gastric behaviour but they are not a routine tool due to their complexity. For more information about the dynamic gastric models readers are referred to Verhoeckx, et al. (2015). On the other hand, static *in vitro* digestion has been designed to be

easy to use on a daily basis (Minekus, *et al.*, 2014), although it does not mimic many relevant factors of gastric physiology such as a progressive acidification and emptying, which might significantly affect the bioaccessibility of nutrients. The importance of the pH dynamics in the protein gastric digestion has been highlighted in previous *in vitro* studies where a pH gradient was considered (Shani-Levi, Levi-Tal, & Lesmes, 2013) (Shani-Levi, et al., 2013; van Aken, Bomhof, Zoet, Verbeek, & Oosterveld, 2011). The semi-dynamic gastric model developed for this study is simple to handle and more physiologically relevant than a static model as it simulates the gradual pH decrease, and it has the novelty to include emptying, and the sequential addition of digestives enzymes and gastric fluid.

In this study we assessed the impact of structure on lipid and protein bioaccessibilty from two dairy based systems. In particular we assessed whether the physical state and spatial distribution of nutrients within the simulated stomach could be a critical factor for the rate of digestion in the small intestine. To this end we used two meals that were isocaloric in terms of fat, protein and carbohydrates but with different structure, liquid vs. semi-solid. We investigated the structural changes in the gastric compartment using a semi-dynamic gastric model simulating *in vivo* conditions including gradual acidification, lipolysis, proteolysis and gastric emptying. Digestion was finally assessed by the amount of absorbable (lipid and protein) species available as a function of time. Lastly, we correlated the absorbable nutrients with the responses observed in a human study (Mackie, et al., 2013) where the same dairy systems were used.

2. Material and Methods

2.1. Materials

Gouda cheese (Waitrose Essential Dutch Gouda), yogurt (Waitrose Essential low-fat yogurt), icing sugar (Tate & Lyle Fairtrade cane sugar) and sunflower oil (Tesco) were purchased from a local supermarket. Sodium caseinate was kindly given by VTT (Finland) and whey protein isolate (WPI) was purchased from Davisco Foods International, USA. Pepsin from porcine gastric mucosa, pancreatin from porcine pancreas 8 x USP specifications and dried un-fractionated bovine bile extract were obtained from Sigma-Aldrich, USA. Lyophilized rabbit gastric extract was purchased from Germe S.A., France. Orlistat ≥ 98 % and phenylmethylsulfonyl fluoride (PMSF) approx. 0.1 M in EtOH were purchased from Sigma-Aldrich. D-leucine (puriss ≥ 99.0 %) was obtained from Fluka analytical, USA. The standards glyceryl triheptadecanoate and heptadecanoic acid were purchased from Sigma-Aldrich, dipentadecanoin and monononadecanoin were from Nu-Check Prep, In. USA. HCl (approx. 37 %, analytical reagent grade) and the solvents hexane, chloroform, acetic acid, methanol, ethyl acetate and toluene were purchased from Fisher Scientific UK. All other chemicals used were of analytical grade and were obtained from Sigma-Aldrich unless specified.

2.2. Preparation of samples

The protocol followed for the preparation of the samples was as described previously by (Mackie, et al., 2013). The liquid sample was an oil in water emulsion. A sodium caseinate solution containing 1.33 g sodium caseinate was dissolved in 110.5 g boiled tap water, the solution was stirred overnight at room temperature. 6.88 g of sunflower oil was mixed with 60.63 g of that sodium caseinate solution in a blender (BL450 series, Kenwood). The shear

cycle comprised 30 s at the low shear setting, 30 s of rest, 30 s at the high shear setting, 30 s of rest and 30 s at high shear setting. Then, the emulsion was mixed with the remaining sodium caseinate solution and 5 g whey protein isolate was added a little at a time. Finally, 1.53 g of icing sugar was also added.

The semi-solid sample was prepared by mixing 23.17 g of finely grated Gouda cheese and 19.41 g yogurt. The sample also comprised 82.66 g water which was added at the start of the gastric digestion to mimic the protocol of the *in vivo* study.

It is important to note that the samples were isocaloric in terms of protein, fat and carbohydrate content, and so the food structure was the main factor influencing the outcome.

2.3. Semi-dynamic *in vitro* gastric digestion

A 20 g freshly prepared sample was placed into a 70 mL glass v-form vessel thermostated at 37 °C after the addition of 3.6 mL of gastric solution simulating the gastric fluid residue in the stomach (fasted state). The gastric solution contained 84.2 % simulated gastric fluid (prepared according the protocol described in Minekus, et al. (2014)) at pH 7, 10 % MilliQ® water, 5.8 % 2 M HCl and 0.0005 % 0.3 M $CaCl_2(H_2O)_2$. Three solutions were added at a constant rate: (1) 15.4 mL of gastric solution was added using a pH-stat (836 Titrando-Metrohm, Switzerland) dosing device at 0.09 mL/min, (2) rabbit gastric extract (13.8 mg in 0.5mL MilliQ® water) containing gastric lipase (58 U/mg solid, using tributyrin as substrate) and pepsin (1,113 U/mg solid, using haemoglobin as substrate) at 0.003 mL/min and (3) pepsin (37.1 mg in 0.5 mL MilliQ® water) from porcine gastric mucosa (3,200 U/mg solid, using haemoglobin as substrate) at 0.003 mL/min was also added because the addition of pepsin from rabbit gastric extract did not fulfil the protease activity required in the stomach

which was 2,000 U/mL final digestion mixture Minekus, et al. (2014). Enzyme solutions were added using a syringe pump (Harvard apparatus, PHD Ultra, USA). A 3D action shaker (Mini-gyro rocker-SSM3-Stuart, Barloworld Scientific limited, UK) at 35 rpm was used for agitation.

The proportions of solutions used were according to the standardized static digestion protocol Infogest Minekus, et al. (2014). The oral phase was not simulated because when extrapolating the *in vivo* data (Mackie, et al., 2013) of gastric volume to this study we did not observe any significant initial dilution apart from the volume of food and residual gastric fluid.

2.4. Gastric emptying simulation

Gastric emptying (GE) was simulated by taking 9 different volumes, referred to as GE points in the text, according to a pre-set curve based on *in vivo* study data using the same dairy systems (Mackie, et al., 2013). Figure *1* shows the volume contained in the gastric vessel at each time point and, the volumes and corresponding times of each GE point are indicated in Table *1*. Samples were taken from the bottom of the vessel using a pipette with a tip internal diameter of 2 mm because it approximates the upper limit of particle size that has been seen to pass through the pyloric opening into the duodenum (Thomas, 2006). It is important to note that another extra volume of the liquid sample was also collected and analysed (referred as GE10). This was the remaining volume of the gastric digestion which mainly contained the fat layer formed as shown below in the results section.

Sufficient 5 M NaOH was added to the samples to increase the pH above 7, inhibiting pepsin activity. Then, samples were snap-frozen with liquid nitrogen and stored at -80 °C until subsequent treatment.

2.5. Small intestinal *in vitro* digestion

Small intestinal digestion was simulated for each GE sample according to a standardised protocol (Minekus, et al., 2014). The pancreatin (trypsin activity 7.18 U/mg and lipase activity 26.5 U/mg) was prepared with 3 x concentrated simulated intestinal fluid in order to keep the system as constant as possible to pH 7 during digestion. The amounts of pancreatin solution, bovine bile (190 mM with water), 0.3 M $CaCl_2(H_2O)_2$ and MilliQ® water were adjusted in each case depending on the gastric sample volume to reach the pancreatin trypsin activity required (100 TAME units per mL of intestinal phase content (Minekus, et al., 2014)). The digestion was performed for 60 min in a shaking incubator (Excella E24, New Brunswick Scientific, USA) at 37 °C, 190 rpm. Centrifuge tubes were placed horizontally in the shaker for better mixing. Samples (0.5 mL) were taken at 0, 1, 30 and 60 min (as shown in Table *1*) and 10 µl of inhibitor mix (1:1 0.1 M PMSF: 10 mM Orlistat in Ethanol) was added. The samples were snap-frozen using liquid nitrogen and stored at -80 °C until further analysis.

2.6. Pre-treatment of digested samples

The samples were treated before the protein hydrolysis analysis. This involved the addition of 5 % trichloroacetic acid (TCA) (0.83 mL) to 0.5 mL of digested sample to cause the precipitation of insoluble protein. The use of TCA in protein hydrolysed samples prior to quantitative analysis has been widely used previously (Flanagan & FitzGerald, 2003; Wu, Chen, & Shiau, 2003). Samples were centrifuged at 10,000 g for 30 min at room temperature

and the supernatant was filtered using syringe filter, 4 mm, 0.45 µm PVDF membrane (GE Healthcare Life Science, UK).

2.7. Protein hydrolysis analysis by o-phthaldialdehyde spectrophotometric assay

The extent of protein hydrolysis was 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 which was finally made up to 100 mL with HPLC grade water.

Different concentrations of standard D-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 at room temperature for 15 min, then the absorbance was measured at 340 nm using a microplate spectrophotometer (Benchmark Plus, BioRad, UK).

2.8. Lipid analysis

2.8.1. Total lipid extraction

Lipid extraction of samples was carried out using the protocol of Bligh and Dyer (1959). The internal standard (IS) method was used, which consisted of 1.6 mg/mL of each lipid standard, i.e. glyceryl triheptadecanoate, heptadecanoic acid, glyceride dipentadecanoin and glyceride

mononononadecanoin, in chloroform. For each 0.5 mL of sample, 0.625 mL IS solution and 1.25 mL methanol was added. Then, 0.625 mL chloroform and 0.625 mL water with 0.9 % NaCl were included obtaining two phases. Thereafter, samples were centrifuged at 3,000 g for 10 min. The lower organic part was taken for lipid extraction.

2.8.2. Extraction of different lipid classes

Fractionation of lipid samples was performed using solid phase extraction allowing the isolation of individual lipid classes: polar lipids namely free fatty acids (FFA) and neutral lipids, namely, triglycerides (TG), diglycerides (DG) and monoglycerides (MG). This was performed by using disposable primary aminopropyl bonded phase cartridges (Varian Bond elute amino propyl 500 mg 10 mL reservoir, Agilent Technologies, US) placed in a sample processing manifold (VacMaster, Biotage, UK). Extraction of lipids from samples after GI digestion was performed using a protocol adapted from Kaluzny, Duncan, Merritt, and Epps (1985).

The cartridge column was equilibrated by rinsing with 4 mL of hexane and allowing it to flow through the cartridge under gravity.

The volume collected in the lipid extraction step was loaded onto the cartridge. Thereafter the column was eluted with chloroform, 4 mL (fraction I, TG and DG) followed by 5 mL of acetone (fraction II, MG) which were eluted under gravity. Methanol (5mL) eluted phospholipids in fraction III and 5 mL of chloroform/methanol/acetic acid (100:2:2 v/v) eluted FFA (fraction IV). Next, the tubes containing fractions I and II were evaporated to dryness in a vortex evaporator (Haakebuchler, Büchi Labortechnik AG, Switzerland) applying vacuum at 40 °C and speed level 4 followed by drying in a vacuum oven

(Gallenkamp, England) connected to a high vacuum pump (Edwards E2M2) for 30 min at room temperature.

A second cartridge was equilibrated in the same manner as above. The fraction I was reconstituted in 0.5 mL of hexane and loaded onto the cartridge. A further 3.5 mL of hexane was applied to the column under gravity (fraction V, TG). Then, a fraction (4 mL) of hexane:ethyl acetate (85:15 v/v) was eluted under gravity (fraction VI, Cholesterol and other sterols). Next, 4 mL of hexane:ethyl acetate (80:20 v/v) was eluted under gravity (fraction VII, DG). Finally, 4 mL of chloroform:methanol (2:1 v/v) was eluted under gravity collecting the total MG in the fraction II tube. The solvent of fractions IV, V and VII were evaporated as previously described.

2.8.3. Derivatization of lipid extraction fractions

Lipids were converted to fatty acid methyl ester (FAME) through methylation to allow subsequent analysis by gas chromatography (GC).

0.5 mL of toluene (containing 0.02 % butylated hydroxytoluene as an antioxidant) and 1 mL of methylation reagent consisted of methanol containing 2 % H_2SO_4 (v/v) was added to the samples. After mixing, tubes were placed in an oven at 50 °C overnight. Thereafter, tubes were removed from the oven to allow them to cool and 1 mL of neutralising solution (12.5 g $KHCO_3$ and 34.55 g K_2HCO_3 dissolved in 500 mL HPLC grade water) was added. Hexane (1 mL) was added and following vigorous mixing samples were centrifuged at 100 g for 5 min. The supernatant (organic phase) was transferred to a vial for analysing by GC.

2.8.4. Analysis of FAMES

Methylated samples were analysed using 7890B GC System (Agilent Technologies, USA), equipped with a model 7694 autosampler, and dual flame ionisation and 5977A mass spectrometry detector (Agilent Technologies, USA) connected by a 1:1 active splitter after the analytical column. The analytical column was a SGE BPX70 capillary column (30 m x 0.25 mm ID x 0.25 µm film thickness) operated in constant flow mode at 30cm sec^{-1} using helium as carrier gas. Samples (1 µL) were injected with the injector in split mode (10:1 split ratio). The oven temperature program consisted of a hold programmed at 115 °C for 1 min, followed by a ramp at 1.5 °C min^{-1} to 240 °C and, thereafter, a ramp at 30 °C min^{-1} to 250 °C with a 10 min hold prior to cooling ready for the next sample.

FAME mix (Supelco 37 Food FAMES) was used to confirm the retention times of FAMES and calculate the relative response factor for the flame ionisation detector which was used to quantify the separated lipid classes. The ion source was held with the electron multiplier voltage at 70 V and scans from 50 to 550 Da were run.

2.9. Confocal laser scanning microscopy (CLSM)

The digested samples were diluted (1/2 in MilliQ® water). Then, 80 µL of sample was mixed with 10 µL 0.1 % (v/v) nile red solution and 10 µL 0.1 % (v/v) fluorescein isothiocyanate. The samples were visualised using CLSM (SP1 CLSM, Leica Microsystems, Mannheim, Germany). Nile red and fluorescein isothiocyanate were used to detect the lipid and protein, respectively. Images were captured using both 40× (N.A. 1.25) oil immersion objective lens. The samples were excited using an argon laser at 488 nm for Nile red and at 633 nm for fluorescein isothiocyanate.

330

331 2.10. Statistics
332

333 All the results are presented as mean ± standard deviation (SD) of three replicates. Statistical
334 significance between the meals was tested by a two-tailed paired *t*-test using GraphPad Prism
335 software (Prism 5 for Windows, Version 5.04). Differences were stated significant at p-value
336 < 0.05.

337

338 **3. Results**
339

340 3.1. Gastric pH profile
341

342 The change in pH during gastric digestion of both samples is illustrated in Figure *2*. They
343 presented similar profiles, with an initial low pH about 1.0 simulating the residual acid in the
344 stomach related to fasting conditions. After meal addition, the pH increased rapidly reaching
345 values of 4.55 ± 0.08 and 5.37 ± 0.25 for semi-solid and liquid samples, respectively. This
346 increase was different between samples due to differences in their buffering capacity even
347 though they had the same protein content. The homogenous distribution of the protein in the
348 liquid sample compared to the semi-solid sample caused the higher pH observed. The pH
349 then decreased in both samples reaching a value below 2.0 due to the constant addition of
350 gastric fluid containing acid. This profile was similar for both samples due to the gradual
351 gastric emptying, hence the pH was modified by the removal of both acid and buffering
352 capacity of food from the gastric compartment.

353

354 3.2. Sample behaviour in the gastric compartment
355

356 Figure *3* shows the appearance of the samples both initially and after 110 minutes of
357 simulated gastric digestion. The semi-solid sample was initially a paste (Figure *3*A) that
358 sedimented to the bottom part of the vessel. The particles formed during digestion remained
359 in the lower part as seen in Figure *3*B. Free oil droplets could be seen floating on the top of
360 the gastric content at the end of digestion. In contrast, the liquid sample was initially a
361 homogenous milky liquid (Figure *3*C). Although some precipitation was observed even in the
362 very early stage of digestion lasting for about 70 min, the solid particles tended to cream to
363 the top and form a boundary layer. An upper cream layer could be clearly seen after
364 approximately 110 min of gastric digestion (Figure *3*D). This appearance remained
365 throughout the latter stages of digestion.

366

367 3.3. Protein hydrolysis analysis
368

369 The extent of protein hydrolysis of both samples at each GE point is shown in Figure *4* and
370 the data is given in Table 1 and 2 of the supplementary material. The samples were analysed
371 during small intestinal digestion at 0 (corresponding to the end of gastric digestion), 1, 30 and
372 60 min. The given values were based on the amount of hydrolysates for 20 g of digested
373 food. The hydrolysis obtained in both meals GE1-9/0 ranged from 4.2 ± 3.4 to 36.9 ± 2.2 mM
374 and from 12.5 ± 3.8 to 32.5 ± 10.2 mM for liquid and semi-solid samples, respectively. This
375 was substantially lower than the subsequent time samples produced by small intestinal
376 digestion, GE1-9/1, GE1-9/30 and GE1-9/60, demonstrating the rapid action of small
377 intestinal proteases. The samples showed different proteolysis behaviour during small

intestinal digestion. The semi-solid sample exhibited a U-shape profile indicating a higher rate of proteolysis in the GE1 and GE9 points and lower levels at intermediate time points. The highest level of proteolysis was achieved in the GE1/60 point, delivering 250.4 ± 35.9 mM of free amine groups. The increase in proteolysis in the last points might be due to the release of protein associated with particles that were only emptied later on. The liquid sample, in contrast, had lower levels of proteolysis in the early GE points which were more constant throughout compared to semi-solid sample. The highest amount of proteolysis was found in the GE10/60 point resulting in 246.7 ± 7.2 mM of free amine groups.

3.4. Lipid analysis

Figure *5* shows the levels (% in w/w) of TG and lipolytic products (FFA, MG and DG) in relation to the total lipid in each sample emptied at the different GE points. Samples were quantified during the small intestinal digestion at 1, 30 and 60 min for each GE point. In general, both samples followed the logical trends of lipolysis during intestinal digestion showing a decrease of TG, an increase of FFA and MG, and about constant levels of the intermediate product DG. However, the rate of lipolysis was different between the samples. The semi-solid sample presented the highest levels of TG in GE1/1, GE2/1 and GE3/1 points, accounting for 58.2 ± 11.7, 59.1 ± 6.2 and 60.3 ± 4.9 %, respectively. By contrast, the liquid sample presented 56.9 ± 8.6 % in the GE1/1 and the highest amount of TG (75.2 ± 16.3 %) was found in the GE10 point corresponding to the residual top cream layer. With regards to FFA, the highest amounts were seen in the semi-solid samples GE4/60, GE5/60 and GE6/60 which contained about 75 %, in contrast to the liquid sample, where the highest levels were found in GE7/60 and GE8/60 points which contained 72.1 ± 12.9 and 71.6 ± 19.6 %,

respectively. The GE10 showed the lowest levels of FFA in the liquid sample representing the 33.1 ± 6.0 %.

In addition, we analysed the individual FFA classes in each GE point for each time of small intestinal digestion (supplementary data Figure 1-3). The data showed a different FFA profile between samples. The semi-solid sample showed a greater variety of FFA types although the most abundant FFAs, i.e. 18:1, 18:0 and 16:0, were present in both samples. No particular trend in their rates of digestion was found.

4. Discussion

4.1. Simulation of human gastric behaviour

The model of gastric digestion used here could closely simulate the structural changes already seen *in vivo* (Mackie, et al., 2013) with the same two meals. This was a result of the inclusion of relevant dynamic aspects of gastric physiology, i.e. gradual acidification, emptying and enzyme secretion.

The pH profile obtained with the samples (Figure *2*) was similar to that seen previously in other *in vivo* studies (Malagelada, Longstreth, Summerskill, & Go, 1976) although some differences can be found depending on the type of the meal digested. Unfortunately, the pH profile for the food matrices studied was not measured *in vivo*. The effect of pH on gastric digestion is important to consider because it affects the protein structure and interactions with other matrix components as well as enzyme activity (Dekkers, Kolodziejczyk, Acquistapace, Engmann, & Wooster, 2016). As a result, gastric pH has important consequences for the rest of digestion and subsequent nutrient bioavailability.

GE plays an important role in the pH profile because it lowers the overall buffering capacity of the gastric contents through the progressive emptying of food and acid contained in the gastric chyme. The importance of GE on pH was observed in some additional experiments using the same samples. The pH of the semi-solid sample was lower than the liquid meal for longer when GE was excluded because of the lower buffering capacity of the semi-solid sample caused by the lower exposure of the protein (see supplementary data Figure 4). However, introducing GE significantly reduced the difference, as seen in Figure *2*. The GE displayed in Figure *1* was obtained by downscaling the clinical data on gastric volume reported by (Mackie, et al., 2013) in which the liquid sample emptied more quickly than the semi-solid sample (the emptying rate of the liquid meal was double that of the semi-solid meal after 25 min of digestion). This differs from other studies (Marciani, et al., 2012; Santangelo, Peracchi, Conte, Fraquelli, & Porrini, 1998) in which a combination of solid and liquid food emptied faster than the same meal homogenised into a liquid form. It is important to note that in these studies the liquid meal stayed homogenous throughout gastric digestion in contrast to the phase separation that occurred in the (Mackie, et al., 2013) study. This highlights the importance of gastric behaviour in controlling the emptying rate. Others studies (Marciani, et al., 2009b; Marciani, et al., 2007) reporting phase separation of emulsions in the stomach showed a faster emptying rate compared to a homogenous system.

4.2. Influence of gastric digestion conditions on food structure

Different gastric behaviour was observed, namely sedimentation and creaming in the semi-solid and liquid samples, respectively (Figure *3*). The liquid sample was an emulsion stabilised by milk proteins. Some precipitation was observed in the early stages of gastric digestion (about pH 5), which remained for about 70 min. This isoelectric precipitation of the

emulsion occurred as a result of the pH approaching the iso-electric point of the casein (pH 4.6) at which point the net charge at the surface becomes zero. This change of charge on the protein led to the loss of electrostatic repulsion and consequently stability as has been shown previously (Day, et al., 2014; Dickinson, 1997). Other aspects of the gastric environment including ionic strength and proteolysis could also have affected the stability of lipid droplets (Helbig, et al., 2012). The salts contained in the simulated gastric fluid could induce flocculation by screening the repulsive forces. In addition, the protective layer of protein absorbed at the interface might be compromised by the proteolytic action of pepsin resulting in the reduction of steric stability. Furthermore, the products of lipolysis, i.e. FFA, MG and DG, are surface active and could displace the protein from the emulsion interface leading to further destabilization. Indeed, these compounds at GE1/1 point accounted for 41.84 and 43.1% of the total lipid in the semi-solid and liquid samples, respectively. All these factors could potentially contribute to the destabilisation of the emulsion causing flocculation and some coalescence of lipid droplets which progressively creamed to the top part during digestion due to their lower density. This process, ultimately, led to phase separation after 110 min of gastric digestion (Figure 3D). Figure 3F confirms the presence of fat droplets in the top layer leaving an aqueous part in the bottom (Figure 3G) and the extent of flocculation and coalescence in that cream layer compared to the stabilised droplets presented in the initial sample (Figure 3E). Phase separation behaviour showing the formation of a cream layer at the top of the stomach has also been shown in *in vivo* (Mackie, et al., 2013; Marciani, et al., 2009b) as a result of destabilisation in gastric conditions.

Conversely, in the semi-solid sample, the density of the cheese-yogurt matrix resulted in the sedimentation of particles to the bottom of the simulated stomach model leaving the top part a more aqueous system. This behaviour was consistent throughout the digestion. Fat from the cheese and yoghurt was trapped in the food matrix that generated the sediment. However, the

combination of gastric conditions including low pH and proteolysis led to the release of some oil droplets seen floating at the top at the end of digestion, although phase separation overall was very limited.

Similar structural behaviour of both samples was seen in the magnetic resonance images of the comparative *in vivo* study using the same dairy systems (Mackie, et al., 2013). The phase separation of the liquid sample was clearly obtained in an earlier stage in the *in vivo* study (after 25min). This might be due to the complex peristaltic movements that were not well simulated in the gastric *in vitro* model used, where the shear rates may have been higher than *in vivo* with regards to the gastric fundus.

4.3. Influence of gastric behaviour on small intestinal protein digestion

Different protein digestion rates were observed between the samples (Figure *4*). In the semi-solid sample there was a higher level of proteolysis in the GE1 and GE2 samples compared to the liquid sample. This might be related to the early emptying of high density particles containing a greater amount of protein which was subsequently digested throughout the small intestinal phase. In addition, the semi-solid sample showed high levels of proteolysis in the GE7, GE8 and GE9 samples which might be due to the emptying of soluble protein released gradually from the matrix. In contrast, the liquid sample showed a more consistent extent of hydrolysis at all GE points because the proteins were more homogeneously distributed within the sample. The highest level of proteolysis in the liquid sample was obtained in the last volume collected, which might again be attributed to the protein associated with the lipid that creamed to the top. However, these results differ from those of van Aken, et al. (2011) in which the protein distribution in the bottom layer was higher than in the cream layer obtained after the gastric digestion of emulsions stabilised by milk proteins. These differences are

likely to be due to the gradual emptying that we carried out throughout the gastric digestion, which was not included in the previous study.

In the present study there was rapid protein hydrolysis after 1 min of small intestinal digestion. This finding is in agreement with the study of Macierzanka et al. (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009), which showed, using β-lactoglobulin and β-casein-stabilized emulsions, that proteins were partially hydrolysed, in particular β-casein, after 1 min into low molecular weight peptides under intestinal conditions. The distinction between the different milk proteins was not assessed because of differences in the nature of the two starting materials. The two samples contained the same amount of protein, although the dairy products used here (yogurt and cheese) usually contain less whey proteins due to the processing, which makes comparison problematic.

Protein digestion has been less well studied than lipid digestion in relation to the impact on colloidal behaviour under GI conditions. However, the understanding of protein digestion and how protein is emptied from the stomach is relevant to study the nutritional impact of foods related to satiety responses (Mackie & Macierzanka, 2010).

4.4. Influence of gastric behaviour on small intestinal lipid digestion

The rate of lipid hydrolysis was controlled by the nutrient composition of the volume emptied into the small intestine which varied because of the different colloidal behaviour within the stomach model. In the case of the semi-solid sample, the lipid availability was much higher in the early stages of digestion as a consequence of the high nutrient content of the sedimented particles. A substantial part of the initial TG was emptied early on i.e. the GE1/1, GE2/1 and GE3/1 time points compared to the rest (Figure *5*).

In contrast, the creaming of the lipid in the liquid sample led to less lipid being emptied at an early stage of digestion. The lipid delivery was quite steady at all the GE points but was substantially higher in the last residual volume analysed (GE10) that consisted almost entirely of the cream layer. This resulted in a delay of lipid delivery into the small intestine. The coalescence and phase separation observed in the liquid sample led to a reduction of the interfacial area available for lipolysis as seen in the limited decrease of TG in GE10 (Figure *5*). The TG percentage in GE10/30 and GE10/60 was 40 and 35% respectively compared to 75% of TG found in GE10/1. This could also be attributed to the saturation of substrate compared to the availability of the enzyme. Similarly, van Aken, et al. (2011) reported a higher fat distribution in the top layer when creaming was observed after the gastric digestion of triolein emulsions stabilised by milk proteins. They also observed that the FFA concentration in the bottom layer was much lower than in the cream layer, probably because FFA were protonated in the low gastric pH therefore they were oil-soluble and remained in the cream layer. In the present study there was also a higher absolute amount of FFA present in the cream layer compared to the lower aqueous layer, even though the relative values in Figure *5* do not reflect it. The levels of FFA in GE1/0 accounted for 17 mg whereas the point GE10/0 contained 54.6 mg. The creaming process led to the concentration of the fat droplets on the top promoting coalescence and decreasing the rate of lipolysis. Another study looking at the lipid digestion of protein stabilised emulsions using a dynamic GI system (Helbig, et al., 2012) also showed the delay of lipid delivery into the small intestine due to creaming of lipid in the stomach. They showed a higher amount of lipid compounds, especially FFA and TG, in the cream layer compared to the bottom part. The authors pointed out that even though different gastric behaviour of the samples was observed (homogeneous vs. creaming), the total amount of FFA released at the end of digestion remained similar, in line with our study.

Lipid digestion occurs mainly in the intestine but we considered the addition of gastric lipase relevant because there is evidence suggesting that it accounts for the 5-40% of total TG lipolysis (Armand, et al., 1997). The gastric lipase used in the present study was from a rabbit gastric extract. This has been reported to be similar to human gastric lipase (HGL) having similar specificity for Sn3 position and optimum pH ranged between 3 and 6 (Carriere, et al., 1991). Moreover, the lipolytic products may facilitate subsequent pancreatic lipolysis (Armand, 2007). The digestion of lipid by the action of pancreatic lipase accounts typically for 30-75%. The levels of lipolysis found in this study were in line with these ranges. The gastric lipase generated significant hydrolysis, accounting for 22% and 33 % in liquid and semi-solid samples, respectively. These values were calculated based on the sum of the total FFA in relation to sum of the total lipid obtained on a weight basis. The extent of lipolysis obtained after an additional 60 minutes in the simulated small intestine was determined and the liquid sample showed 63% whereas the semi-solid sample reached 82%. These values were calculated taking into account the sum of the total FFA and MG in relation to the sum of the total lipid obtained on a weight basis. It can be observed that semi-solid sample showed higher lipolysis than liquid sample along GI tract. This could be attributed to the presence of larger surface area of the semi-solid particles whereas the reduced area available in the phase separated and coalesced liquid sample decreased the available surface area for lipase action.

It is important to state that the sampling in this study was quite complex due to the heterogeneity of the matrixes. This could lead to some variability of the total initial and final lipid content and therefore the underestimation of lipid values.

4.5. Possible link to physiological responses

Since satiety related physiological responses such as CCK release and gastric emptying are linked to the rate and extent of lipid and protein sensing by intestinal endocrine cells, we can expect different satiety responses between the two samples. Lipid and, in particularly, protein have been seen to be the most satiating macronutrients (Fiszman, et al., 2013). To provide a better understanding of the physiological trends in our study, the previous data for protein and lipid was replotted in a form representing the absorbable nutrient as a function of linear time. We assumed the protein hydrolysates quantified were absorbable since the protein digestion by intestinal proteases have been seen to be efficient to further protein breakdown into amino acids and small peptides (2-3 amino acids) which are absorbable. The absorbable lipid referred to the FFA and MG fractions that can be absorbed by enterocytes (Armand, 2007). Figure *6*A shows a similar absorbable protein profile for both samples. The semi-solid sample presented statistically higher levels of absorbable protein (p = 0.0341, paired, two-sided t-test) in the first time point (i.e. 10 min). The samples were also statistically different (p = 0.0356, paired, two-sided t-test) in the last time point (i.e. > 170 min) with the liquid sample having a higher concentration of absorbable protein. On the other hand, the samples differed statistically in all the time points with regards to absorbable lipid (i.e. FFA+MG), which is illustrated in Figure *6*B. The semi-solid sample presented higher levels of absorbable lipid than the liquid sample in all the time points except in the last (i.e. > 170 min). These patterns can be linked with the different gastric behaviour of the samples.

Sedimentation of the semi-solid sample led to the early detection of higher concentrations of both protein and lipid seen in Figure *6*A and B in the first time points. The early delivery of a higher amount of nutrients to the small intestine might trigger an increase of negative hormonal feedback by slowing GE, which could promote the feeling of fullness. It could also result in increasing the period of time that food remained in the stomach leading to a greater gastric distension and enhancing sensations of fullness (Delzenne, et al., 2010). Conversely,

599 the effect of creaming observed in the liquid sample caused a delay of the nutrient release in
600 the small intestine, seen in the last time point (i.e. > 170 min) of Figure *6*A and B. Since the
601 amount of nutrient delivered during digestion was lower, especially in the case of lipid, we
602 can assume that this would cause the release of low levels of CCK. Conversely, Mackie, et al.
603 (2013) found the CCK levels of the liquid emulsion were higher than those in semi-solid
604 sample for the first 40 min. The authors suggested that the lower viscosity of liquid sample
605 induced the rapid emptying and delay of CCK regulation. Nevertheless, Marciani, et al.
606 (2009b) showed a decrease of fullness and less CCK released from an emulsion that layered
607 in the stomach compared to another emulsion which remained homogenous (Marciani, et al.,
608 2007). The faster GE rate of the liquid sample observed in the parallel clinical study can now
609 be explained with the lower nutrient concentration in the aqueous layer that emptied first
610 from the stomach.

611 Mackie, et al. (2013) also showed differences in fullness and hunger between the samples.
612 The semi-solid sample induced substantially more fullness than the liquid sample after just 15
613 min of digestion. This could potentially be due to the higher levels of protein and lipid
614 released in the small intestine after the first 10 min from the semi-solid sample compared to
615 liquid sample as shown. The *in vivo* study also found that these differences in fullness were
616 prolonged after 2 hours suggesting that the impact of the high caloric chyme initially emptied
617 was not only on satiation but satiety could also be affected. However, we could not correlate
618 the high levels of nutrients in the last point of digestion from liquid sample with the satiety
619 responses seen in *in vivo* (Mackie, et al., 2013) because the clinical measurements were not
620 taken for long enough to detect any distinct peak related to this high caloric-content fraction.
621 In accordance with the present study, Golding, et al. (2011) showed a delay in blood TG
622 presenting a distinct peak after 180 min of ingestion when using sodium stearyl lactylate-
623 stabilised emulsion which phase separated in gastric conditions.

5. Conclusions

This work shows the successful development of a simple semi-dynamic model based on available physiological data (Mackie, et al., 2013) to mimic human gastric digestion. The experiments showed that the gastric digestion of the two dairy meals was affected by their macrostructure. The different behaviour of samples, creaming vs. sedimentation, determined the composition of chyme delivery into the small intestinal phase. In the liquid system, the change of interfacial composition during gastric digestion was the main driver for destabilisation of lipid droplets and formation of cream layer which led to the delay in nutrient release. In contrast, the sedimented particles of the semi-solid samples in the gastric phase caused the early emptying of high nutrient concentrations. The results showed differences in protein and lipid digestion between the two meals. The patterns of digestion observed *in vitro* provides a plausible explanation for the satiety responses seen in *in vivo* showing a decrease in appetite for the more structured meal.

This work contributes to the understanding of how to control nutrient digestion and uptake, which may help to develop functional foods with particular physiological properties.

Abbreviations

GI, gastrointestinal; GE, gastric emptying; CCK, cholecystokinin; GPL-1, glucagon-like peptide 1; GIP, gastric inhibitory polypeptide; PYY, peptide YY; TG, triglycerides; DG, diglycerides; MG, monoglycerides; FFA, free fatty acids; TCA, trichloroacetic acid; OPA, o-phthaldialdehyde; FAME, fatty acid methyl ester; GC, gas chromatography.

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767

Table 1. Time (min) and target volume (mL) corresponded in each gastric emptying point.

Gastric Emptying Point	Semi-solid Sample		Liquid Sample	
	Time (min)	Emptied Volume (mL)	Time (min)	Emptied Volume (mL)
GE1	7.1	1.1	5.9	2.4
GE2	29.7	6.9	29.0	5.7
GE3	50.1	4.0	50.0	6.8
GE4	70.0	3.7	69.9	3.8
GE5	89.4	3.8	89.5	4.0
GE6	111.1	3.5	110.3	3.9
GE7	132.4	3.8	131.9	3.7
GE8	152.0	3.4	150.8	3.1
GE9	171.8	3.0	171.4	3.0
GE10			residual gastric content	

778 **Figure captions**

779

780 Figure 1. Volume (mL) contained in the stomach model as a function of time (min) of the
781 semi-solid (solid line) and liquid (broken line) samples. The data was obtained by
782 downscaling the *in vivo* data of the referred study (Mackie, *et al.*, 2013). Each gastric
783 emptying (GE) point is indicated in the graph. The table (right hand side) presents the sample
784 names and their corresponding GE points in each time point.

785

786 Figure 2. pH profile during gastric digestion of the semi-solid (solid line) and liquid (broken
787 line) samples using the semi-dynamic gastric model. Errors bars represent the SD values
788 (n=3).

789

790 Figure 3. Images of semi-solid (A-B) and liquid (C-D) samples in the initial state (A and C)
791 and after 111.1 min (B) and after 110.3 min (D) of gastric digestion using the semi-dynamic
792 gastric model. Representation of microstructure in the liquid sample before gastric digestion
793 (E) and, the upper cream layer (F) and the bottom aqueous layer (G) after gastric digestion.
794 Protein and lipid are present in green and red, respectively. To note that the yellow block seen
795 in images B and D corresponds to the pH probe.

796

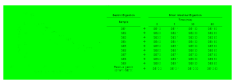
797 Figure 4. A surface plot representation of concentration of free amine groups (mM) for each
798 gastric emptying point (GE) at 0 (referred to end point of gastric digestion), 1, 30 and 60 min
799 after small intestinal digestion for both semi-solid (Figure 4 A) and liquid samples (Figure 4
800 B). The data from the 3 replicates was averaged and is given in Table 1 of the supplementary
801 material.

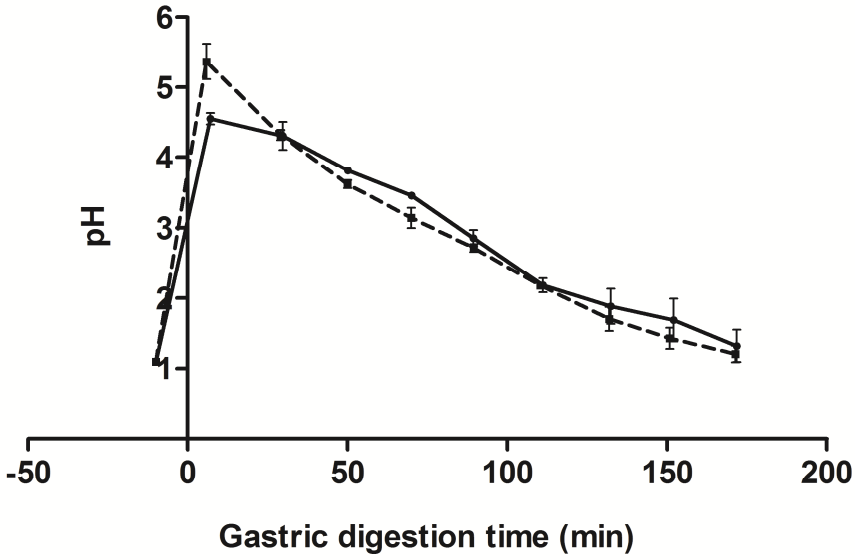
802 Figure 5. Levels (expressed as mass percentage) of lipid classes (TG, DG, MG and FFA) in
803 each gastric emptying (GE) point at 1, 30 and 60 min after small intestinal digestion for both
804 semi-solid and liquid samples (average of 3 replicates). The SD averages for semi-solid
805 sample are 2.5, 5.3, 4.5 and 1.6 % for MG, FFA, TG and DG respectively. The SD averages
806 for liquid sample are 1.7, 7.6, 7.3 and 2.4 % for MG, FFA, TG and DG respectively.

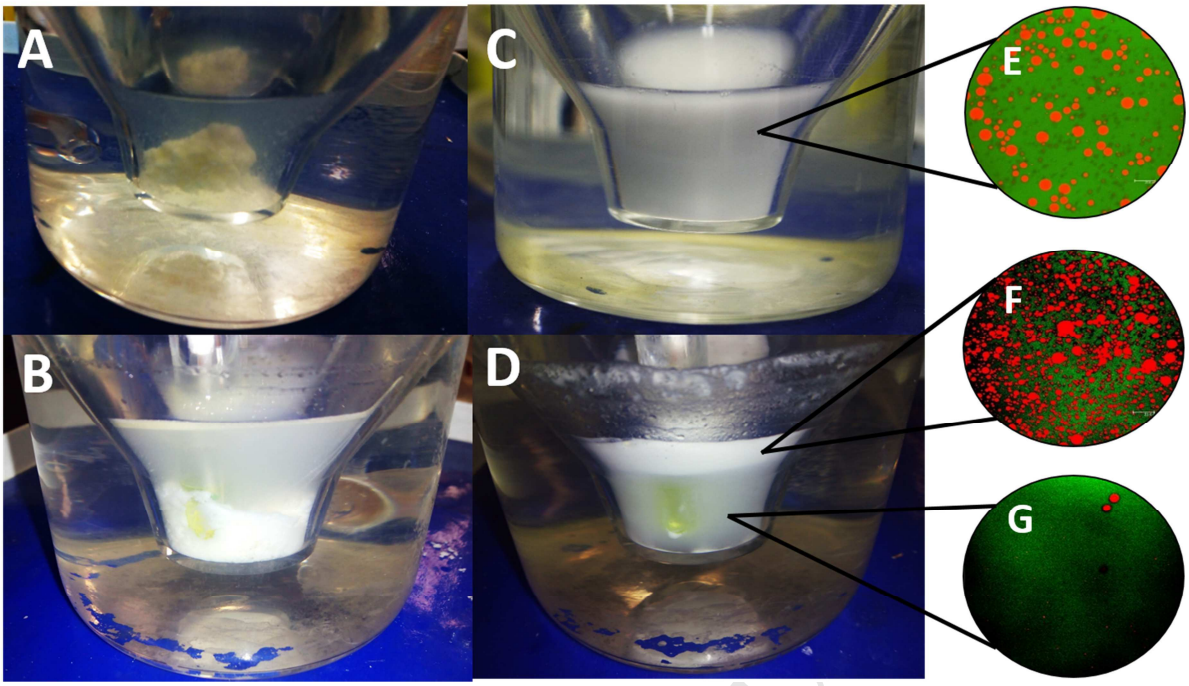
807

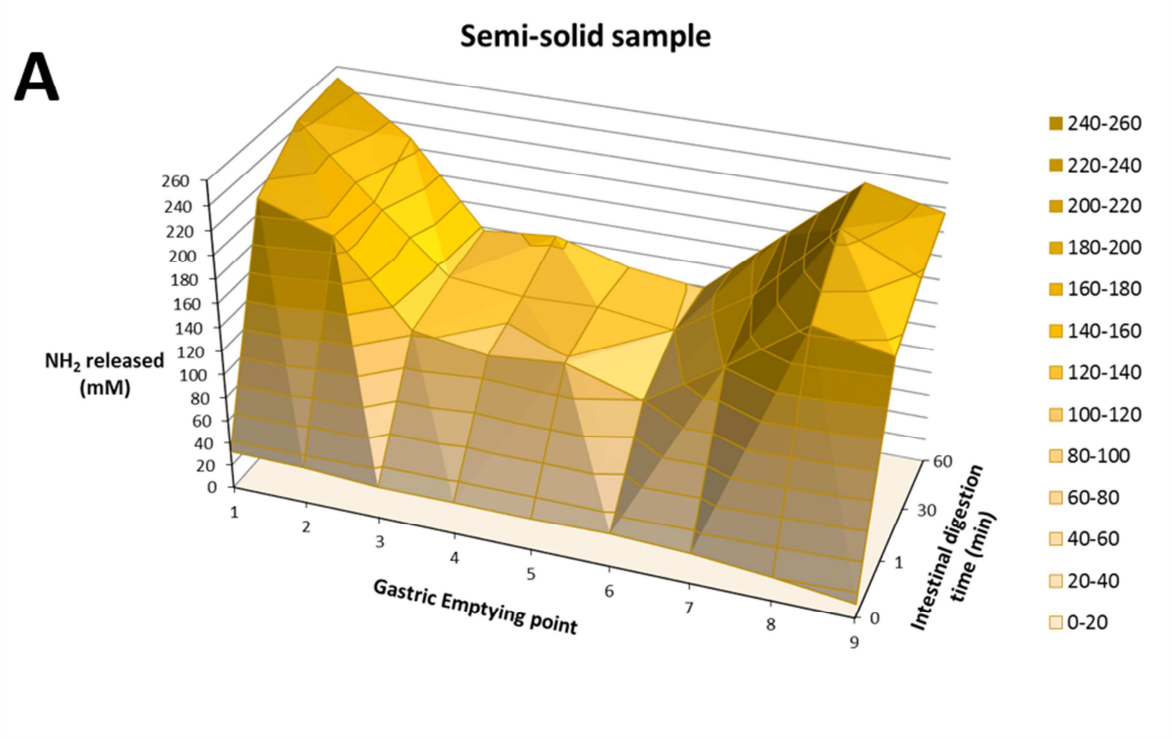
808 Figure 6. Representation of potentially absorbable nutrients, protein (A) and lipid (B), during
809 the digestion time (average of 3 replicates). Absorbable protein refers to the free amine group
810 levels obtained and absorbable lipid refers to the sum of the amount of FFA and MG
811 obtained. This representation is based on the data in Figure 4 and Figure 5 but expressed in
812 linear time. $p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*).

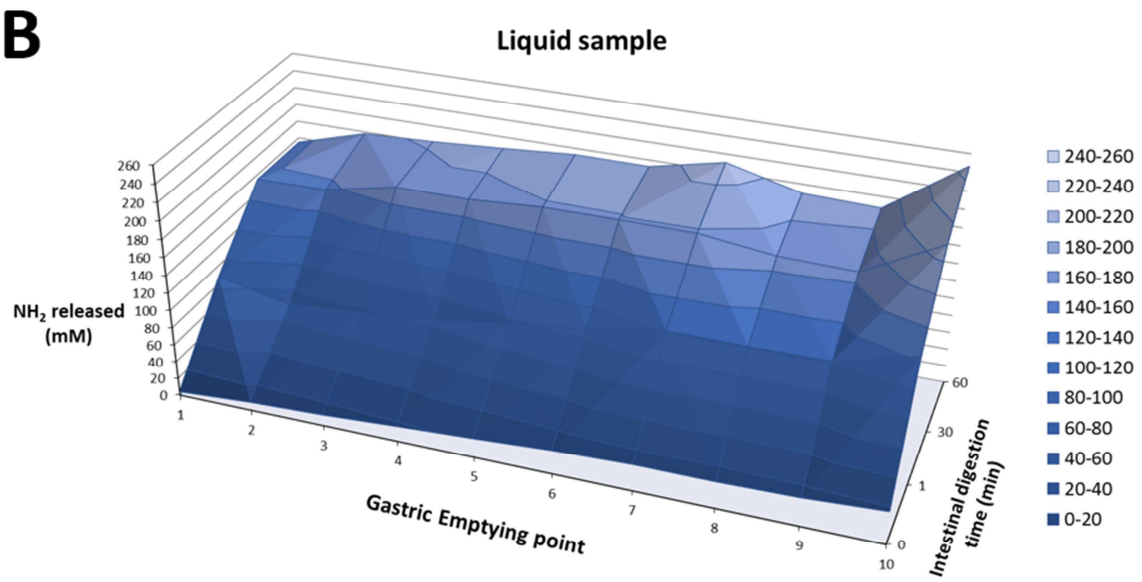
813

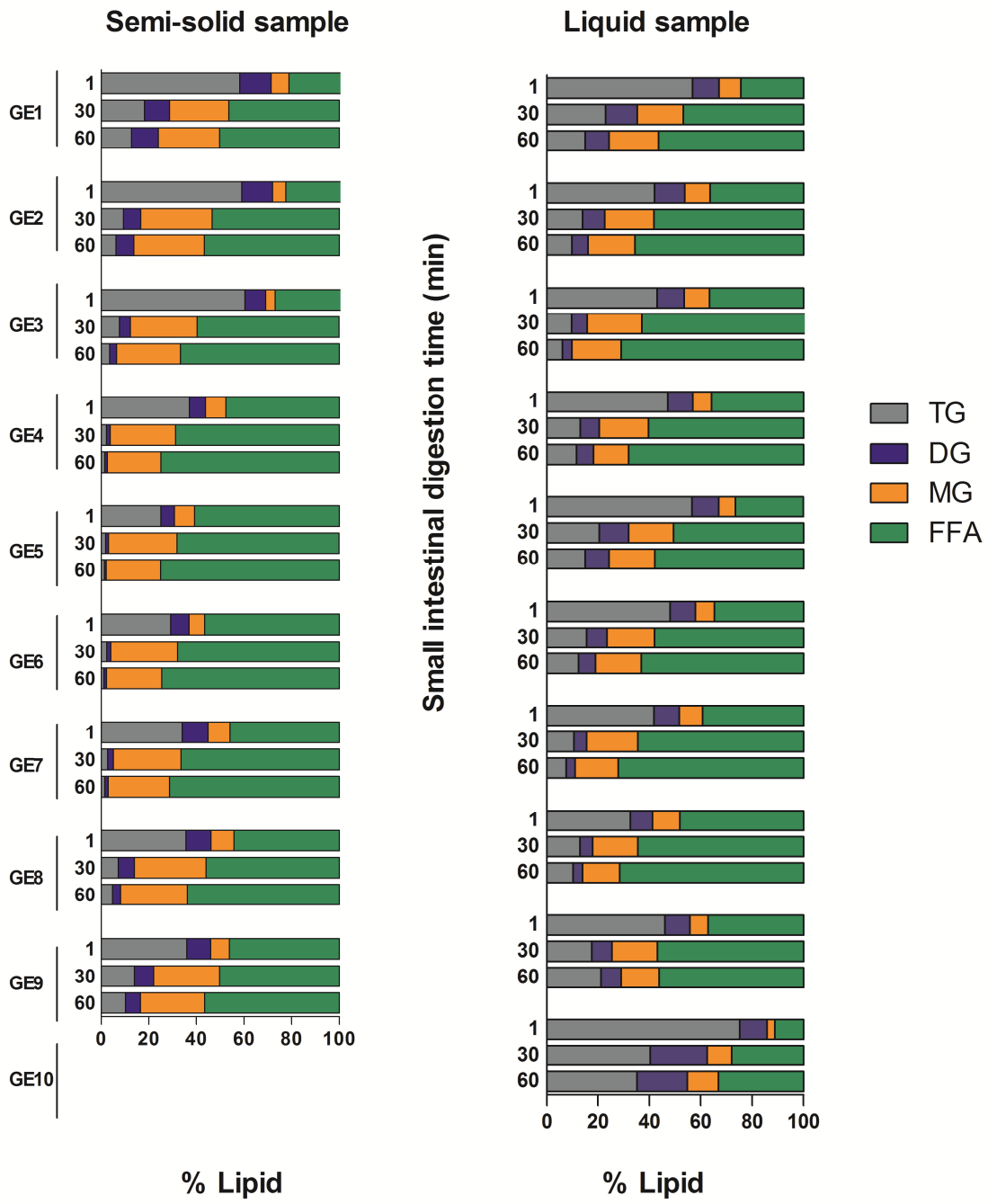


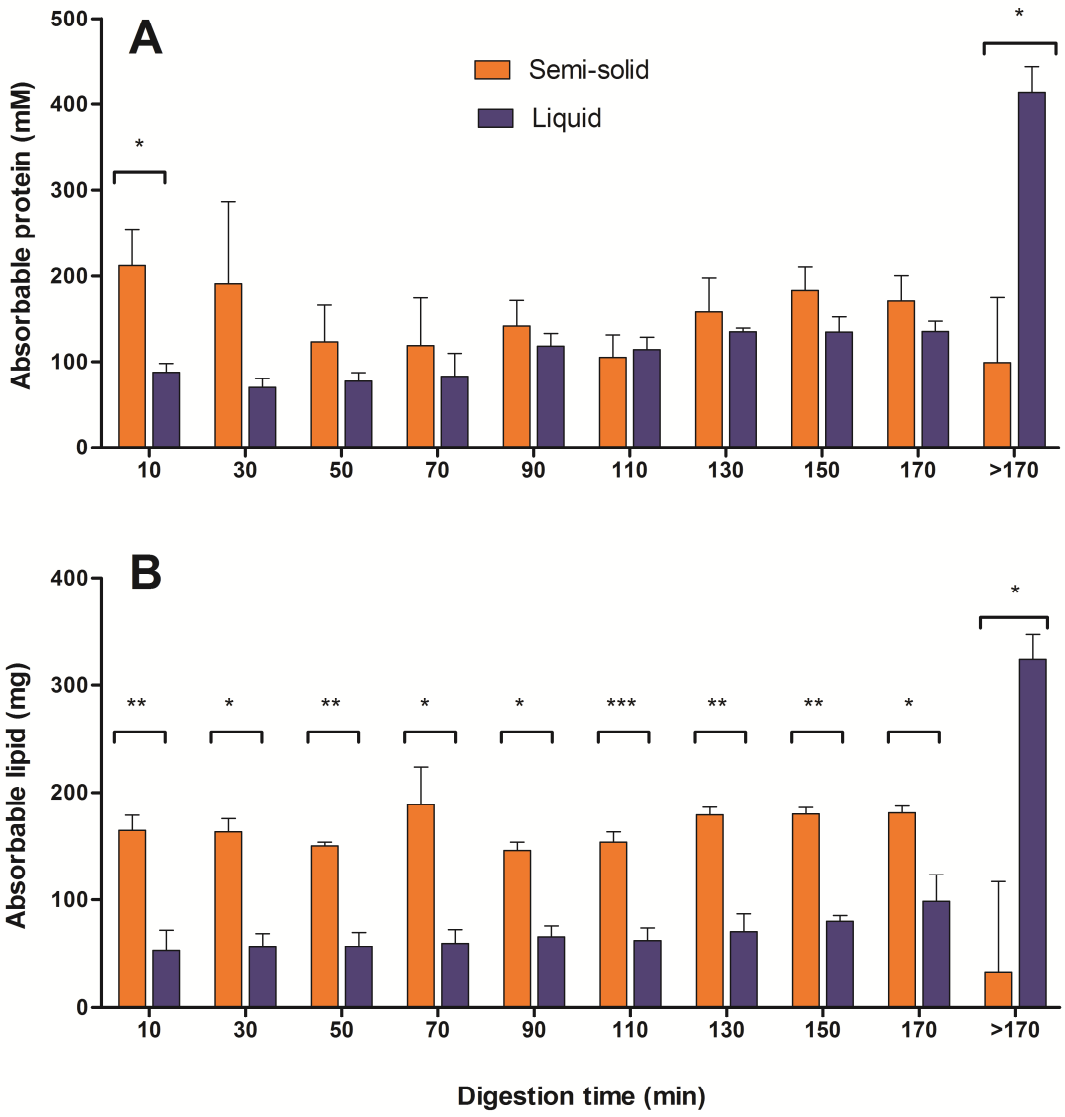





A
Semi-solid sample
NH2 released (mM)
260
240
220
200
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
Gastric Emptying point
60
30
1
0
Intestinal digestion time (min)
240-260
220-240
200-220
180-200
160-180
140-160
120-140
100-120
80-100
60-80
40-60
20-40
0-20


B
Liquid sample
NH2 released (mM)
260
240
220
200
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Gastric Emptying point
60
30
1
0
Intestinal digestion time (min)
240-260
220-240
200-220
180-200
160-180
140-160
120-140
100-120
80-100
60-80
40-60
20-40
0-20


Semi-solid sample
Liquid sample
Small intestinal digestion time (min)
GE1
GE2
GE3
GE4
GE5
GE6
GE7
GE8
GE9
GE10
1
30
60
0
20
40
60
80
100
% Lipid
% Lipid
TG
DG
MG
FFA


A
Semi-solid
Liquid
Absorbable protein (mM)
500
400
300
200
100
0
*
*
10
30
50
70
90
110
130
150
170
>170
B
Absorbable lipid (mg)
400
300
200
100
0
**
*
**
*
*
***
**
**
*
*
10
30
50
70
90
110
130
150
170
>170
Digestion time (min)

- Development of a simple and physiologically relevant gastric digestion model
- Specific dairy structures led to a different behaviour in the gastric phase
- Gastric behaviour affected nutrient release and digestion in the small intestine
- Differences in bioaccessibility were correlated with previous physiological data