



In vivo digestion of bovine milk fat globules: Effect of processing and interfacial structural changes. I. Gastric digestion



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ABSTRACT

The aim was to study the *in vivo* gastric digestion of fat globules in bovine cream from raw, pasteurised or pasteurised and homogenised milk. Fasted rats were gavaged once and chyme samples were collected after 30, 120 and 180 min post-gavage. Proteins from raw (RC) and pasteurised (PC) creams appeared to be digested faster and to a greater extent. Free fatty acids (FAs) increased throughout the 3 h postprandial period. Short and medium chain FAs were released more rapidly than long chain FAs which were hydrolysed to a greater degree from PC. The size of the fat globules of all creams increased in the stomach. Protein aggregates were observed in pasteurised and homogenised cream chyme. Protrusions, probably caused by the accumulation of insoluble lipolytic products, appeared at the surface of the globules in RC and PC chyme. Overall, PC proteins and lipids appeared to be digested to a greater extent.

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1. Introduction

For mammals, milk is the sole source of nutrition at birth. For many decades, researchers have been investigating the composition, structure and digestion of milk, mainly of bovine and human origin. Pioneering studies from Patton's (Patton & Carey, 1979; Rigler, Honkanen, & Patton, 1986; Rigler & Patton, 1983) and Hamosh's (Bitman et al., 1985; Hamosh, Bitman, Wood, Hamosh, & Mehta, 1985; Hamosh et al., 1999) groups have unravelled interesting insights into the process of milk digestion. Most of the work on the structure of the fat globules during gastrointestinal digestion has been carried out on human or bovine milk in infants (Armand et al., 1996; Hamosh et al., 1999), and by using a range of *in vitro* models (Gallier, Ye, & Singh, 2012; Patton & Carey, 1979; Ye, Cui, & Singh, 2011) or *in vivo* models such as the suckling rat (Berendsen & Blanchettmackie, 1979; Bitman et al., 1985). Recently, work from our laboratory has focused on the *in vitro*

digestion of bovine milk and the importance of the milk fat globule interface (Gallier, Gragson, Jiménez-Flores, & Everett, 2012; Ye et al., 2011).

The native bovine milk fat globule is composed of a triglyceride core surrounded by a complex interface, the milk fat globule membrane (MFGM), a trilayer of phospholipids embedding proteins and cholesterol. Its diameter varies between 0.2 and 15 µm, with an average of 4 µm (Michalski, Michel, Sainmont, & Briard, 2002). The structure of the MFGM has been the subject of numerous recent studies (Gallier, Gragson, Jimenez-Flores, & Everett, 2010; Gallier et al., 2012; Vanderghem et al., 2011). The main MFGM proteins are mucin I (MUC I; 200 kDa), xanthine oxidase (XO; 150 kDa), periodic acid Schiff (PAS) III (95–100 kDa), cluster of differentiation (CD) 36 (76–78 kDa), butyrophilin (BTN; 67 kDa), adipophilin (ADPH; 52 kDa), PAS 6/7 (48–54 kDa) and fatty acid binding protein (FABP; 13 kDa) (Singh, 2006). The main MFGM phospholipids are phosphatidylcholine (36%), phosphatidylethanolamine (27%), sphingomyelin (22%), phosphatidylinositol (11%), and phosphatidylserine (4%) (Keenan & Mather, 2002). Both MFGM proteins and phospholipids have been shown to have beneficial physiological properties such as anticarcinogenic properties and antibacterial activities (Singh, 2006). However, milk is not often consumed raw, and pasteurisation and homogenisation processes are commonly used. These two processes have a dramatic impact on the structure and composition of the MFGM. Thermal treatment denatures some MFGM proteins, like PAS 7 and PAS 6 (Ye, Singh, Taylor, & Anema, 2004), and increases complexation of butyrophilin with xanthine oxidase (Ye, Singh, Taylor, & Anema, 2002). It also results

Abbreviations: RC, raw cream; PC, pasteurised cream; PHC, pasteurised and homogenised cream; MFGM, milk fat globule membrane; MUC1, mucin1; XO, xanthine oxidase; PAS, periodic acid Schiff; CD, cluster of differentiation; BTN, butyrophilin; ADPH, adipophilin; FABP, fatty acid binding protein; FA, fatty acid; SCFA, short chain fatty acid; MCFA, medium chain fatty acid; LCFA, long chain fatty acid; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; Rd-DHPE, rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; DIC, differential interference contrast; CLSM, confocal laser scanning microscopy; TEM, transmission electron microscopy.

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in the association of serum proteins, mainly whey proteins, via disulphide bonds with the native MFGM. The protein κ -casein binds directly to the MFGM or indirectly by interaction with β -lactoglobulin (Ye et al., 2004). Homogenization reduces the size of the fat globules to less than 1 μm . The rupture of the native MFGM during homogenisation results in a newly formed membrane, mostly composed of adsorbed caseins (Lopez, 2005). Therefore, the composition and structure of the MFGM is completely different after homogenisation. About 10% of the native MFGM is still present at the surface of the fat globules after homogenisation (Keenan, 1983).

Lipolysis is an interfacial process. Therefore the “interfacial quality” and the size of the lipid droplets may affect the way lipids are digested (Reis, Holmberg, Watzke, Leser, & Miller, 2009). The stomach acts as an emulsifier organ through mechanical energy and shear and the presence of emulsifiers such as phospholipids from the gastric mucosa and free fatty acids from lipolysis (Wickham, Faulks, & Mills, 2009). But because of its acidic pH, leading to reduced ionisation, it can cause breakage of emulsions (Armand et al., 1994). During gastric digestion, the gastric lipase adsorbs onto the oil–water interface, and structures rich in free long chain fatty acids (LCFA) and phospholipids, forming spherical clusters at the surface of the oil droplets, trap the gastric lipase limiting its activity (Pafumi et al., 2002). The gastric lipase is able to penetrate the MFGM (Hamosh & Scow, 1973) while pepsin hydrolyses some of the MFGM proteins from the outer layer of the MFGM trilayer (Ye et al., 2011). The free fatty acids from gastric lipolysis, when reaching the duodenum, stimulate the release of cholecystokinin, which slows down gastric emptying and stimulates gastric, pancreatic and hepatic secretions (Michalski, 2009). Gastric lipase contributes to 5–40% of lipolysis in the stomach and an additional 7.5% in the duodenum. In rats, gastric lipase is absent but the acid-stable lingual lipase, produced by Von Ebner glands, is present, acting similarly to the human gastric lipase in the stomach with a preference for fatty acids on *sn*-3 positions and short (SCFA) and medium (MCFA) chain fatty acids and has an optimal activity at pH 5.0–5.4 (Hamosh & Scow, 1973).

Taking this into consideration, it is reasonable to postulate that processing milk, and therefore changing the size and interfacial composition of the fat globules, will have an impact on the digestion and absorption of the fat globules. Michalski (2009) has reviewed the current knowledge on the impact of processing on the digestion and absorption of milk lipids. Michalski et al. (2006) have shown that the milk fat ultrastructure in dairy products affects the fatty acid profile of plasma lipids in rats. While *in vitro* models have been used to investigate the structural changes that occur during the digestion of food emulsions, they often omit the gastric digestion step (Berton, Sebban-Kreuzer, Rouvellac, Lopez, & Crenon, 2009; Patton & Carey, 1979), rendering the latter models physiologically less relevant. Moreover, *in vivo* studies that describe the impact of processing on the behaviour of milk fat globules in the gastrointestinal tract are scarce. The aim here was to investigate the impact of processing on the structural changes that occur to milk fat globules in the stomach. To that end, cream was prepared from raw milk, pasteurised milk or pasteurised and homogenised milk and the growing rat was used as an *in vivo* model for humans. This allowed us to compare the *in vivo* gastric digestion of milk fat globules with the same fatty acid profile but different surface composition and size. The *in vivo* intestinal digestion was studied separately and this work is presented in a subsequent publication (Gallier et al., 2013, Unpublished results).

2. Materials and methods

2.1. Samples and reagents

Milli-Q water (18.2 M Ω cm, purified by treatment with a Milli-Q apparatus; Millipore Corporation, Bedford, MA) was used. All

chemicals were of analytical grade and were purchased from Sigma–Aldrich Corporation unless specified otherwise. Fresh raw bovine milk from pasture-fed (with grass silage) Friesian cows was collected from the Massey University No. 1 Dairy Farm (Palmerston North, New Zealand). The milk was divided into two batches. One batch of raw milk was refrigerated at 4 °C overnight. The other batch was pasteurised at 63 °C for 30 min. Half of the pasteurised milk was further homogenised at 10/5 MPa by two passes. The pasteurised milk and pasteurised and homogenised milk were also kept overnight at 4 °C. A Malvern Mastersizer MSE (Malvern Instruments Ltd.) was used to determine the particle size distribution of the milk samples. Raw milk had a d_{43} of 4.7 μm , a d_{32} of 3.1 μm , and a surface area of 2.07 m² g^{−1}, pasteurised milk had a d_{43} of 4.7 μm , a d_{32} of 3.1 μm , and a surface area of 2.07 m² g^{−1}, and pasteurised and homogenised milk had a d_{43} of 0.63 μm , a d_{32} of 0.23 μm , and a surface area of 28.1 m² g^{−1}.

Raw cream (RC) and pasteurised cream (PC) were obtained by centrifuging the raw milk and pasteurised milk, respectively, at 3000g for 15 min at 4 °C. Pasteurised and homogenised cream (PHC) was obtained by centrifuging pasteurised and homogenised milk at 20,000g for 20 min at 4 °C.

2.2. Rat study

Ethics approval for the study was granted by the Massey University Animal Ethics Committee.

Fifty-four male Sprague Dawley rats of approximately 280 g bodyweight were obtained from the Small Animal Unit, Massey University. The rats were housed individually in stainless steel wire-bottomed cages in a room maintained at 22 $\pm$ 2 °C, with a 12 h light/dark cycle. The rats were fasted for 24 h prior to gavaging to ensure that no residual food material was present in the stomach. During the fasting period the rats had access to a solution containing 10% dextrose. On the sampling day, the rats were allocated to the dietary treatments such that 54 rats (6 rats per cream preparation per time point) were gavaged with either RC, PC or PHC. Water was removed 2 h before the rats were gavaged with 2 mL of their respective cream preparation. At either 30 min, 2 or 3 h after gavaging, the rats were euthanised by asphyxiation with carbon dioxide gas and then decapitated. The rats were opened and their stomachs carefully removed, rinsed with reverse osmosis water and gently dried with a paper towel. The stomachs were dissected open and the chyme carefully collected such that the chyme material underwent minimal disturbance.

The chyme samples were then examined using microscopy techniques without any further treatment. Additionally an aliquot of each chyme sample was heated at 95 °C for 10 min to inactivate the enzymes and then kept at −20 °C prior to analysis for fatty acids using fatty acid methyl ester analysis and for proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE).

2.3. Fatty acid analysis

Total and bound fatty acids in creams and chyme samples were determined as described elsewhere (Zhu, Svendsen, Jaepelt, Moughan, & Rutherford, 2011). Sodium methoxide-catalysed transesterification followed by gas chromatography analysis was used to quantify the bound fatty acids while the total fatty acids were determined by saponification and gas chromatography analysis. Bound fatty acids are fatty acids attached to the glycerol backbone and free fatty acids are obtained by subtraction of bound fatty acids from total fatty acids.

2.4. Protein hydrolysis

Cream and chyme samples were extracted three times with petroleum ether (300 mg of chyme or 150 mg of cream in 150 μL

of Milli-Q water mixed with 1 mL of petroleum ether) vortexed for 20 s and centrifuged at 16,000g for 1 min. The lower aqueous phase and the interface were pooled. The fat extraction step was repeated 3 times. The protein composition of the pooled aqueous phases and interfaces was determined by 10–20% tricine–SDS–PAGE. The defatted samples were mixed with tricine sample buffer (0.2 M Tris–HCl buffer, pH 6.8, 40% glycerol, 2% SDS, 0.04% Coomassie Brilliant Blue G-250, β -mercaptoethanol (19:1, v:v)) at a ratio 1:2 and were heated at 95 °C for 5 min. The samples were centrifuged for 20 min at 16,000g at 4 °C; the residual fat layer, if any, was discarded, and 40 μ L of sample was loaded onto a precast Criterion 10–20% gradient tricine gel (Bio-Rad Laboratories Pty, Auckland, New Zealand). The gels were stained with either Coomassie Blue as described elsewhere (Gallier & Singh, 2012) or with PAS as described in Zachariu, Zell, Morrison, and Woodlock (1969). Precision Plus protein unstained standards (10–250 kDa) were obtained from Bio-Rad Laboratories Pty.

2.5. Confocal laser scanning microscopy (CLSM)

The microstructure of the chyme samples was studied using a confocal laser scanning microscope (Leica DM6000B; Heidelberg, Germany) with a 63 mm oil immersion objective lens. The fluorescent probes were the same as those used by Gallier et al. (2012). Nile Red was used to stain the triglycerides and Fast Green FCF, the proteins. The Alexa Fluor® 488 conjugate of wheat germ agglutinin (WGA) was used for the localisation of glycoproteins and glycolipids at the surface of the fat globules and the fluorescent head group-labelled phospholipid analogue Lissamine™ rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (Rd-DHPE) was used to investigate the interfacial organisation of the phospholipids. When the fluorescence emission of the probes was not efficient or when interesting features were observed, the scan differential interference contrast (DIC) mode was used to acquire images of the chyme samples.

2.6. Transmission electron microscopy (TEM)

The structure of the chyme samples (after 3 h) was investigated using a Philips 201C transmission electron microscope (Philips, The Netherlands). The chyme samples were injected into freshly made 3% agarose tubes. The tubes were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), then in 1% osmium tetroxide in cacodylate buffer, dehydrated in acetone and embedded in fresh 100% resin (Procure 812). The resin blocks were cut using a diamond knife and an Ultramicrotome (Leica, Austria) and stained with Toluidine Blue for observation under a light microscope (Olympus BX51, Japan). Ultrathin sections (100 nm) were deposited onto a copper grid and stained with saturated uranyl acetate in 50% ethanol and then with lead citrate.

2.7. Statistical analysis

The proportions of bound fatty acids to total fatty acids in the gastric chyme for the different treatments were compared using a two-way ANOVA with cream preparation, postprandial time and the interaction between preparation and time as the model variables (GLM Procedure; SAS 2007). Where overall significant ($P < 0.05$) differences were observed, individual treatment means were compared using the Tukey test. Outlier data points were excluded from the statistical analysis based on the Grubbs test. The normality of distributions was tested using the Shapiro–Wilkes test.

3. Results and discussion

3.1. Fatty acid analysis

The proportions of bound fatty acids (as a percentage of total fatty acids) present in the gastric chyme of rats receiving the RC, PC and PHC preparations during the first 3 h after gavaging are presented in Table 1. The proportion of bound fatty acids in the stomach chyme was significantly ($P < 0.05$) different over time post-gavage for all of the determined fatty acids with the exception of decanoic acid. For the shorter chain fatty acids (≤ 16 carbons units), the proportion of bound fatty acids in the chyme was generally not different ($P < 0.05$) between 30 min and 2 h post-gavage but decreased ($P < 0.05$) between 30 min and 3 h. For the longer chain fatty acids (> 16 carbon units), the proportion of bound fatty acids in the gastric chyme decreased ($P < 0.05$) between 30 min and 2 h post-gavage but was not different ($P > 0.05$) between 2 and 3 h. The proportion of bound fatty acids in the chyme was not significantly ($P > 0.05$) different across cream preparations for octanoic, decanoic and lauric acids but was significantly ($P < 0.05$) different for caproic, myristic, palmitic, stearic, oleic and linoleic acids. For the latter fatty acids, the proportion of bound fatty acids was highest ($P < 0.05$) for PHC and lowest ($P < 0.05$) for PC. The proportion of the bound latter fatty acids for RC was not different ($P > 0.05$) from that for PC and PHC. No significant ($P > 0.05$) interaction between cream preparation and time post-gavage for the proportion of bound fatty acids in the gastric chyme was observed for any of the fatty acids.

Less bound fatty acids, thus more free fatty acids in relation to total fatty acids, were present in the chyme with longer postprandial times, which means that, even if the pH in the stomach was lower than that required for optimal lingual lipase activity, triglycerides were still being hydrolysed. Furthermore, the gastric fatty acid data did not take into account gastric emptying, so the proportion of free fatty acids in the stomach will underestimate the total amount of hydrolysed fatty acids. SCFA and MCFA are predominantly in the *sn*-3 position on the glycerol backbone (Michalski et al., 2006). They are hydrophilic and are hydrolysed rapidly due to the *sn*-3 position preference of the rat lingual lipase (Hamosh & Scow, 1973), similar to the human gastric lipase, and they have a low pK_a of around 4.8 (Carey, Small, & Bliss, 1983) so they are solubilised more readily and are likely to exit the stomach more rapidly once hydrolysed since the aqueous phase of the stomach empties more rapidly than the solid phase (Borgstrom & Patton, 1991). The method of fatty acid analysis did not allow us to measure the amounts of butyric acid, due to its solubility in the solvent and its volatility, but it is most likely hydrolysed at a fast rate, as 36% of both acids are found on the *sn*-3 position in milk triglycerides (Michalski, 2009).

3.2. Protein hydrolysis

The SDS–PAGE patterns of RC, PC, PHC and their respective chyme samples are presented in Fig. 1. The cream samples presented similar protein profiles, but as expected, RC had more MFGM proteins and PHC had more caseins. The three cream samples contained residual serum proteins (i.e. caseins and whey proteins) as the milk fat globules were not washed. After 3 h of gastric digestion, more intact proteins and peptides were present in the stomach chyme for PHC, but this could be due to either slower proteolysis of PHC or slower gastric emptying. Gastric emptying was not determined as part of this study. After 30 min and 2 h of gastric digestion of RC, some MFGM proteins, caseins and β -lactoglobulin were still intact, however hydrolysis had occurred. A main band around 18 kDa was detected after 3 h and could be due to the

Table 1
Fatty acid composition (wt%) of the cream preparations and the least squares mean ($n = 6$) proportion of bound fatty acids^A in the stomach contents of rats for the first 3 h after gavaging with cream derived from raw milk (RC), pasteurised milk (PC), or pasteurised and homogenised milk (PHC).

		Fatty acid							
		Caproic C6:0	Caprylic C8:0	Capric C10:0	Lauric C12:0	Myristic C14:0	Palmitic C16:0	Stearic C18:0	Oleic C18:1 Linoleic C18:2
Fatty acid composition^B									
	RC	1.7(0.06)	1.3(0.04)	3.0(0.11)	3.6(0.14)	12.3(0.52)	34.1(1.20)	14.7(0.56)	25.7(1.00)
	PC	1.7(0.10)	1.3(0.06)	3.0(0.05)	3.5(0.06)	12.2(0.07)	34.2(0.10)	14.6(0.21)	25.7(0.17)
	PHC	1.7(0.03)	1.3(0.04)	2.9(0.16)	3.4(0.19)	12.1(0.27)	34.6(0.12)	14.8(0.16)	25.5(0.19)
Proportion of bound fatty acids									
Cream	RC	73.0 ^{a,b}	78.5	82.1	82.6	92.5 ^{a,b}	93.4 ^{a,b}	93.4 ^{a,b}	92.6 ^{a,b}
	PC	59.3 ^b	78.0	79.3	83.4	89.6 ^b	89.8 ^b	86.6 ^b	90.0 ^b
	PHC	81.0 ^a	79.3	80.8	87.0	96.8 ^a	97.9 ^a	97.7 ^a	96.6 ^a
Overall SE ^C		5.69	3.39	2.91	2.65	1.65	1.78	2.13	1.67
Time	30 min	81.4 ^a	83.9 ^a	84.0	89.2 ^a	96.7 ^a	99.0 ^a	98.9 ^a	99.3 ^a
	2 h	82.1 ^a	80.7 ^{a,b}	80.5	86.0 ^{a,b}	93.4 ^{a,b}	93.1 ^{a,b}	91.2 ^b	92.2 ^b
	3 h	49.7 ^b	71.3 ^b	77.7	77.7 ^b	88.9 ^b	89.1 ^b	87.7 ^b	87.7 ^b
Overall SE ^C		5.69	3.39	2.91	2.65	1.65	1.78	2.13	1.67
Cream × time	RC, 30 min	88.8	88.0	86.3	90.8	96.0	98.5	100.5	99.3
	RC, 2 h	81.8	76.6	78.6	84.6	92.1	92.2	91.4	91.1
	RC, 3 h ^D	48.4	71.0	81.4	72.3	89.3	89.6	88.4	87.3
	PC, 30 min	74.8	85.5	86.2	92.1	97.0	99.4	96.5	100.0
	PC, 2 h ^D	71.6	77.6	76.2	80.8	88.4	87.6	84.1	87.4
	PC, 3 h	31.6	71.0	75.6	77.3	83.5	82.4	79.2	82.7
	PHC, 30 min	80.7	78.1	79.6	84.8	97.1	99.0	99.9	98.5
	PHC, 2 h	93.1	87.8	86.6	92.6	99.5	99.4	98.3	98.3
	PHC, 3 h	69.1	72.0	76.2	83.5	93.8	95.6	95.4	93.0
Overall SE ^C		9.79	5.86	4.96	4.58	2.86	2.52	3.65	2.70
Statistics									
Cream		*	NS	NS	NS	*	**	**	*
Time		***	*	NS	*	**	**	**	***
Cream × time		NS	NS	NS	NS	NS	NS	NS	NS

NS Not significant, $P > 0.05$, $^{*}0.05 > P > 0.01$, $^{**}0.01 > P > 0.001$, $^{***}P < 0.001$.

Means with different superscript letters (a and b) within each column for cream type and time post-gavage separately are significantly ($P < 0.05$) different.

^A The proportion of bound fatty acids present in the stomach chyme was calculated as follows: Proportion of bound fatty acids (%) = bound fatty acids (mg g⁻¹ wet weight)/total fatty acids (mg g⁻¹ wet weight) × 100.

^B Values in brackets are standard deviations.

^C Overall standard error of the mean.

^D $n = 5$, due to insufficient chyme material for analysis.

presence of intact β -lactoglobulin or peptides from higher molecular weight proteins. β -lactoglobulin in the chyme for RC is mostly in solution and is known to be resistant to pepsin when in solution (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). Few peptides appeared after 30 min of gastric digestion of PC. After 2 and 3 h, most proteins were digested with the exception of some α_{s1} - and α_{s2} -caseins and a 150 kDa band was detected being either intact XO or partially hydrolysed MUC1. Few peptides were detected in the gastric chyme for PHC after 30 min, 2 and 3 h, but many proteins were still intact after 3 h. It seems that the caseins, adsorbed at the surface of the PHC globules, were resistant to pepsin, which is contrary to Maldonado-Valderrama et al.'s results (2011). Two reasons may explain the apparent resistance of caseins to digestion after pasteurisation and homogenisation of milk. Firstly, large protein aggregates, trapping fat globules, were seen in the gastric chyme for PHC (as shown later). Thus it may be more difficult for pepsin to break down these protein aggregates and access MFGM proteins. In addition, whey proteins and caseins form aggregates when milk is heated (Singh, 1995), leading to an increased resistance of caseins to *in vitro* digestion (Dupont, Mandalari, Molle, Jardin, Rolet-Repecaud, et al., 2010) in simulated infant gastrointestinal conditions and a faster *in vitro* degradation of raw milk than heat-treated milk by human proteases (Almaas et al., 2006).

On the SDS-PAGE patterns stained with PAS (results not shown), chyme for RC, PC and PHC at the 3 different digestion

times presented 2 bands between 75 and 85 kDa indicating the presence of glycoproteins and glycosylated peptides of high molecular weights. The likely candidates are glycosylated peptides from MUC1 and CD36 as they have been shown to be resistant to pepsin hydrolysis *in vivo* (Hamosh et al., 1999) and *in vitro* (Le et al., 2012).

The fasting basal pH in the human stomach is 1.9. The pH of a meal after 30 min ingestion can increase up to 6.1 and then returns to its basal value after 3 h (Armand et al., 1999). Pepsin is most active at pH 2 (Boyer, 1971) so it may be expected that little protein hydrolysis occurs during the first hour after a meal. In the present study, we did not correct for gastric emptying and therefore the hydrolysis of some proteins may be overlooked.

Some of the MFGM glycoproteins are resistant to hydrolysis by pepsin (Hamosh et al., 1999; Le et al., 2012); the glycosylation helps the proteins to maintain their integrity in acidic conditions. Moreover, the glycoproteins and glycolipids of native milk fat globules form the glycocalyx, protecting the milk fat globule from digestive enzymes (Shimizu, Miyaji, & Yamauchi, 1982). The pasteurisation of milk causes interactions of whey proteins with the MFGM proteins, resulting in the presence of whey proteins at the surface of the globules. The presence of proteins, which are easily digested by pepsin especially in their absorbed or adsorbed form, may facilitate the access of gastric lipase to the triglyceride core by destabilising the surface structure of the membrane. This may explain the earlier appearance of free fatty acids at the surface of PC chyme fat globules than in RC chyme. Intramolecular disulphide

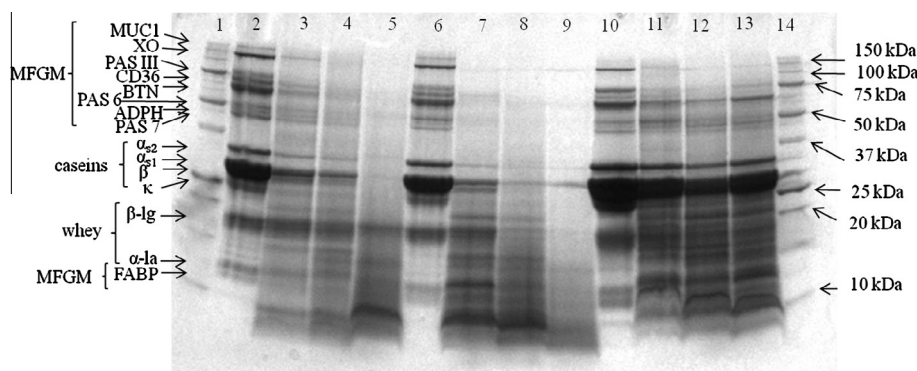


Fig. 1. SDS–PAGE of cream derived from raw milk (RC), pasteurised milk (PC), pasteurised and homogenised milk (PHC), and stomach chyme samples obtained from rats after gavaging with RC, PC or PHC. Lanes 1 and 14: protein standards; Lanes 2–5: RC and RC chyme after 30, 120 and 180 min of gastric digestion, respectively; Lanes 6–9: PC and PC chyme after 30, 120 and 180 min of gastric digestion, respectively; Lanes 10–13: PHC and PHC chyme after 30, 120 and 180 min of gastric digestion, respectively. MFGM, milk fat globule membrane; MUC1, mucin 1; XO, xanthine oxidase; PAS, periodic acid Schiff; CD36, cluster of differentiation 36; BTN, butyrophilin; ADPH, adipophilin; β -lg, β -lactoglobulin; α -la, α -lactalbumin; FABP, fatty acid binding protein.

bridges give β -lactoglobulin and α -lactalbumin a highly compact conformation, responsible for their resistance to digestion (Bouzerzour et al., 2012). However, when adsorbed, as in the case after pasteurisation and homogenisation, a change in the protein conformation exposes sites susceptible to attack by digestive enzymes (Maldonado-Valderrama et al., 2011) which may explain the greater apparent digestion of whey proteins for the PHC and PC compared to the RC (Fig. 1). The effect of heat treatment on the *in vitro* digestion of β -lactoglobulin was also reported by Dupont, Mandalari, Molle, Jardin, Leonil, et al. (2010). They explained the increased digestibility of β -lactoglobulin in terms of denaturation of the protein during heat treatment suppressing the protective effect of gastric mucosa phospholipids present in the stomach against proteolysis. Indeed, β -lactoglobulin, in its native form, binds to physiological phosphatidylcholine, leading to a greater resistance of the protein during digestion (Mandalari, Mackie, Rigby, Wickham, & Mills, 2009).

3.3. Microstructural changes

3.3.1. CLSM

Figs. 2–4 show the microstructural changes taking place during the gastric digestion of the cream samples at different times using CLSM. One common characteristic was the increase in size of the globules in the stomach. Globules in the chyme of the rats gavaged

with RC (RC chyme globules; Fig. 2) and PC (PC chyme globules; Fig. 3) were of similar size, being 1–35 and 1–40 μ m in size respectively, while the globules in the chyme of the rats gavaged with PHC (PHC chyme globules; Fig. 4) were smaller (between less than 0.5 and 25 μ m in size). Gastric conditions led to an increase in size of the globules in all cases, but the PHC chyme globules remained smaller. This is consistent with other studies on emulsions with different particle sizes (Armand et al., 1999; Borel et al., 1994). Few protein aggregates with entrapped small globules were observed after 3 h of digestion of PC (Fig. 3) and throughout the digestion of PHC (Fig. 4). After 30 min and 2 h of digestion, some liquid-ordered domains were observed at the surface of the PHC chyme globules (Fig. 4), as described for the native milk fat globules by Gallier et al. (2010). The destabilisation of the PHC when reaching the stomach may have led to a competitive rearrangement at the surface of the globules between caseins and MFGM fragments. The acidic gastric conditions made it difficult to use Rd-DHPE and WGA in most samples. The presence of liquid-ordered domains in chymes indicates that the MFGM phospholipid trilayer may remain intact in the stomach and the liquid-ordered domains may in turn play a physiological role in the intestine similar to the role of lipid rafts in cells (Brown & London, 1998). MFGM proteins were still detected on the SDS–PAGE patterns of chyme for the PHC treatment after 3 h post-gavage (Fig. 1), thus MFGM fragments may still be intact at the surface of PHC chyme globules.

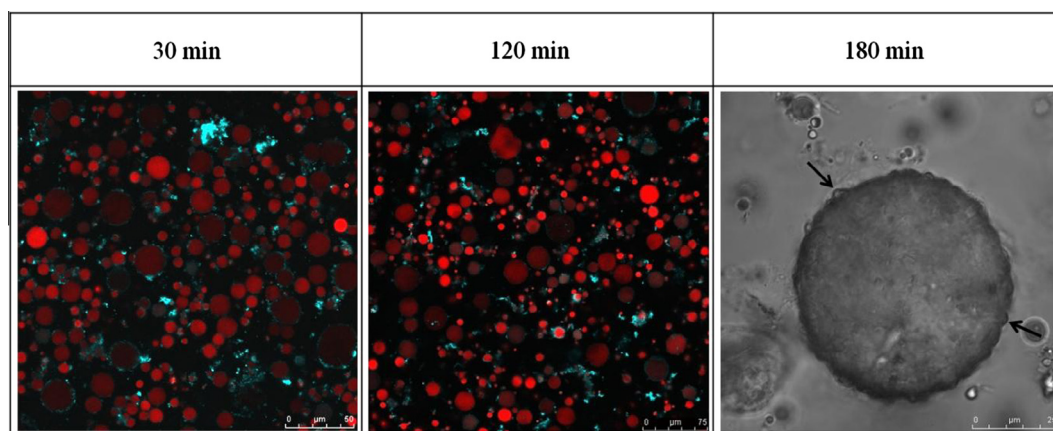


Fig. 2. CLSM and DIC images of the gastric chyme collected from rats 30, 120 and 180 min after gavaging with cream derived from raw milk. The lipids were stained with Nile red (red) and the proteins were stained with Fast Green FCF (blue). The black arrows are pointing at spherical amorphous lipid protrusions. Scale bars = 50 μ m (30 min), 75 μ m (120 min) and 25 μ m (180 min). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

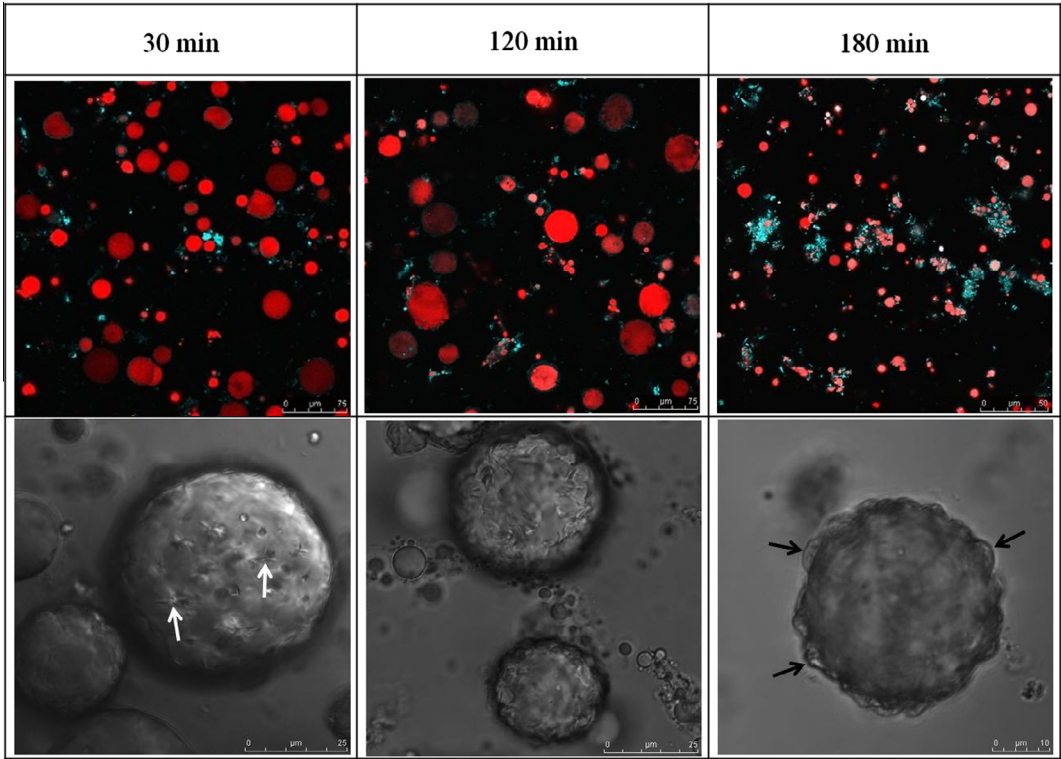


Fig. 3. CLSM and DIC images of the gastric chyme collected from rats 30, 120 and 180 min after gavaging with cream derived from pasteurised milk. The lipids were stained with Nile Red (red) and the proteins were stained with Fast Green FCF (blue). The white arrows are pointing at needle-shaped crystal structures and the black arrows are pointing at spherical amorphous lipid protrusions. Scale bars = 75 μm (30 min and 120 min top), 25 μm (30 min and 120 min bottom), 50 μm (180 min top) and 10 μm (180 min bottom). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

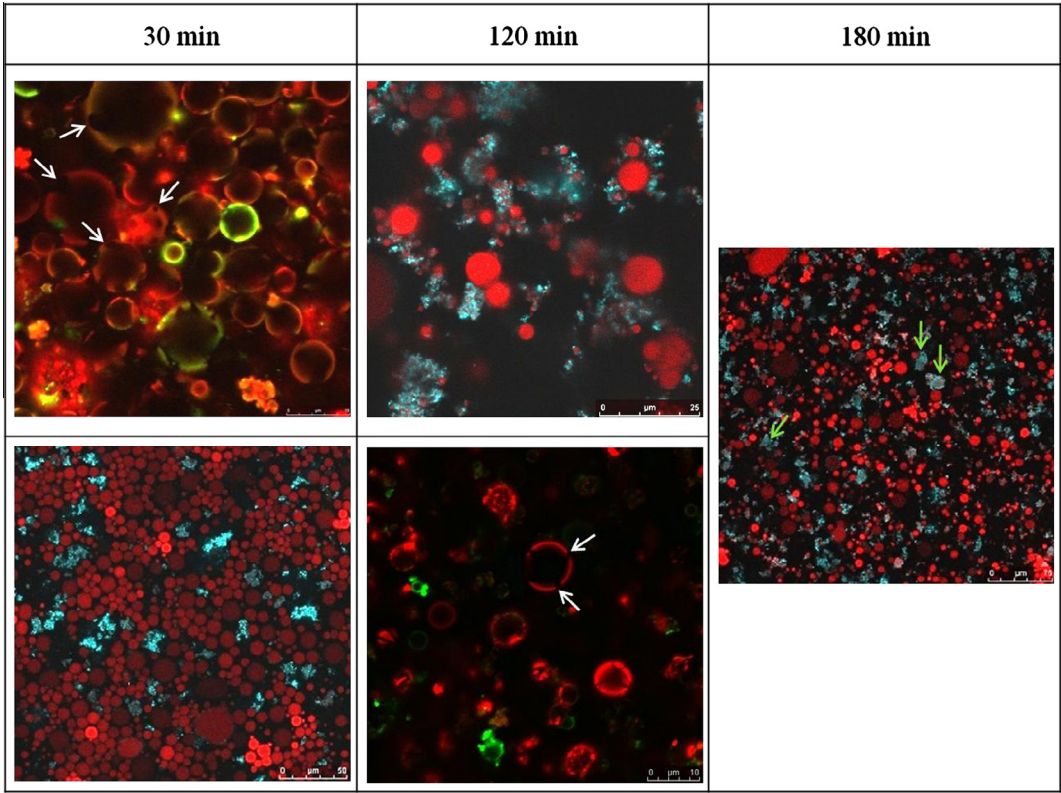


Fig. 4. CLSM images of the gastric chyme collected from rats 30, 120 and 180 min after gavaging with cream derived from pasteurised and homogenised milk. The lipids were stained with Nile Red (red), the proteins with Fast Green FCF (blue), the glycoproteins and glycolipids with WGA (green) and the phospholipids with Rd-DHPE (red at the oil–water interface). The white arrows are pointing at liquid-ordered domains and the green arrows are pointing at protein aggregate containing fat globules. Scale bars = 10 μm (30 min top and 120 min bottom), 25 μm (30 min bottom), 50 μm (120 min top) and 75 μm (180 min). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MFGM glycoproteins and phospholipids are thought to also play a role in maintaining the integrity of the MFGM during gastric digestion (Hamosh et al., 1999).

3.3.2. DIC

Using DIC, interesting features were observed on the surface of the RC and PC chyme globules (Figs. 2 and 3 respectively). Some needle-shaped crystals appeared on the surface of the PC chyme globules after 30 min (Fig. 3). After 2 and 3 h, the surface of the globules became irregular, with small protrusions. This was also observed on the surface of the RC chyme globules after 3 h (Fig. 2), but no protrusions were observed on the surface of the PHC chyme globules within the 3 h period. The protrusions are similar to the 200 nm spherical clusters, trapping the gastric lipase and being responsible for its inhibition, observed by Pafumi et al. (2002). Pafumi et al. (2002) studied the *in vitro* digestion of triolein droplets emulsified with phospholipids and cholesterol and

hydrolysed with human gastric lipase. Using scanning electron microscopy, they observed spherical protrusions at the surface of hydrolysed oil droplets, and they confirmed, using fluorescence microscopy, the presence of fluorescently-labelled gastric lipase inside the protrusions composed mostly of fluorescently-labelled free fatty acids.

3.3.3. TEM

The chyme samples taken after 3 h post-gavage for the 3 cream preparations were analysed using TEM (Fig. 5). Some globules had an intact interface (Fig. 5B, D and F). Fig. 5F shows some globules in a protein matrix as seen in Fig. 4. Lamellar phases were seen at the surface of some RC and PC chyme globules (Fig. 5A and C respectively) and are the protrusions seen in Figs. 2 and 3. They are most likely to be composed of the lipolytic products and phospholipids from the MFGM and the gastric mucosa, which are swelling amphiphiles and tend to form lamellar phases at the oil–water interface.

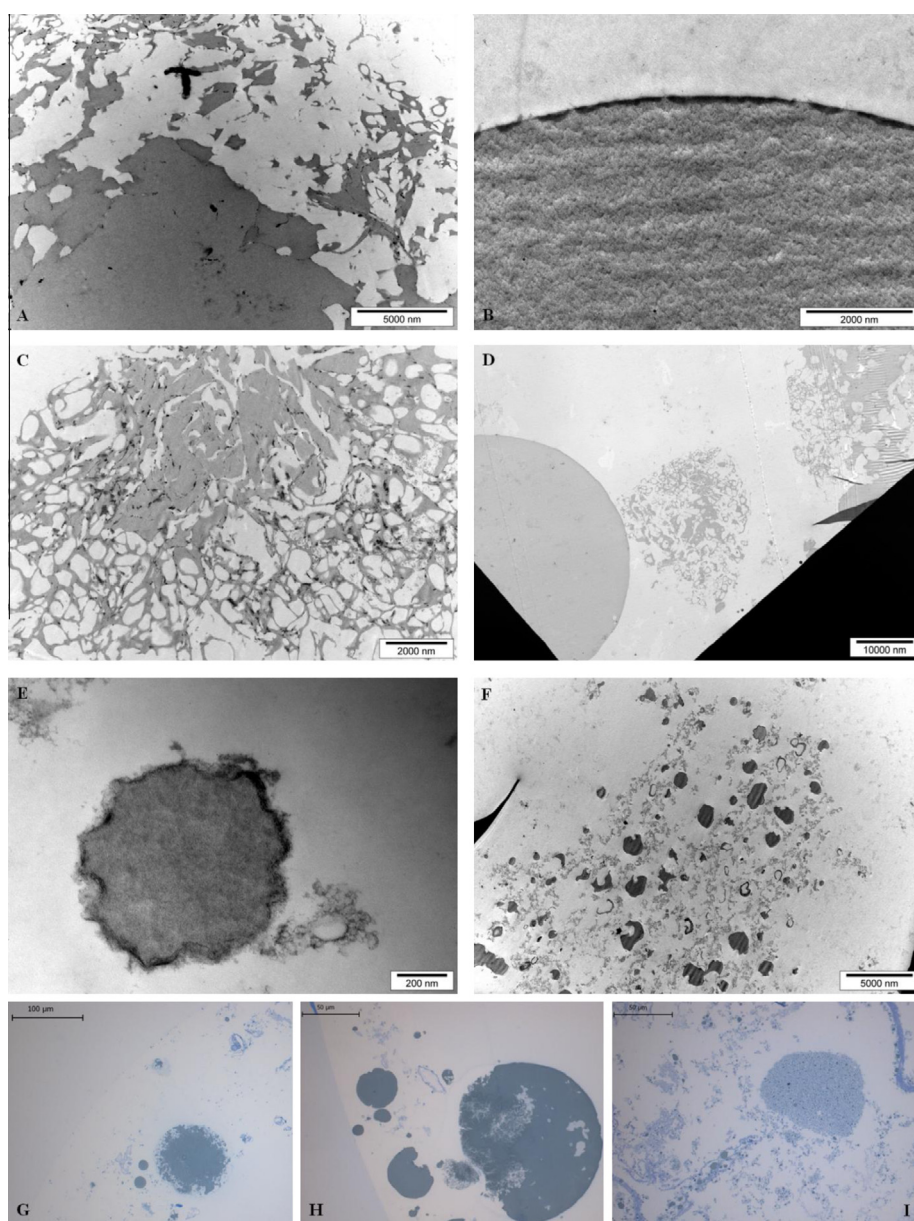


Fig. 5. TEM (A–F) and light microscopy (G–I) images of the gastric chyme collected from rats 180 min after gavaging with cream derived from raw milk (A, B and G), pasteurised milk (C, D and H) or pasteurised and homogenised milk (E, F and I). Scale bars = 200 nm (E), 2000 nm (B and C), 5000 nm (A and F), 10,000 nm (D), 50 μm (H and I), and 100 μm (G).

Similarly, Berendsen and Blanchettemackie (1979) observed lamellar structures at the surface of milk globules in the stomach of 10-day-old suckled rats. Some dark crystals (Fig. 5A and C) were observed in the lamellar phase and could be due to the presence of high melting point fatty acid-containing lipolytic products.

Overall, milk processing and the ultrastructure of the milk fat globules appeared to affect the lipolysis of the milk fat globules. The hydrolysis of fat and proteins of the cream samples in the stomach led to different microstructures of the chyme samples. The formation of the lamellar structures or spherical protrusions at the surface of fat globules during digestion not only inhibits the lipolysis process by gastric lipase but also displaces some interfacial material by competition of the free fatty acids accumulating at the interface with phospholipids and proteins originally surrounding the fat globules. Pafumi et al. (2002) reported the protrusions to be mainly composed of protonated free LCFA (73.7%) but also phospholipids, monoglycerides, diglycerides, free cholesterol and triglycerides. LCFA and monoglycerides have a low aqueous solubility (Hernell, Staggers, & Carey, 1990) and accumulate at the interface in gastric conditions. Pafumi et al. (2002) explained that, as lipolysis proceeds, the free fatty acid-rich interfacial areas form spherical particles, trapping the gastric lipase and preventing its access to the triglyceride core. A smaller size of globules leads to a larger specific surface area and thus a delay in the inhibition of gastric lipase, possibly explaining why no protrusions were observed at the surface of the PHC chyme globules. The faster appearance of the protrusions on the PC chyme globules (Fig. 3) is consistent with the greater amounts of free LCFA (Table 1) and greater apparent protein hydrolysis (Fig. 1). Under acidic conditions, free fatty acids are protonated and are not expected to complex significantly with calcium to form soaps (Carey et al., 1983).

In vitro and *in vivo* studies have shown that small droplets are digested to a greater extent than larger droplets because of an increase in surface area (Armand et al., 1999; Borel et al., 1994) and a delayed inhibition of gastric lipase activity (Pafumi et al., 2002). In the presently reported study however, it was evident that the ultrastructure of the fat, including the interfacial composition and structure of the fat globules, affected the digestion of the cream samples. The PHC, which contained smaller fat globules, appeared to be digested to a lesser extent than RC and PC, which contained larger fat globules. The latter result may have been due to the formation of protein aggregates trapping very small PHC fat globules and preventing the gastric lipase from accessing them. A similar finding has been reported by Armand et al. (1996) who compared the digestion of human milk fat globules with the digestion of smaller droplets from infant formula. The digestion of the former was more efficient because of the presence of the MFGM possibly facilitating the access of gastric lipase to the triglyceride core or specific MFGM phospholipid–gastric lipase interactions (Michalski, 2009). The bovine and human native milk fat globules have a similar interfacial structure with a phospholipid trilayer being the backbone of the membrane with embedded proteins and glycoproteins. The presence of mostly proteins at the surface of infant milk-formula lipid droplets and homogenised milk fat globules may slow down their gastric digestion.

4. Conclusions

The composition and structure of stomach chyme from rats which had been gavaged with cream from raw, pasteurised or pasteurised and homogenised milk revealed differences in their digestion, possibly as a function of milk fat globule ultrastructure and interfacial composition. Processing of milk had an influence on the digestibility of milk fat globules in the rat stomach. Cream proteins appeared to be less digested after pasteurisation and

homogenisation, whereas pasteurisation appeared to lead to greater apparent lipolysis. However, the protein and fatty acid analyses did not take into account potential differences in the gastric emptying rate of each cream preparation. The size of the globules in all samples increased in acidic gastric conditions, and accumulation of lipolytic products was visible at the surface of the fat globules in the chyme of rats gavaged with raw or pasteurised creams. The inhibition of gastric lipase was delayed by a reduction in size of the globules. Gastric digestion is an important step in the gastrointestinal digestion of milk fat globules and can be delayed or accelerated by changing the size or the oil–water interface respectively.

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