TITLE 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

Submitted and corrected Word version Dec.2016, Mulet-Cabero, et al. 2017, doi:10.1016/j.foodhyd.2016.12.039

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.

Submitted and corrected Word version Dec.2016, Mulet-Cabero, et al. 2017, doi:10.1016/j.foodhyd.2016.12.039

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, *et al.*, 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

Submitted and corrected Word version Dec.2016, Mulet-Cabero, et al. 2017, doi:10.1016/j.foodhyd.2016.12.039

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, *et al.* (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, *et al.*, 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

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89 delayed gastric emptying of caseins introducing the concept of ‘fast’ and ‘slow’ protein,
90 respectively (Boirie, *et al.*, 1997).

91

92 Lipid is another important nutrient playing a key role in satiety. There are several *in vivo*
93 studies looking at the impact of emulsion structure on lipid digestion rate (Keogh, *et al.*,
94 2011; Marciani, *et al.*, 2009a; Marciani, *et al.*, 2007). They have shown that lipid droplets can
95 be designed to exert specific behaviours in the stomach taking into account different physical
96 processes (i.e. flocculation, coalescence and creaming) that they might undergo under the
97 gastric conditions due to changes in the interfacial properties (Dickinson, 1997). Marciani, *et*
98 *al.* (2009a) compared two emulsions with different acid stabilities. They showed that the
99 acid-stable emulsion, homogenous in the stomach, provided a slower and more consistent
100 gastric emptying. In contrast, the acid-unstable emulsion that broke into two phases upon
101 gastric acidification presented a more rapid initial gastric emptying of the aqueous layer
102 followed by the emptying of the upper fat layer in a slower rate.

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

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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, *et al.*, 2013; van Aken, *et al.*, 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

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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

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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

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(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.

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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, *et al.*, 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).

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237 2.7. Protein hydrolysis analysis by o-phthaldialdehyde spectrophotometric assay
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239 The extent of protein hydrolysis was determined using the standardised o-phthaldialdehyde
240 (OPA) spectrophotometric assay in micro-titre plates. OPA reagent consisted of 3.81 g
241 sodium tetraborate dissolved in approximately 80 mL water. Once dissolved, 0.088 g
242 dithiothreitol and 0.1 g sodium dodecyl sulphate were added. Then, 0.080 g OPA dissolved in
243 2-4 mL ethanol was placed in the solution which was finally made up to 100 mL with HPLC
244 grade water.

245 Different concentrations of standard D-leucine solution (made with phosphate buffer
246 solution) ranged from 0 to 10 mM were used to obtain a calibration curve. 10 µl of
247 standard/sample was placed into each well and mixed with 200 µl of OPA reagent. The
248 reaction was allowed to proceed at room temperature for 15 min, then the absorbance was
249 measured at 340 nm using a microplate spectrophotometer (Benchmark Plus, BioRad, UK).

250

251 2.8. Lipid analysis
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253 2.8.1. Total lipid extraction
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255 Lipid extraction of samples was carried out using the protocol of Bligh and Dyer (1959). The
256 internal standard (IS) method was used, which consisted of 1.6 mg/mL of each lipid standard,
257 i.e. glyceryl triheptadecanoate, heptadecanoic acid, glyceride dipentadecanoin and glyceride
258 monononadecanoin, in chloroform. For each 0.5 mL of sample, 0.625 mL IS solution and
259 1.25 mL methanol was added. Then, 0.625 mL chloroform and 0.625 mL water with 0.9 %

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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, *et al.* (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.

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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

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306 Methylated samples were analysed using 7890B GC System (Agilent Technologies, USA),
307 equipped with a model 7694 autosampler, and dual flame ionisation and 5977A mass
308 spectrometry detector (Agilent Technologies, USA) connected by a 1:1 active splitter after
309 the analytical column. The analytical column was a SGE BPX70 capillary column (30 m x
310 0.25 mm ID x 0.25 μm film thickness) operated in constant flow mode at 30cm sec^{-1} using
311 helium as carrier gas. Samples (1 μL) were injected with the injector in split mode (10:1 split
312 ratio). The oven temperature program consisted of a hold programmed at 115 °C for 1 min,
313 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
314 with a 10 min hold prior to cooling ready for the next sample.

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

319

320 2.9. Confocal laser scanning microscopy (CLSM)
321

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

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2.10. Statistics

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

3. Results

3.1. Gastric pH profile

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

3.2. Sample behaviour in the gastric compartment

Submitted and corrected Word version Dec.2016, Mulet-Cabero, et al. 2017, doi:10.1016/j.foodhyd.2016.12.039

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

3.3. Protein hydrolysis analysis

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

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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 58.16 ± 11.67, 59.05 ± 6.22 and 60.31 ± 4.91 %, respectively. By contrast, the liquid sample presented 56.90 ± 8.61 % in the GE1/1 and the highest amount of TG (75.15 ± 16.25 %) 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 72.11 ± 12.93 and 71.58 ± 19.57 %, respectively. The GE10 showed the lowest levels of FFA in the liquid sample representing the 33.07 ± 5.99 %.

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

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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, *et al.*, 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, *et al.*, 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).

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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, *et al.*, 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

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455 flocculation by screening the repulsive forces. In addition, the protective layer of protein
456 absorbed at the interface might be compromised by the proteolytic action of pepsin resulting
457 in the reduction of steric stability. Furthermore, the products of lipolysis, i.e. FFA, MG and
458 DG, are surface active and could displace the protein from the emulsion interface leading to
459 further destabilization. Indeed, these compounds at GE1/1 point accounted for 41.84 and
460 43.1% of the total lipid in the semi-solid and liquid samples, respectively. All these factors
461 could potentially contribute to the destabilisation of the emulsion causing flocculation and
462 some coalescence of lipid droplets which progressively creamed to the top part during
463 digestion due to their lower density. This process, ultimately, led to phase separation after
464 110 min of gastric digestion (Figure 3D). Figure 3F confirms the presence of fat droplets in
465 the top layer leaving an aqueous part in the bottom (Figure 3G) and the extent of flocculation
466 and coalescence in that cream layer compared to the stabilised droplets presented in the initial
467 sample (Figure 3E). Phase separation behaviour showing the formation of a cream layer at
468 the top of the stomach has also been shown in *in vivo* (Mackie, *et al.*, 2013; Marciani, *et al.*,
469 2009b) as a result of destabilisation in gastric conditions.

470 Conversely, in the semi-solid sample, the density of the cheese-yogurt matrix resulted in the
471 sedimentation of particles to the bottom of the simulated stomach model leaving the top part
472 a more aqueous system. This behaviour was consistent throughout the digestion. Fat from the
473 cheese and yoghurt was trapped in the food matrix that generated the sediment. However, the
474 combination of gastric conditions including low pH and proteolysis led to the release of some
475 oil droplets seen floating at the top at the end of digestion, although phase separation overall
476 was very limited.

477 Similar structural behaviour of both samples was seen in the magnetic resonance images of
478 the comparative *in vivo* study using the same dairy systems (Mackie, *et al.*, 2013). The phase
479 separation of the liquid sample was clearly obtained in an earlier stage in the *in vivo* study

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(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, *et al.*, 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

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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

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530 5). The TG percentage in GE10/30 and GE10/60 was 40.33 and 35.09% respectively
531 compared to 75.15% of TG found in GE10/1. This could also be attributed to the saturation of
532 substrate compared to the availability of the enzyme. Similarly, van Aken, *et al.* (2011)
533 reported a higher fat distribution in the top layer when creaming was observed after the
534 gastric digestion of triolein emulsions stabilised by milk proteins. They also observed that the
535 FFA concentration in the bottom layer was much lower than in the cream layer, probably
536 because FFA were protonated in the low gastric pH therefore they were oil-soluble and
537 remained in the cream layer. In the present study there was also a higher absolute amount of
538 FFA present in the cream layer compared to the lower aqueous layer, even though the relative
539 values in Figure 5 do not reflect it. The levels of FFA in GE1/0 accounted for 16.98 mg
540 whereas the point GE10/0 contained 54.58 mg. The creaming process led to the concentration
541 of the fat droplets on the top promoting coalescence and decreasing the rate of lipolysis.
542 Another study looking at the lipid digestion of protein stabilised emulsions using a dynamic
543 GI system (Helbig, *et al.*, 2012) also showed the delay of lipid delivery into the small
544 intestine due to creaming of lipid in the stomach. They showed a higher amount of lipid
545 compounds, especially FFA and TG, in the cream layer compared to the bottom part. The
546 authors pointed out that even though different gastric behaviour of the samples was observed
547 (homogeneous vs. creaming), the total amount of FFA released at the end of digestion
548 remained similar, in line with our study.

549 Lipid digestion occurs mainly in the intestine but we considered the addition of gastric lipase
550 relevant because there is evidence suggesting that it accounts for the 5-40% of total TG
551 lipolysis (Armand, *et al.*, 1997). The gastric lipase used in the present study was from a rabbit
552 gastric extract. This has been reported to be similar to human gastric lipase (HGL) having
553 similar specificity for Sn3 position and optimum pH ranged between 3 and 6 (Carriere, *et al.*,
554 1991). Moreover, the lipolytic products may facilitate subsequent pancreatic lipolysis

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555 (Armand, 2007). The digestion of lipid by the action of pancreatic lipase accounts typically
556 for 30-75%. The levels of lipolysis found in this study were in line with these ranges. The
557 gastric lipase generated significant hydrolysis, accounting for 22% and 33 % in liquid and
558 semi-solid samples, respectively. These values were calculated based on the sum of the total
559 FFA in relation to sum of the total lipid obtained on a weight basis. The extent of lipolysis
560 obtained after an additional 60 minutes in the simulated small intestine was determined and
561 the liquid sample showed 63% whereas the semi-solid sample reached 82%. These values
562 were calculated taking into account the sum of the total FFA and MG in relation to the sum of
563 the total lipid obtained on a weight basis. It can be observed that semi-solid sample showed
564 higher lipolysis than liquid sample along GI tract. This could be attributed to the presence of
565 larger surface area of the semi-solid particles whereas the reduced area available in the phase
566 separated and coalesced liquid sample decreased the available surface area for lipase action.

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

570

571 ### 4.5. Possible link to physiological responses

572

573 Since satiety related physiological responses such as CCK release and gastric emptying are
574 linked to the rate and extent of lipid and protein sensing by intestinal endocrine cells, we can
575 expect different satiety responses between the two samples. Lipid and, in particularly, protein
576 have been seen to be the most satiating macronutrients (Fiszman & Varela, 2013). To provide
577 a better understanding of the physiological trends in our study, the previous data for protein
578 and lipid was replotted in a form representing the absorbable nutrient as a function of linear
579 time. We assumed the protein hydrolysates quantified were absorbable since the protein

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580 digestion by intestinal proteases have been seen to be efficient to further protein breakdown
581 into amino acids and small peptides (2-3 amino acids) which are absorbable. The absorbable
582 lipid referred to the FFA and MG fractions that can be absorbed by enterocytes (Armand,
583 2007). Figure 6A shows a similar absorbable protein profile for both samples. The semi-solid
584 sample presented statistically higher levels of absorbable protein (p = 0.0341, paired, two-
585 sided t-test) in the first time point (i.e. 10 min). The samples were also statistically different
586 (p = 0.0356, paired, two-sided t-test) in the last time point (i.e. > 170 min) with the liquid
587 sample having a higher concentration of absorbable protein. On the other hand, the samples
588 differed statistically in all the time points with regards to absorbable lipid (i.e. FFA+MG),
589 which is illustrated in Figure 6B. The semi-solid sample presented higher levels of absorbable
590 lipid than the liquid sample in all the time points except in the last (i.e. > 170 min). These
591 patterns can be linked with the different gastric behaviour of the samples.

592 Sedimentation of the semi-solid sample led to the early detection of higher concentrations of
593 both protein and lipid seen in Figure 6A and B in the first time points. The early delivery of a
594 higher amount of nutrients to the small intestine might trigger an increase of negative
595 hormonal feedback by slowing GE, which could promote the feeling of fullness. It could also
596 result in increasing the period of time that food remained in the stomach leading to a greater
597 gastric distension and enhancing sensations of fullness (Delzenne, *et al.*, 2010). Conversely,
598 the effect of creaming observed in the liquid sample caused a delay of the nutrient release in
599 the small intestine, seen in the last time point (i.e. > 170 min) of Figure 6A and B. Since the
600 amount of nutrient delivered during digestion was lower, especially in the case of lipid, we
601 can assume that this would cause the release of low levels of CCK. Conversely, Mackie, *et al.*
602 (2013) found the CCK levels of the liquid emulsion were higher than those in semi-solid
603 sample for the first 40 min. The authors suggested that the lower viscosity of liquid sample
604 induced the rapid emptying and delay of CCK regulation. Nevertheless, Marciani, *et al.*

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(2009b) showed a decrease of fullness and less CCK released from an emulsion that layered in the stomach compared to another emulsion which remained homogenous (Marciani, *et al.*, 2007). The faster GE rate of the liquid sample observed in the parallel clinical study can now be explained with the lower nutrient concentration in the aqueous layer that emptied first from the stomach.

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

Submitted and corrected Word version Dec.2016, Mulet-Cabero, et al. 2017, doi:10.1016/j.foodhyd.2016.12.039

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.

Acknowledgements

This work has funded by the Irish Dairy Levy Research Trust (project number MDDT6261). Ana-Isabel Mulet-Cabero was funded under Teagasc Walsh Fellowship scheme and BBSRC in the UK (grant BB/J004545/1). The authors gratefully acknowledge George van Aken for

651 the design of the original meals and Balazs Bajka for his help in confocal microscopy and
652 Marianne Defernez for help in preparing some of the figures.

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660 **References**

661 Armand, M. (2007). Lipases and lipolysis in the human digestive tract: where do we stand?
662 *Current Opinion in Clinical Nutrition & Metabolic Care, 10*(2), 156-164.

663 Armand, M., Pasquier, B., Borel, P., Andre, M., Senft, M., Peyrot, J., Salducci, J., & Lairon,
664 D. (1997). Emulsion and absorption of lipids: the importance of physicochemical properties.
665 *Oleagineux Corps Gras Lipides (France)*.

666 Barber, W. D., & Burks, T. F. (1983). Brain stem response to phasic gastric distension. *Am J*
667 *Physiol, 245*(2), G242-248.

668 Benelam, B. (2009). Satiation, satiety and their effects on eating behaviour. *Nutrition*
669 *Bulletin, 34*(2), 126-173.

670 Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and putification.
671 *Canadian Journal of Biochemistry and Physiology, 37*(8), 911-917.

Submitted and corrected Word version Dec.2016, Mulet-Cabero, et al. 2017, doi:10.1016/j.foodhyd.2016.12.039

672 Boirie, Y., Dangin, M., Gachon, P., Vasson, M.-P., Maubois, J.-L., & Beaufrère, B. (1997).
673 Slow and fast dietary proteins differently modulate postprandial protein accretion.
674 *Proceedings of the National Academy of Sciences, 94*(26), 14930-14935.

675 Carriere, F., Moreau, H., Raphel, V., Laugier, R., Benicourt, C., Junien, J. L., & Verger, R.
676 (1991). Purification and biochemical characterization of dog gastric lipase. *European Journal*
677 *of Biochemistry, 202*(1), 75-83.

678 Day, L., Golding, M., Xu, M., Keogh, J., Clifton, P., & Wooster, T. J. (2014). Tailoring the
679 digestion of structured emulsions using mixed monoglyceride–caseinate interfaces. *Food*
680 *Hydrocolloids, 36*, 151-161.

681 Dekkers, B. L., Kolodziejczyk, E., Acquistapace, S., Engmann, J., & Wooster, T. J. (2016).
682 Impact of gastric pH profiles on the proteolytic digestion of mixed beta lg-Xanthan
683 biopolymer gels. *Food & Function, 7*(1), 58-68.

684 Delzenne, N., Blundell, J., Brouns, F., Cunningham, K., De Graaf, K., Erkner, A., Lluch, A.,
685 Mars, M., Peters, H., & Westerterp-Plantenga, M. (2010). Gastrointestinal targets of appetite
686 regulation in humans. *Obesity reviews, 11*(3), 234-250.

687 Dickinson, E. (1997). Properties of Emulsions Stabilized with Milk Proteins: Overview of
688 Some Recent Developments. *Journal of Dairy Science, 80*(10), 2607-2619.

689 Feinle, C., Christen, M., Grundy, D., Faas, H., Meier, O., Otto, B., & Fried, M. (2002).
690 Effects of duodenal fat, protein or mixed-nutrient infusions on epigastric sensations during
691 sustained gastric distension in healthy humans. *Neurogastroenterology & Motility, 14*(2),
692 205-213.

Submitted and corrected Word version Dec.2016, Mulet-Cabero, et al. 2017, doi:10.1016/j.foodhyd.2016.12.039

693 Fiszman, S., & Varela, P. (2013). The satiating mechanisms of major food constituents – An
694 aid to rational food design. *Trends in Food Science & Technology, 32*(1), 43-50.

695 Flanagan, J., & FitzGerald, R. J. (2003). Characterisation and quantification of the reaction(s)
696 catalysed by transglutaminase using the o-phthaldialdehyde reagent. *Nahrung, 47*(3), 207-
697 212.

698 Golding, M., Wooster, T. J., Day, L., Xu, M., Lundin, L., Keogh, J., & Clifton, P. (2011).
699 Impact of gastric structuring on the lipolysis of emulsified lipids. *Soft Matter, 7*(7), 3513-
700 3523.

701 Guo, M., Fox, P., Flynn, A., & Kindstedt, P. (1995). Susceptibility of β-lactoglobulin and
702 sodium caseinate to proteolysis by pepsin and trypsin. *Journal of Dairy Science, 78*(11),
703 2336-2344.

704 Halford, J. C. G., & Harrold, J. A. (2012). Satiety-enhancing products for appetite control:
705 science and regulation of functional foods for weight management. *Proceedings of the*
706 *Nutrition Society, 71*(02), 350-362.

707 Helbig, A., Silletti, E., Aken, G. A., Oosterveld, A., Minekus, M., Hamer, R. J., & Gruppen,
708 H. (2012). Lipid Digestion of Protein Stabilized Emulsions Investigated in a Dynamic In
709 Vitro Gastro-Intestinal Model System. *Food Digestion, 4*(2), 58-68.

710 Kaluzny, M. A., Duncan, L. A., Merritt, M. V., & Epps, D. E. (1985). Rapid separation of
711 lipid classes in high yield and purity using bonded phase columns. *Journal of Lipid Research,*
712 *26*(1), 135-140.

713 Keogh, J. B., Wooster, T. J., Golding, M., Day, L., Otto, B., & Clifton, P. M. (2011). Slowly
714 and rapidly digested fat emulsions are equally satiating but their triglycerides are

Submitted and corrected Word version Dec.2016, Mulet-Cabero, et al. 2017, doi:10.1016/j.foodhyd.2016.12.039

715 differentially absorbed and metabolized in humans. *The Journal of nutrition, 141*(5), 809-
716 815.

717 Macierzanka, A., Sancho, A. I., Mills, E. C., Rigby, N. M., & Mackie, A. R. (2009).
718 Emulsification alters simulated gastrointestinal proteolysis of β-casein and β-lactoglobulin.
719 *Soft Matter, 5*(3), 538-550.

720 Mackie, A. R., & Macierzanka, A. (2010). Colloidal aspects of protein digestion. *Current*
721 *Opinion in Colloid & Interface Science, 15*(1), 102-108.

722 Mackie, A. R., Rafiee, H., Malcolm, P., Salt, L., & van Aken, G. (2013). Specific food
723 structures supress appetite through reduced gastric emptying rate. *Am J Physiol Gastrointest*
724 *Liver Physiol, 304*(11), G1038-1043.

725 Malagelada, J.-R., Longstreth, G. F., Summerskill, W. H. J., & Go, V. L. W. (1976).
726 Measurement of Gastric Functions During Digestion of Ordinary Solid Meals in Man.
727 *Gastroenterology, 70*(2), 203-210.

728 Marciani, L., Faulks, R., Wickham, M. S., Bush, D., Pick, B., Wright, J., Cox, E. F., Fillery-
729 Travis, A., Gowland, P. A., & Spiller, R. C. (2009a). Effect of intragastric acid stability of fat
730 emulsions on gastric emptying, plasma lipid profile and postprandial satiety. *British Journal*
731 *of Nutrition, 101*(6), 919.

732 Marciani, L., Faulks, R., Wickham, M. S., Bush, D., Pick, B., Wright, J., Cox, E. F., Fillery-
733 Travis, A., Gowland, P. A., & Spiller, R. C. (2009b). Effect of intragastric acid stability of fat
734 emulsions on gastric emptying, plasma lipid profile and postprandial satiety. *British Journal*
735 *of Nutrition, 101*(06), 919-928.

Submitted and corrected Word version Dec.2016, Mulet-Cabero, et al. 2017, doi:10.1016/j.foodhyd.2016.12.039

736 Marciani, L., Hall, N., Pritchard, S. E., Cox, E. F., Totman, J. J., Lad, M., Hoad, C. L., Foster,
737 T. J., Gowland, P. A., & Spiller, R. C. (2012). Preventing Gastric Sieving by Blending a
738 Solid/Water Meal Enhances Satiation in Healthy Humans. *The Journal of nutrition, 142*(7),
739 1253-1258.

740 Marciani, L., Wickham, M., Singh, G., Bush, D., Pick, B., Cox, E., Fillery-Travis, A., Faulks,
741 R., Marsden, C., Gowland, P. A., & Spiller, R. C. (2007). Enhancement of intragastric acid
742 stability of a fat emulsion meal delays gastric emptying and increases cholecystokinin release
743 and gallbladder contraction. *American Journal of Physiology - Gastrointestinal and Liver*
744 *Physiology, 292*(6), G1607-G1613.

745 Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F.,
746 Boutrou, R., Corredig, M., & Dupont, D. (2014). A standardised static in vitro digestion
747 method suitable for food–an international consensus. *Food & function, 5*(6), 1113-1124.

748 Santangelo, A., Peracchi, M., Conte, D., Fraquelli, M., & Porrini, M. (1998). Physical state of
749 meal affects gastric emptying, cholecystokinin release and satiety. *Br J Nutr, 80*(6), 521-527.

750 Shani-Levi, C., Levi-Tal, S., & Lesmes, U. (2013). Comparative performance of milk
751 proteins and their emulsions under dynamic in vitro adult and infant gastric digestion. *Food*
752 *Hydrocolloids, 32*(2), 349-357.

753 Thomas, A. (2006). GastroGut motility, sphincters and reflex control. *Anaesthesia &*
754 *Intensive Care Medicine, 7*(2), 57-58.

755 van Aken, G. A., Bomhof, E., Zoet, F. D., Verbeek, M., & Oosterveld, A. (2011). Differences
756 in in vitro gastric behaviour between homogenized milk and emulsions stabilised by Tween
757 80, whey protein, or whey protein and caseinate. *Food Hydrocolloids, 25*(4), 781-788.

Submitted and corrected Word version Dec.2016, Mulet-Cabero, et al. 2017, doi:10.1016/j.foodhyd.2016.12.039

758 Verhoeckx, K., Cotter, P., López-Expósito, I., Kleiveland, C., Lea, T., Mackie, A., Requena,
759 T., Swiatecka, D., & Wichers, H. (2015). *The Impact of Food Bioactives on Health*: Springer
760 International Publishing.

761 Wang, C., McPherson, K., Marsh, T., Gortmaker, S., & Brown, M. (2011). Health and
762 economic burden of the projected obesity trends in the USA and the UK. *The Lancet,*
763 *378*(9793), 815-825.

764 Wilde, P. J. (2009). Eating for Life: Designing Foods for Appetite Control. *Journal of*
765 *Diabetes Science and Technology, 3*(2), 366-370.

766 Wren, A. M., & Bloom, S. R. (2007). Gut hormones and appetite control. *Gastroenterology,*
767 *132*(6), 2116-2130.

768 Wu, H.-C., Chen, H.-M., & Shiau, C.-Y. (2003). Free amino acids and peptides as related to
769 antioxidant properties in protein hydrolysates of mackerel (Scomber austriasicus). *Food*
770 *Research International, 36*(9–10), 949-957.

771

Submitted and corrected Word version Dec.2016, Mulet-Cabero, et al. 2017, doi:10.1016/j.foodhyd.2016.12.039

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	

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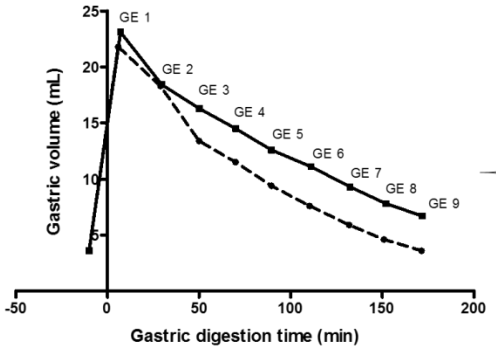
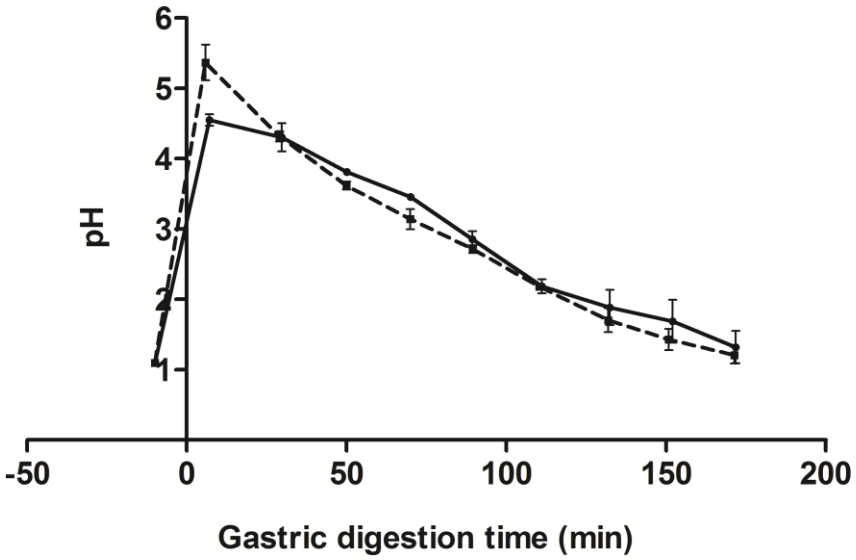
Figures



Gastric Digestion Sample		Small Intestinal Digestion Time (min) 0	1	30	60
GE1	→	GE1/0	GE1/1	GE1/30	GE1/60
GE2	→	GE2/0	GE2/1	GE2/30	GE2/60
GE3	→	GE3/0	GE3/1	GE3/30	GE3/60
GE4	→	GE4/0	GE4/1	GE4/30	GE4/60
GE5	→	GE5/0	GE5/1	GE5/30	GE5/60
GE6	→	GE6/0	GE6/1	GE6/30	GE6/60
GE7	→	GE7/0	GE7/1	GE7/30	GE7/60
GE8	→	GE8/0	GE8/1	GE8/30	GE8/60
GE9	→	GE9/0	GE9/1	GE9/30	GE9/60
Residual gastric content (GE10)	→	GE10/0	GE10/1	GE10/30	GE10/60

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



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

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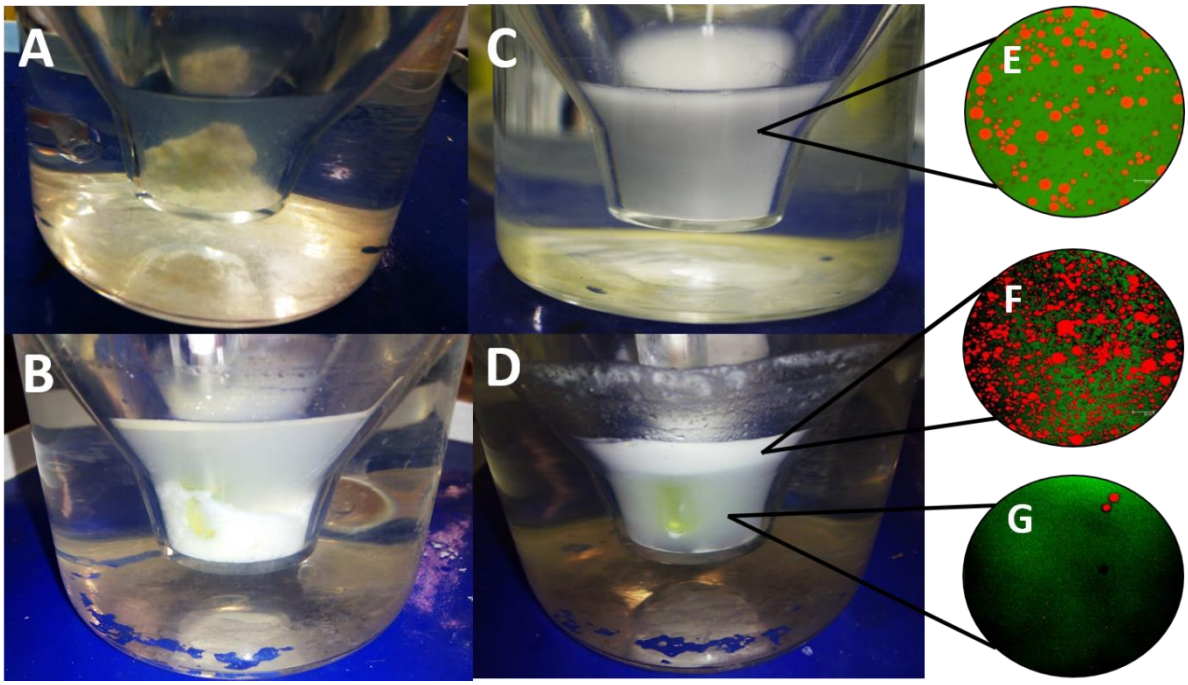

796

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

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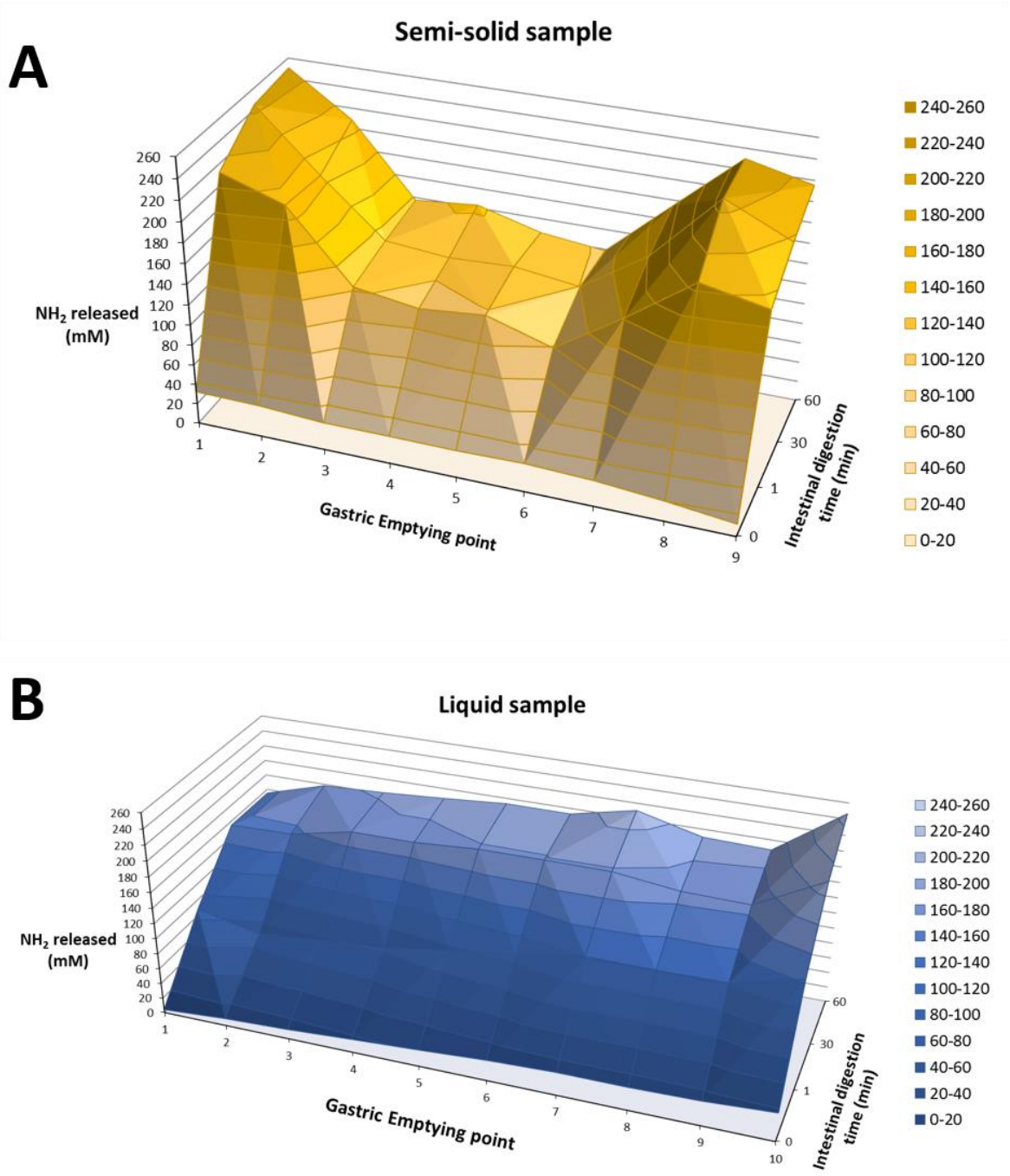

Figure 4. Surface representation of concentration of free amine groups (mM) for each gastric emptying point (GE) at 0 (referred to end point of gastric digestion), 1, 30 and 60 min after small intestinal digestion for both semi-solid (Figure 4 A) and liquid samples (Figure 4 B). The data from the 3 replicates was averaged and plotted in Matlab (The Mathworks, Cambridge, UK).

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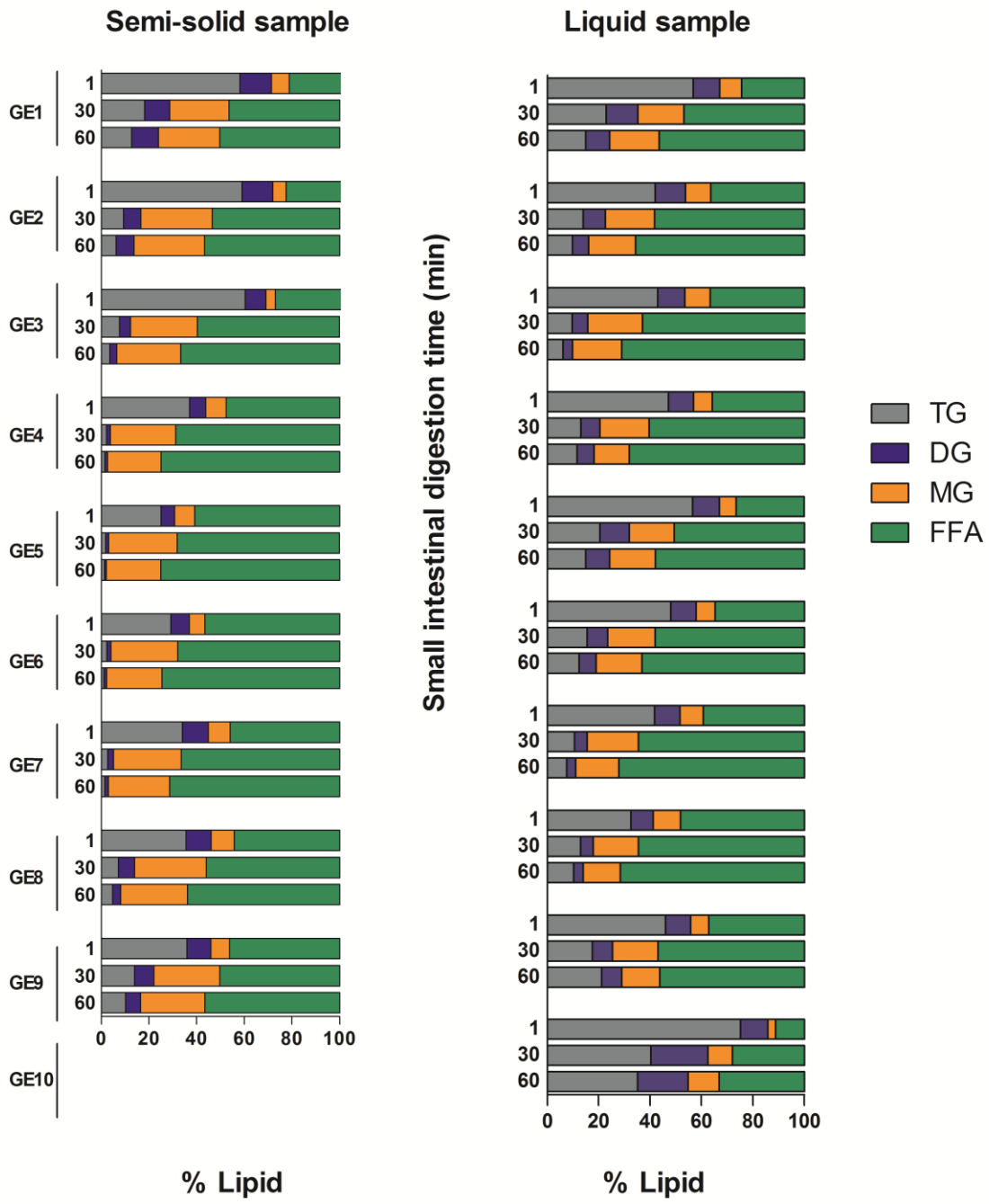

Figure 5. Levels (expressed as mass percentage) of lipid classes (TG, DG, MG and FFA) in each gastric emptying (GE) point at 1, 30 and 60 min after small intestinal digestion for both semi-solid and liquid samples (average of 3 replicates). The SD averages for semi-solid sample are 2.5, 5.3, 4.5 and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

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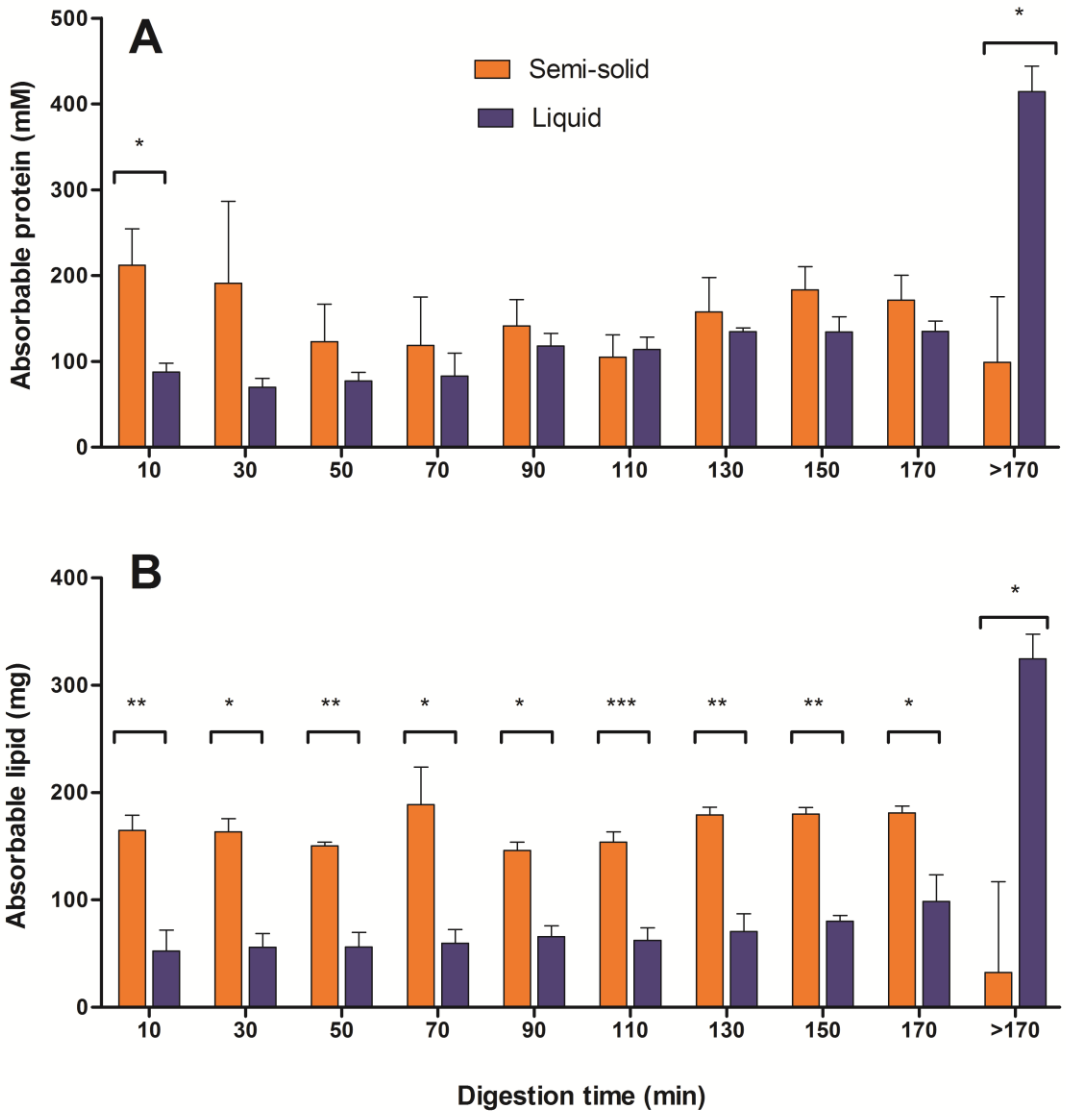

817

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

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