Food Chemistry 141 (2013) 3215–3223

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

In vivo digestion of bovine milk fat globules: Effect of processing and interfacial structural changes. II. Upper digestive tract digestion

Sophie Gallier *, Xiang Q. Zhu, Shane M. Rutherfurd, Aiqian Ye, Paul J. Moughan, Harjinder Singh

Riddet Institute, Massey University, Private Bag 11 222, Palmerston North 4442, New Zealand

ARTICLE INFO

Article history:
Available online 14 June 2013

Keywords:
Bovine cream
Milk fat globule membrane
In vivo intestinal digestion
Confocal microscopy
Transmission electron microscopy

ABSTRACT

The aim of this research was to study the effect of milk processing on the *in vivo* upper digestive tract digestion of milk fat globules. Fasted rats were serially gavaged over a 5 h period with cream from raw, pasteurised, or pasteurised and homogenised milk. Only a few intact dietary proteins and peptides were present in the small intestinal digesta. Significantly ($P < 0.05$) more longer chain ($C \geqslant 10$) fatty acids were present in the digesta of rats gavaged with raw (448 mg g^{-1} digesta dry matter (DDM)) and homogenised creams (528 mg g^{-1} DDM), as compared to pasteurised and homogenised cream (249 mg g^{-1} DDM). Microscopy techniques were used to investigate the structural changes during digestion. Liquid–crystalline lamellar phases surrounding the fat globules, fatty acid soap crystals and lipid–mucin interactions were evident in all small intestinal digesta. Overall, the pasteurised and homogenised cream appeared to be digested to a greater extent.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Milk is an important food in the human diet, not only as the sole source of nutrition for infants but also as a source of energy in the form of fat, protein and carbohydrate for children and adults. Milk fat has a highly diverse fatty acid profile containing more than 400 different fatty acids (Michalski, 2009). Milk contains 98% of its triglycerides in the form of globules surrounded by a complex interface, the milk fat globule membrane (MFGM), a trilayer of phospholipids with proteins, glycoproteins, cholesterol, glycolipids and vitamins. The diameter of the milk fat globules ranges between 0.2 and 15 μm, with an average diameter of 4 μm (Michalski, Michel, Sainmont, & Briard, 2002). Recent studies have focused on the structure and composition of the MFGM (Dewettinck et al., 2008; Gallier, Gragson, Jimenez-Flores, & Everett, 2010; Gallier, Gragson, Jiménez-Flores, & Everett, 2012; Vanderghem et al., 2011). The main MFGM proteins are mucin 1 (MUC 1; 200 kDa), xanthine oxidase (XO; 150 kDa), cluster of differentiation (CD) 36 (76–78 kDa), butyrophilin (BTN; 67 kDa), adipophilin (52 kDa) co-migrating with periodic acid Schiff (PAS) 6/7 (48–54 kDa) and fatty acid binding protein (13 kDa). MUC1 plays an important role in the intestinal immune system. It is able to inhibit infection by rotavirus and common enteropathogenic bacteria and prevent mother–child transmission of HIV (Le et al., 2012). PAS 6/7 and CD36 also improve the intestinal immune system and bind to pathogens. Therefore, the resistance of MFGM glycoproteins to digestion or their partial degradation into bioactive peptides during digestion is critical for their action in the gastrointestinal tract.

Raw milk is usually processed for safe human consumption and extended shelf-life. Commercially available milk is commonly pasteurised and homogenised. Thermal treatments such as pasteurisation and ultra-high temperature processing lead to denaturation of some MFGM proteins (Ye, Singh, Taylor, & Anema, 2004) and interactions between the MFGM and whey proteins (Michalski, 2009). homogenisation involves the rupture of the MFGM and a concomitant reduction in the size of the globules to less than 1 μm, thus creating a larger oil–water interface. As the MFGM is not present in sufficient quantity to cover this new interface, caseins are able to adsorb onto the interface (Michalski, 2009). The changes in composition and structure of the milk fat globule surface that occur during heat treatment or homogenisation will also have an impact on interfacial processes such as interactions with the food matrix and the process of lipolysis.

The process of triglyceride digestion in the gastrointestinal tract begins with lingual or gastric lipase (depending on the species) which hydrolyses 5–40% of the dietary lipids in the stomach and 7.5% in the duodenum. Pancreatic lipase then hydrolyses much of

Abbreviations: DDM, digesta dry matter; 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; FABP, fatty acid binding protein; LCFA, long chain fatty acid; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; DIC, differential interference contrast; CLSM, confocal laser scanning microscopy; TEM, transmission electron microscopy.

* Corresponding author. Tel.: +64 (0)6 356 9099x81612; fax: +64 (0)6 350 5655.
E-mail address: S.Gallier@massey.ac.nz (S. Gallier).

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.06.019

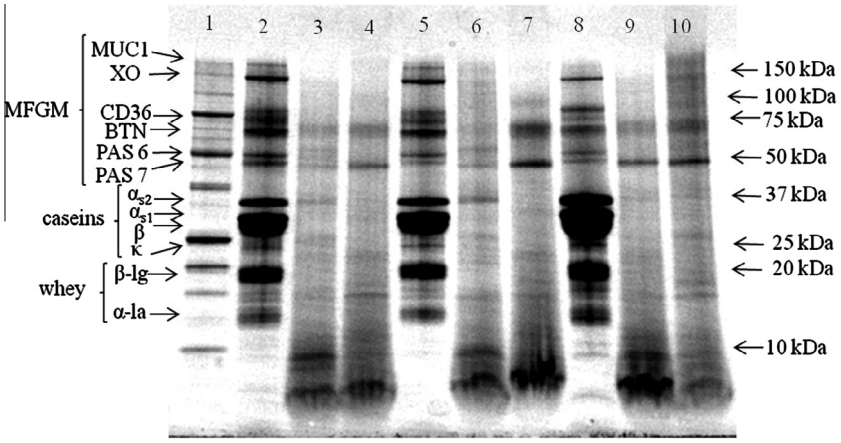

Fig. 1. SDS–PAGE patterns of cream prepared from raw milk (RC), pasteurised milk (PC) and pasteurised and homogenised milk (PHC) and the intestinal digesta of rats after being gavaged with one of each of the cream preparations. Proteins were stained with Coomassie Blue. Lane 1, protein standards; lane 2, RC; lane 3, upper small intestinal contents of the rats receiving RC; lane 4, lower small intestinal contents of the rats receiving RC; lane 5, PC; lane 6, upper small intestinal contents of the rats receiving PC; lane 7, lower small intestinal contents of the rats receiving PC; lane 8, PHC; lane 9, upper small intestinal contents of the rats receiving PHC; lane 10, lower small intestinal contents of the rats receiving PHC. MFGM, milk fat globule membrane, MUC1, mucin 1; XO, xanthine oxidase; PAS, periodic acid Schiff; CD36, cluster of differentiation 36; BTN, butyrophilin; β-lg, β-lactoglobulin; α-la, α-lactalbumin.

the remaining dietary lipids (40–70%) in the small intestine. Lipids remaining undigested may be subjected to fermentation in the large intestine. Other active lipases are present in pancreatic secretions, such as human pancreatic lipase-related protein 2, carboxyl ester lipase and phospholipase A_2 (Carey, Small, & Bliss, 1983). The pancreatic secretion also contains proteases such as trypsin and α-chymotrypsin. Gastric lipase acts preferentially on fatty acids in the *sn*-3 position of the triglyceride backbone whereas pancreatic lipase is specific to the *sn*-1 and *sn*-3 positions (N'Goma, Amara, Dridi, Jannin, and Carrière, 2012). In the stomach, lipolytic products accumulate at the oil–water interface and are responsible for the emulsification of fat droplets. In the small intestine, bile salts adsorb onto the oil–water interface and solubilise lipolytic products into mixed micelles. Bile salts are able to displace proteins from an oil–water interface by orogenic mechanisms, interact with polar lipids at the interface (Wilde & Chu, 2011) and act as stabilizers of fat droplets in the duodenum (Michalski, 2009). Conjugated bile salts in physiological concentrations are able to displace the MFGM from the surface of the milk fat globule (Patton, Borgstrom, Stemberger, & Welsch, 1986). The formation of free fatty acids from gastric lipolysis promotes the activation of the pancreatic lipase–colipase complex (Michalski, 2009). Vesicles and mixed micelles transport lipolytic products to be absorbed by the enterocytes (Michalski, 2009). Short and medium chain fatty acids are absorbed into the portal system, whereas long chain fatty acids (LCFA) are assembled into lipoproteins and are transported through the lymphatic system. However, some LCFA may be precipitated into insoluble calcium soaps, reducing their absorption (Michalski, 2009).

As lipolysis is an interfacial process, the ultrastructure of the milk fat globules is likely to play a key role in the digestion and absorption of milk fat. Most studies investigating the digestion of milk fat globules have used *in vitro* digestion models with (Gallier, Ye, & Singh, 2012) or without (Berton, Sebban-Kreuzer, Rouvellac, Lopez, & Crenon, 2009) the gastric digestion step. However, *in vitro* models are a crude simulation of the complex process of digestion that occurs in the gastrointestinal tract and at best provide a simplified but standardised experimental system. The aim here was to use an *in vivo* model (the growing rat) to allow an initial investigation of the effect of milk processing on the digestion of milk fat globules in the upper digestive tract (mouth to terminal ileum). To that end, creams obtained from raw milk, pasteurised milk, and pasteurised and homogenised milk were used as concentrated sources of milk fat globules that had undergone different processing conditions. A study describing the gastric digestion of milk fat globules alone is the subject of a related study (Gallier et al., 2013).

2. Materials and methods

2.1. Samples and reagents

Milli-Q water (18.2 MΩ cm) was purified by treatment with a Milli-Q apparatus (Millipore Corporation, Bedford, MA). 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). One half of the milk was kept at 4 °C overnight. The other half was subjected to pasteurisation at 63 °C for 30 min. 1 L of the pasteurised milk was homogenised at 10/5 MPa by two passes. The pasteurised milk and pasteurised and homogenised milk were then kept overnight at 4 °C. The particle size distribution of the three milk preparations was determined using a Malvern Mastersizer MSE (Malvern Instruments Ltd.). Raw milk had a d_{43} of 4.7 μm, a d_{32} of 3.1 μm, and a surface area of 2.07 $m^2\,g^{-1}$, pasteurised milk had a d_{43} of 4.7 μm, a d_{32} of 3.1 μm, and a surface area of 2.07 $m^2\,g^{-1}$, 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 $m^2\,g^{-1}$.

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

2.2. Rat study

Ethics approval for the study was obtained from the Massey University Animal Ethics Committee.

Eighteen male Sprague Dawley rats of approximately 300 g bodyweight were obtained from the Small Animal Unit, Massey University, and housed individually in stainless steel wire-bottomed cages in a room maintained at 22 ± 2 °C, with a 12 h light/dark cycle. The rats were fasted for 24 h prior to taking small intestinal digesta samples to minimise residual food material present in

the small intestine. 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 6 rats per lot were gavaged with either RC, PC or PHC. An indigestible marker was not included in the cream preparations, since it was found that even the gentle mixing required to evenly distribute the marker into the cream preparations caused the cream to turn into butter and thus become unsuitable for the study. Water was removed 2 h prior to the rats being gavaged hourly, over a total period of five hours, with 2 ml of their respective cream preparation (10 ml gavaged in total). After the 5 h gavaging period, the rats were euthanised by asphyxiation with carbon dioxide gas and then decapitated. The body cavity was opened and the small intestine carefully removed. The small intestine was rinsed with reverse osmosis water and dried with a paper towel. The small intestine was dissected at the midpoint into two equal segments. The resulting intestinal segments (upper small intestine and lower small intestine) were dissected open and the digesta carefully removed with a spatula making sure not to disturb the mucosal layer nor disrupt the digesta macrostructure. An aliquot of each fresh digesta sample was then immediately examined by microscopy. The remaining digesta were mixed and heated at 95 °C for 10 min to inactivate digestive enzymes, freeze-dried and stored at −20 °C prior to analysis for fatty acids and proteins.

2.3. Fatty acid release

Total and bound fatty acids in the cream samples and small intestinal digesta samples were determined as described previously (Zhu, Svendsen, Jaepelt, Moughan, & Rutherfurd, 2011). The *in vivo* concentration data are very tentative, given there was no marker and concentrations cannot be related back to the diet.

2.4. Protein hydrolysis

Cream samples (150 mg) and intestinal digesta (50 mg) were defatted with petroleum ether. The proteins of the defatted samples were analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) as described by Gallier and Singh (2012). The gels were stained with Coomassie Blue which stains proteins in general (Gallier & Singh, 2012) or PAS which stains glycoproteins (Zachariu, Zell, Morrison, & Woodlock, 1969).

2.5. Confocal laser scanning microscopy (CLSM)

The microstructure of intestinal contents was studied using a confocal laser scanning microscope (Leica DM6000B; Heidelberg, Germany) with a 63 mm oil immersion objective lens and using the scan differential interference contrast (DIC) mode.

2.6. Transmission electron microscopy (TEM)

The structure of the intestinal digesta was investigated using a Philips 201C transmission electron microscope (Philips, The Netherlands). After injection into freshly made 3% agarose, the intestinal samples were prepared as described by Gallier, Tate, and Singh (2013).

2.7. Statistical analysis

Fatty acid contents were compared across cream preparations using a repeated measures analysis of variance where the gastrointestinal tract segment (upper small intestine and lower small intestine) was the repeated factor (GLM Procedure; SAS 2007). Where overall significant ($P < 0.05$) differences were observed individual treatment means were compared using the Tukey test.

Table 1
Mean ($n = 6$) total fatty acid concentrations (mg g^{-1} digesta dry matter) in upper and lower small intestinal (SI) digesta samples collected from rats gavaged with either raw cream (RC), pasteurised cream (PC) or pasteurised and homogenised cream (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
Cream	RC	3.75[b]	2.65[b]	9.51[a]	15.93[a]	59.03	179.65	62.60[ab]	104.85	15.94
	PC	7.29[a]	4.84[a]	13.57[a]	20.13[a]	71.11	206.58	69.87[a]	124.01	18.37
	PHC	1.07[b]	0.86[b]	3.46[b]	6.65[b]	28.05	110.76	42.22[b]	46.52	8.82
Overall SE[A]		0.85	0.56	1.37	2.01	6.77	16.97	4.71	11.43	1.55
Location	Upper SI	4.55	3.42[a]	10.29[a]	16.23[a]	57.58	170.86	55.16	95.28	14.58
	Lower SI	3.52	2.32[b]	7.40[b]	12.25[b]	47.87	160.67	61.22	88.30	14.19
Overall SE[A]		0.53	0.36	0.88	1.27	4.27	10.89	3.35	7.49	1.00
Cream x location	RC, upper SI	4.57	3.21	11.51	19.11	68.45[ac]	197.22[ab]	63.85	115.34[ab]	17.17[ab]
	RC, lower SI[B]	2.93	2.09	7.51	12.76	49.61[bd]	162.08[abc]	61.36	94.36[ab]	14.72[abc]
	PC, upper SI	7.64	5.28	14.66	21.91	69.60[abc]	190.27[ab]	59.95	112.96[ab]	16.56[ab]
	PC, lower SI	6.96	4.39	12.48	19.35	72.61[ab]	222.89[a]	77.79	135.06[a]	20.20[a]
	PHC, upper SI[C]	1.45	1.22	4.70	8.65	34.71[cd]	125.08[bc]	46.37	57.56[bc]	10.00[bc]
	PHC, lower SI[B]	0.66	0.49	2.23	4.65	21.40[d]	96.44[c]	44.07	35.49[c]	7.64[c]
Overall SE[A]		0.92	0.62	1.51	2.20	7.37	18.80	5.77	12.91	1.72
Statistics										
Cream		***	***	***	**	**	**	***	**	**
Location		NS	*	**	**	*	NS	NS	NS	NS
Cream x location		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 within each column for cream type, intestinal location and the interaction between cream type and location separately denote significantly different means.
[A] Overall standard error of the mean.
[B] $n = 5$ due to insufficient digesta material.
[C] $n = 4$ due to insufficient digesta material.

3. Results and discussion

3.1. Protein hydrolysis

The SDS–PAGE patterns for the cream samples and upper and lower small intestinal contents of rats gavaged with the three cream preparations are presented in Fig. 1. The RC contained a slightly higher amount of MFGM proteins whereas the PHC appeared to have greater amounts of caseins and whey proteins. The bands for most of the proteins present in the original cream preparations were not detected in the intestinal digesta samples. The protein bands that were observed in the upper small intestinal digesta had molecular weights of 70, 50, 52, 48, 35 and 8 kDa (rats gavaged with RC), 70, 52, 48, 35, 15 and 8 kDa (rats gavaged with PC) and 70, 48 and 8 kDa (rats gavaged with PHC), whereas in the lower small intestinal digesta the molecular weights of the protein bands were 70, 48 and 15 kDa (rats gavaged with RC), 100, 70 and 48 kDa (rats gavaged with PC) and 150, 70, 48, 27, 18 and 15 kDa (rats gavaged with PHC). Some of these bands most likely represent endogenous proteins, including digestive enzymes such as pepsin (35 kDa), α-amylase (51–54 kDa), trypsin (23.3 kDa) and chymotrypsin (26 kDa) (Boyer, 1971). The protein band at 48 kDa could have been intact PAS 7 for which the band intensity was higher in the lower small intestinal digesta of rats gavaged with PC and PHC compared to those gavaged with RC. During an *in vitro* gastrointestinal digestion of MFGM fragments, Le et al. (2012) detected two protein bands at 43 and 46 kDa, related to PAS 6 and 7. The protein band at 70 kDa could be partially hydrolysed MUC1. Overall, the gastric resistant proteins and peptides formed in stomach chyme samples from rats fed the RC, PC and PHC preparations (Gallier et al., 2013. Unpublished results) appeared to be almost fully hydrolysed by the intestinal proteases in the upper and lower small intestines regardless of the cream preparation the rats received (Fig. 1).

For the SDS–PAGE patterns stained with PAS (results not shown), high molecular weight proteins appeared on the top of the gel (above 150 kDa) for all intestinal digesta samples but could not be well resolved; they are likely to be either mucins from the MFGM (intact or partially hydrolysed MUC1) or endogenous mucin secreted into the gastrointestinal tract, or a combination of the two. MUC1 has been shown to resist gastrointestinal digestion both *in vivo* (infants) (Hamosh et al., 1999) and *in vitro* (Le et al., 2012). Moreover, 200 kDa fragments of MUC1 have also been found in the faeces of infants (Ward, German, & Corredig, 2006) fed human milk but not when fed infant-milk formula, which has smaller lipid droplets covered by milk proteins. All small intestinal digesta samples contained an intense band at 70–80 kDa with higher intensity in the digesta from the lower half of the small intestine of rats gavaged with PHC (results not shown). This band is possibly a peptide derived from the digestion of CD36. However, a 70 kDa peptide band derived from CD36 has been detected during the *in vitro* digestion of MFGM fragments using simulated gastric conditions but not using simulated intestinal conditions, thus the observed 70 kDa protein band on the PAS-stained SDS–PAGE (results not shown) is likely associated with the presence of endogenous proteins.

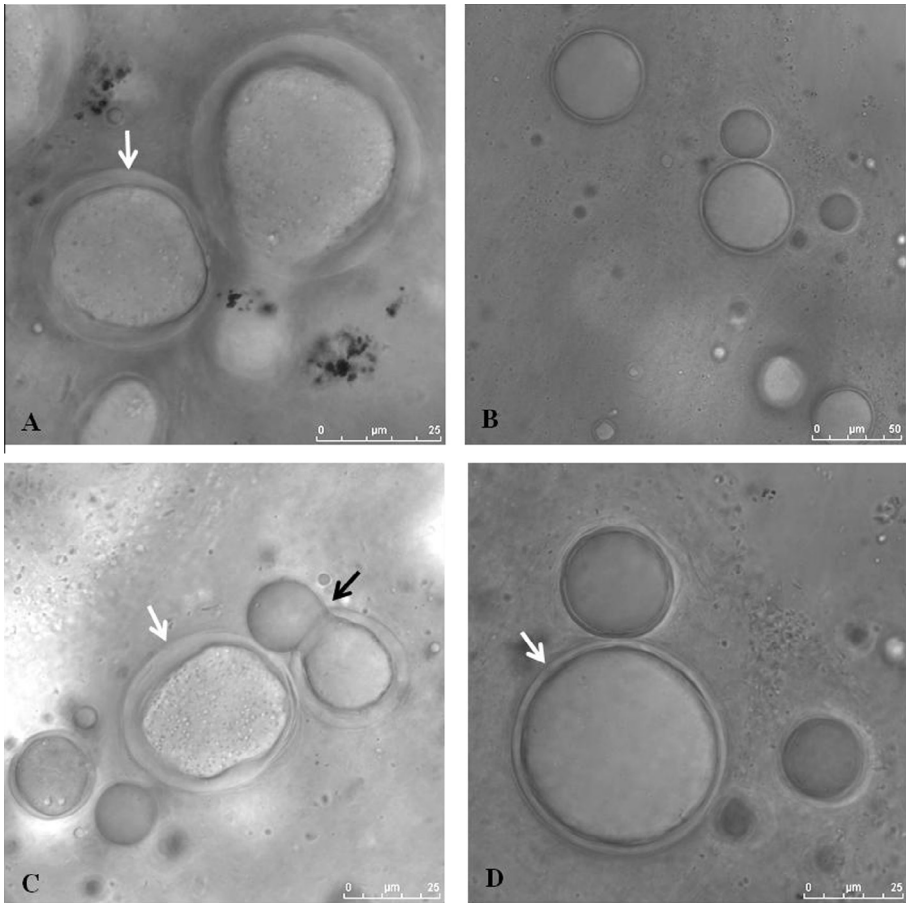

Fig. 2. DIC images of upper (A and C) and lower (B and D) small intestinal digesta of rats gavaged with cream prepared from raw milk. The white arrows demark liquid–crystalline lamellar phases and the black arrows are pointing at the oil coming out of the cracked liquid–crystalline lamellae. Scale bars = 25 µm (A, C and D) and 50 µm (B).

3.2. Intestinal fatty acid content

The total fatty acid content of dry matter for digesta collected from rats gavaged with either RC, PC or PHC is given in Table 1. There was a significant ($P < 0.01$) effect of cream preparation on the fatty acid concentration in the small intestinal digesta for all of the fatty acids examined. The concentrations of caprylic, capric and lauric acids were significantly ($P < 0.05$) higher (32–47% higher) in the upper small intestine compared to the lower small intestine, but there was no significant ($P > 0.05$) effect of intestinal location for caproic, palmitic, stearic, oleic and linoleic acids. The concentrations of caproic and caprylic acids in the small intestinal digesta were significantly ($P < 0.05$) higher (83–94% higher) for the PC as compared to the RC. The concentrations of capric, lauric and stearic acids were not ($P > 0.05$) different between the RC and PC treatments. There was a significant ($P < 0.05$) interaction between cream preparation and intestinal location for myristic, palmitic, oleic and linoleic acids, and the contrast comparison for these data is given in Table 1. The concentrations of myristic, palmitic, oleic and linoleic acids were generally not different ($P > 0.05$) across treatments for the digesta collected from the upper small intestine but were different in digesta collected from the lower small intestine. In the lower small intestine, myristic, palmitic, oleic and linoleic acids concentrations were significantly ($P < 0.05$) lower (43–71% lower) for PHC as compared to PC. Moreover, the concentrations of caproic, caprylic, capric, lauric and stearic acids were significantly ($P < 0.05$) lower (39–85% lower) for PHC compared to PC across both small intestinal regions. Butyric acid was not determined as it is readily absorbed in the small intestine (Michalski, 2009), and if present in the digesta, it would have been lost during freeze-drying of the samples due to its volatility.

In the presently reported study, small intestinal fatty acid concentrations, rather than small intestinal fatty acid flows (where concentrations are related back to dietary intake), were determined due to difficulties in incorporating an indigestible marker (titanium dioxide) into the cream preparations, especially for the PC. Consequently, drawing conclusions about how the preparation of the cream samples impacted on the digestion of fatty acids should be done with caution, as it rests on several assumptions and further work needs to be conducted to confirm the tentative conclusions reached here. Despite the latter important caveat, it would appear that the extent of digestion of longer chain fatty acids, but not shorter chain fatty acids, in the PC was similar to that of the RC. In addition, homogenisation appeared to improve the digestion of fatty acids, as evidenced by the much lower small intestinal fatty acid concentrations observed for the PHC as compared to the PC. The latter result is not unexpected since lipolysis is an oil–water interfacial process, and the larger the surface area (the smaller the oil droplets), the greater the degree of lipolysis. This is in agreement with studies showing that smaller droplets experience greater lipolysis than larger ones (Armand, 2007), but these tentative conclusions need to be corroborated in a more robust digestion study, where the amounts of digesta fatty acids are related to the dietary intakes.

3.3. Microstructural changes

3.3.1. CLSM

The microstructure of the upper and lower small intestinal digesta for the rats gavaged with RC, PC and PHC (RC, PC and PHC upper and lower small intestinal digesta respectively) was revealed using CLSM (Figs. 2–4). Fat globules of RC and PC upper and lower small intestinal contents (Figs. 2 and 3 respectively) were similar in size (5–50 μm) and microstructure, while PHC intestinal contents presented fat globules of a smaller size (2–20 μm). These size distributions for RC, PC and PHC were similar to those observed in the gastric chyme of rats gavaged with equivalent cream preparations (Gallier et al., 2013. Unpublished results) and is also consistent with other *in vivo* studies reporting that the size of the fat droplets in the small intestine was similar to that present in the stomach for human adults fed emulsions of different droplet size and infants fed human milk or infant milk formula, respectively (Armand, 2008). RC, PC and PHC upper small intestinal contents (Figs. 2A, 2C, 3A, 3C, and 4A) showed the expelling of oil droplets, leaving behind a crystalline shell, as also reported by Patton and Carey (1979) studying intestinal digestion of emulsified olive oil droplets using *in vitro* models. The expelled oil is possibly stabilized by the adsorption of biliary salts and phospholipids at the interface. Very few fat globules were found to be present in the PHC lower small intestinal contents (Fig. 4B), possibly due to a greater apparent digestion and absorption of fatty acids from PHC compared to PC and RC.

Most fat globules (Figs. 2–4) were surrounded by liquid–crystalline lamellae likely composed of bile salts, phospholipids and lipolytic products. These lamellae are formed at low concentrations of bile salts and are due to swelling of the amphiphilic lipolytic products, creating water spacing between the lamellae. Lipolytic products can then be solubilised into phospholipid vesicles (Carey et al., 1983). When sufficient bile salts are present (near their critical micellar concentration), they are able to solubilise the lipolytic products into mixed micelles of only a few nanometers in size, which is below the resolution limit of the CLSM. LCFA have poor water solubility at intestinal pH and therefore accumulate at the oil–water interface, forming a liquid–crystalline phase until they are solubilised into micelles or vesicles or precipitated into insoluble soaps in the presence of calcium (Carey et al., 1983). LCFA at the *sn*-1 and *sn*-3 positions are less absorbed as they are saponified as calcium soaps which are excreted in stools, but LCFA at the *sn*-2 position are easily absorbed as 2-monoglycerides (Michalski, 2009). Short and medium chain fatty acids are readily solubilised in vesicles and absorbed through the intestinal walls (Michalski et al., 2006).

In all digesta samples, some dark round spots (as seen in Figs. 2A and 4B) and needle-shaped crystals between 2 and 8 μm in size (as seen in Figs. 3D and 4B) were present. The presence of crystals was predominant in the RC, PC and PHC lower small intestinal contents with a higher concentration of crystals (qualitative observation) in the PHC lower small intestinal contents as compared to the RC and PC lower small intestinal contents. The crystals are likely free fatty acids released after triglyceride digestion and their predominance in the lower small intestine may be due to the higher pH. Needle-shaped crystals of $\geqslant$20 μm in size were also observed by Knutson et al. (2010) in human jejunum aspirates after ingestion of vegetable oil emulsions and were found, based on Raman microscopy, to be composed of LCFA in their acid form. Indeed, at physiological pH, LCFA are fully or at least partially (near half ionisation) protonated (Cistola, Hamilton, Jackson, & Small, 1988). Moreover, saturated LCFA ($\geqslant$16 carbon units) form crystals at body temperature. Therefore, the crystals observed in Figs. 3D and 4B are possibly a mixture of fatty acid crystals and fatty acid soap crystals, as both can coexist at physiological pH (Cistola et al., 1988). The presence of galactolipids and phospholipids, both MFGM components, are known to play a role in crystal formation (Knutson et al., 2010). The formation of calcium-fatty acid soaps has been shown to reduce calcium absorption and is influenced by fatty acid chain length and degree of saturation (Gacs & Barltrop, 1977). Knutson et al. (2010) hypothesised that the presence of crystals in the ileum is involved in increased satiety by activation of the ileal brake. Therefore the apparent greater presence of crystals in the PHC lower small intestinal contents could be associated with an increased activation of the ileal brake, which in turn could lead to an increase in the gastrointestinal transit time of PHC.

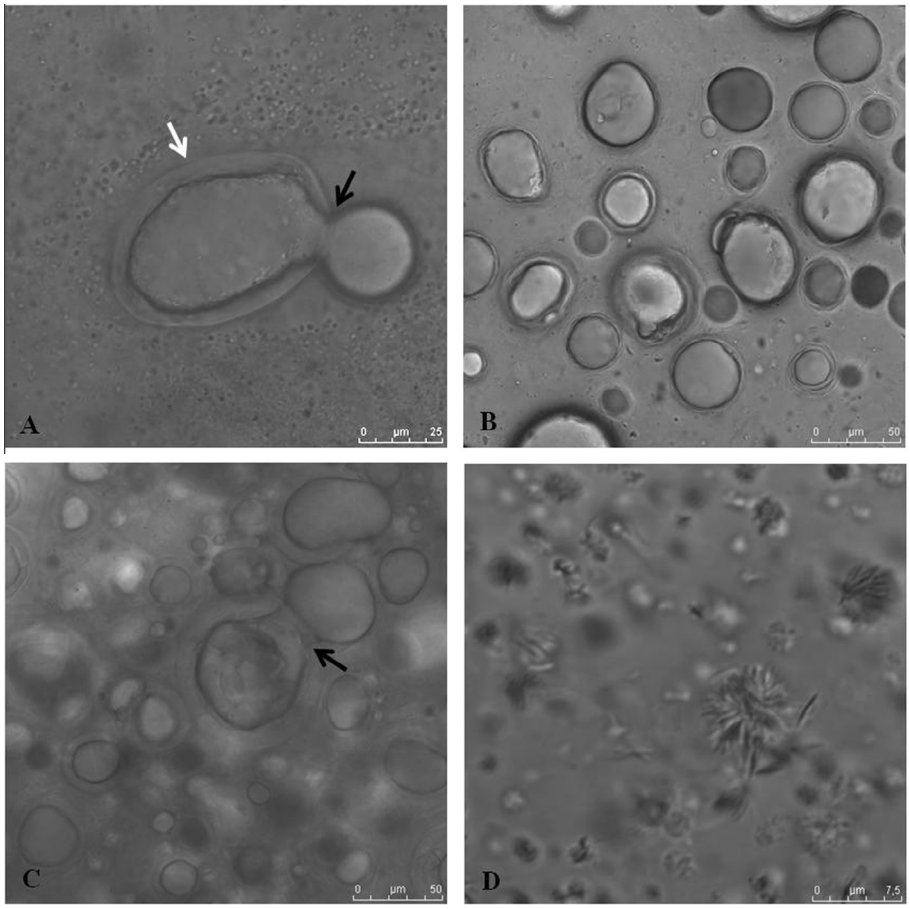

Fig. 3. DIC images of upper (A and C) and lower (B and D) small intestinal digesta of rats gavaged with cream prepared from pasteurised milk. The white arrows demark at liquid–crystalline lamellar phases and the black arrows oil coming out of the cracked liquid–crystalline lamellae. Scale bars = 25 µm (A), 50 µm (B and C) and 7.5 µm (D).

3.3.2. TEM

The upper and lower small intestinal contents of the rats gavaged with either RC, PC or PHC were also analysed using TEM (Fig. 5). The liquid–crystalline phase observed at the oil–water interface of fat globules with CLSM (Figs. 2–4) was also observed with TEM (Fig. 5A, D, G–J). These TEM images showed the formation of multilayers of lipolytic products. Using an *in vitro* model, Rigler, Honkanen, and Patton (1986) reported a layer thickness of 4–5 nm with water spacing of 0–8 nm. In Fig. 5A, D, G–J, the space between layers is much larger possibly because of the harsh drying conditions during the sample preparation. A remnant crystalline shell was observed in Fig. 5J similar to the shell remaining after the fat was expelled as observed in Fig. 4A. The presence of a liquid–crystalline lamellar phase at the surface of lipid droplets during *in vitro* intestinal digestion, due to the accumulation of lipolytic products at the oil–water interface, was first reported by Patton and Carey (1979). Recently, we have reported the presence of a similar liquid–crystalline phase at the surface of milk fat globules during the intestinal digestion of raw milk using an *in vitro* model (Gallier et al., 2012). During the gastric digestion of the cream samples, a liquid–crystalline phase of lipolytic products, possibly trapping the gastric lipase (Pafumi et al., 2002), was observed (Gallier et al., 2013. Unpublished results). Upon reaching the duodenum, this phase is likely to be rapidly solubilised by biliary phospholipids and salts, and pancreatic lipase starts hydrolysing triglycerides and diglycerides leading to a newly formed liquid–crystalline phase of lipolytic products, such as that observed in the present study.

The multilamellar and unilamellar vesicles transporting the lipolytic products were observed in Fig. 5B, C and L, and had a size in the range of 50–200 nm. Some vesicles, formed from the liquid–crystalline layer surrounding the fat globules, had a size of between 40 and 100 nm (Fig. 5G). It was not possible to observe the mixed bile salt micelles using TEM due to limitations in resolution. The sizes of the micelles and multilamellar and unilamellar vesicles determined *in vivo*, and reported in the literature, vary from 3 to 20 nm for the micelles (Armand et al., 1999; Carey et al., 1983), up to 600 nm for the multilamellar vesicles (Muellertz, Fatouros, Smith, Vertzoni, & Reppas, 2012) and 13–200 nm for the unilamellar vesicles (Armand et al., 1999; Carey et al., 1983; Muellertz et al., 2012). It is believed that the multilamellar vesicles are shed from the liquid–crystalline lamellar phase, then form smaller unilamellar vesicles which form mixed micelles in the presence of bile salts (Rigler et al., 1986). Some dark elongated crystals, often layered (Fig. 5D, G–I), were present at the oil–water interface, and could be composed of calcium–fatty acid soaps (Sassene et al., 2010). However, further investigation is necessary to confirm their nature.

Round electron-dense structures (Figs. 5E, F and I), associated with the interface of the fat globules in the small intestinal digesta of rats fed with PC and PHC, are possibly mucins and may also be the dark round spots observed in Figs. 2A and 4B. They appear to be composed of small subunits. The mucins have a size ranging from 200 and 800 nm, and the subunits were in the range of 10–40 nm (Fig. 5F). Mucins are highly glycosylated (about 80%) proteins with a high molecular weight ranging from 0.5 to 20 MDa (Fefelova, Nurkeeva, Mun, & Khutoryanskiy, 2007). Fefelova et al. (2007) analysed mucin particles in water at pH 6.8 using TEM and reported a similar size and shape as those observed in the PC and PHC small intestinal digesta (Fig. 5E, F and I). Electrostatic interactions and

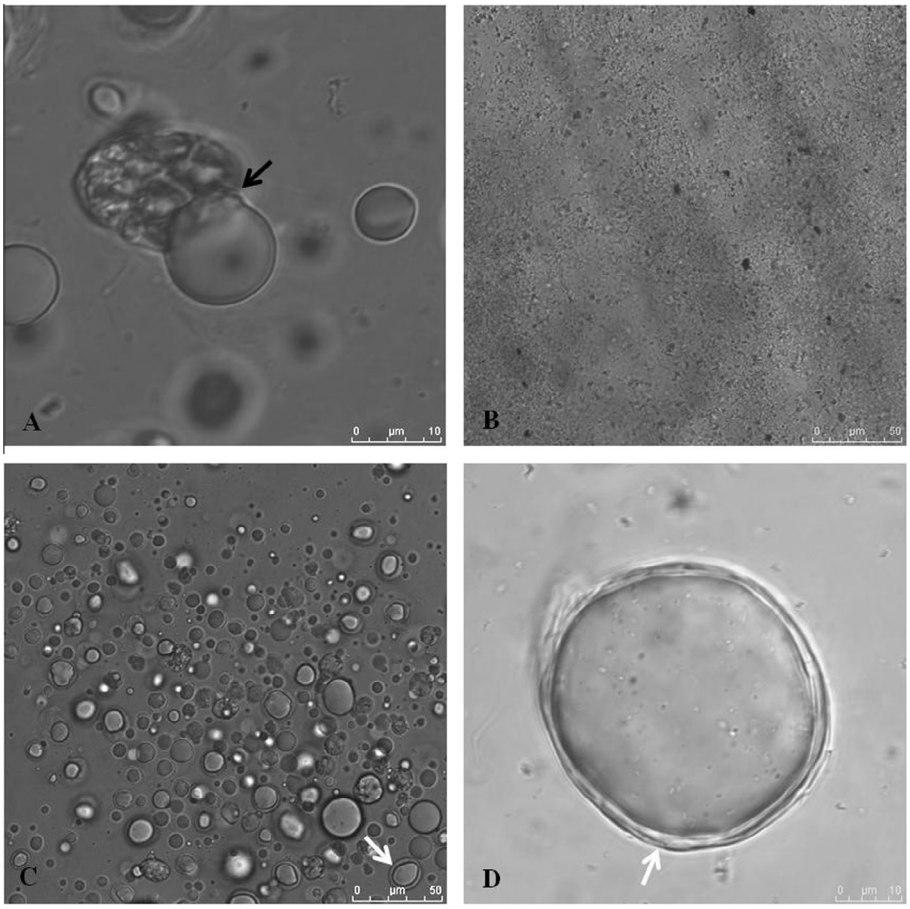

Fig. 4. DIC images of upper (A and C) and lower (B and D) small intestinal digesta of rats gavaged with cream prepared from pasteurised and homogenised milk. The white arrows demark at liquid–crystalline lamellar phases and the black arrows oil coming out of the cracked liquid–crystalline lamellae. Scale bars = 10 µm (A and D) and 50 µm (B and C).

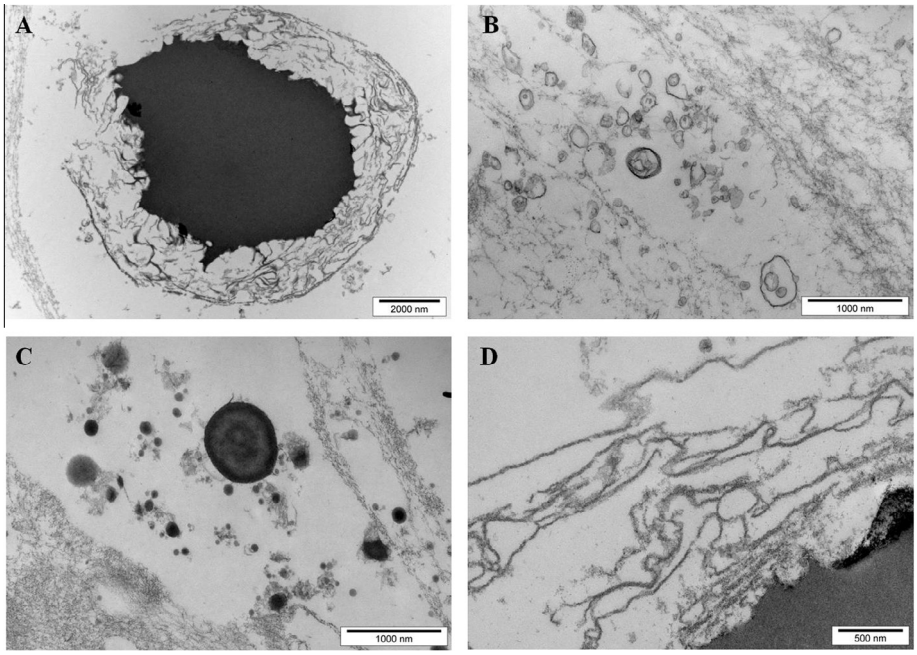

Fig. 5. TEM images of upper (A, B, E, F, I and J) and lower (C, D, G, H, K and L) small intestinal digesta of rats gavaged with cream prepared from raw milk (A–D), pasteurised milk (E–H) or pasteurised and homogenised milk (I–L). Scale bars = 2000 nm (A, F, G and K), 500 nm (D), 1000 nm (B and C), 5000 nm (E, H and I), 10,000 nm (J) and 200 nm (L).

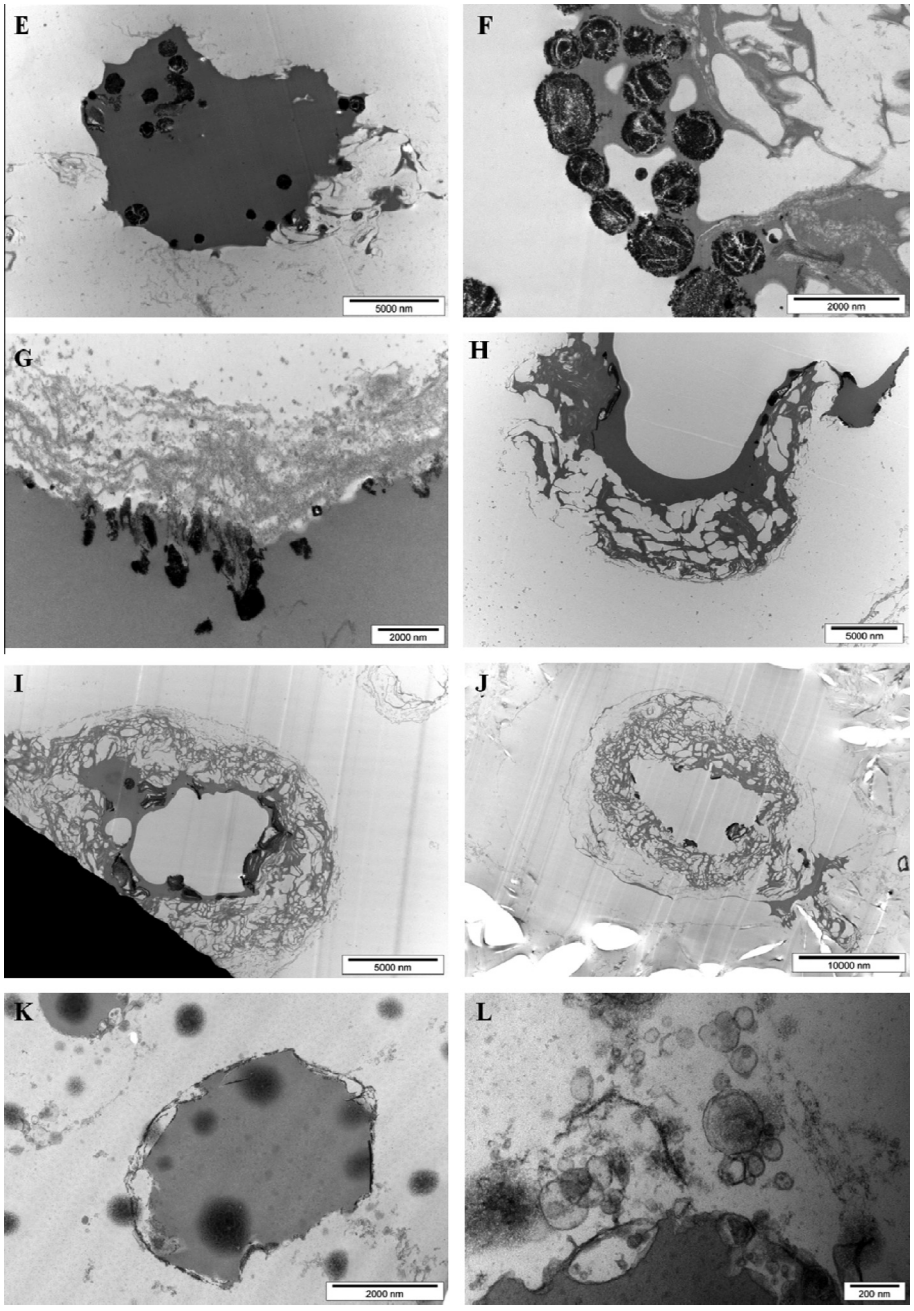

Fig. 5 (*continued*)

hydrophobic adsorption are the main driving factors involved in the mucoadhesive properties of mucins and therefore mucin–lipid interactions (Bansil & Turner, 2006; Fefelova et al., 2007). The intestinal mucins could also interact with the MFGM glycoproteins that are fully or partially resistant to gastrointestinal proteases such as MUC1 and PAS 6/7 (Le et al., 2012). Mucins are rarely introduced in *in vitro* models and perhaps should be included, as mucin–lipid interactions appear to be of physiological importance in the digestion of lipids.

4. Conclusions

Investigation of the composition and microstructure of the small intestinal digesta of rats gavaged with cream from raw, pasteurised, or pasteurised and homogenised milk suggests differences in the digestion of fat globules which may in turn be a function of milk processing. Protein digestion appeared to be complete and similar across cream preparations. It appeared that pasteurisation and homogenisation of the milk fat globules led to a greater degree of lipolysis. Microscopic studies showed the formation of liquid–crystalline layers due to the accumulation of lipolytic products at the surface of the fat globules, the formation of insoluble crystals possibly consisting of a mixture of fatty acids and fatty acid soaps and the interactions of what appear to be mucins with the lipid interface. However all of the latter features were observed in the small intestinal digesta regardless of the cream preparation. From the above results, pasteurisation appeared to decrease the extent of lipolysis of short chain fatty acids, but homogenisation led to an apparently greater lipolysis as well as an increase in crystal formation in the lower half of the small intestine. The latter conclusions are tentative, and further *in vivo* studies

are required whereby fatty acid flows related to food dry matter intake rather than fatty acid concentration point estimates are determined.

Acknowledgements

The authors would like to thank Jack Cui for conducting the SDS–PAGE analysis and Trent Olson for running the animal trial. The authors would like to acknowledge the Manawatu Microscopy and Imaging Centre (MMIC), New Zealand, for access to CLSM and TEM instruments, and Jianyu Chen (MMIC) for the TEM sample preparation. The work was supported by a Centre of Research Excellence fund from the Tertiary Education Commission and the Ministry of Education, New Zealand.

References

Armand, M. (2007). Lipases and lipolysis in the human digestive tract: Where do we stand? *Current Opinion in Clinical Nutrition and Metabolic Care, 10*, 156–164.
Armand, M. (2008). Milk fat digestibility. *Sciences Des Aliments, 28*, 84–98.
Armand, M., Pasquier, B., Andre, M., Borel, P., Senft, M., Peyrot, J., et al. (1999). Digestion and absorption of 2 fat emulsions with different droplet sizes in the human digestive tract. *American Journal of Clinical Nutrition, 70*, 1096–1106.
Bansil, R., & Turner, B. S. (2006). Mucin structure, aggregation, physiological functions and biomedical applications. *Current Opinion in Colloid and Interface Science, 11*, 164–170.
Berton, A., Sebban-Kreuzer, C., Rouvellac, S., Lopez, C., & Crenon, I. (2009). Individual and combined action of pancreatic lipase and pancreatic lipase-related proteins 1 and 2 on native versus homogenized milk fat globules. *Molecular Nutrition and Food Research, 53*, 1592–1602.
Boyer, P. D. (1971). *The enzymes*. New York, NY: Academic Press.
Carey, M. C., Small, D. M., & Bliss, C. M. (1983). Lipid digestion and absorption. *Annual Review of Physiology, 45*, 651–677.
Cistola, D. P., Hamilton, J. A., Jackson, D., & Small, D. M. (1988). Ionization and phase behavior of fatty acids in water: Application of the Gibbs phase rule. *Biochemistry, 27*, 1881–1888.
Dewettinck, K., Rombaut, R., Thienpont, N., Le, T. T., Messens, K., & Van Camp, J. (2008). Nutritional and technological aspects of milk fat globule membrane material. *International Dairy Journal, 18*, 436–457.
Fefelova, N. A., Nurkeeva, Z. S., Mun, G. A., & Khutoryanskiy, V. V. (2007). Mucoadhesive interactions of amphiphilic cationic copolymers based on 2-(methacryloyloxy)ethyl trimethylammonium chloride. *International Journal of Pharmaceutics, 339*, 25–32.
Gacs, G., & Barltrop, D. (1977). Significance of Ca-soap formation for calcium absorption in rat. *Gut, 18*, 64–68.
Gallier, S., Cui, J., Olson, T. D., Rutherfurd, S. M., Ye, A., Moughan, P. J. and Singh, H. 2013. In vivo digestion of bovine milk fat globules: effect of processing and interfacial structural changes. I. Gastric digestion. Unpublished results.
Gallier, S., Gragson, D., Jimenez-Flores, R., & Everett, D. (2010). Using confocal laser scanning microscopy to probe the milk fat globule membrane and associated proteins. *Journal of Agricultural and Food Chemistry, 58*, 4250–4257.
Gallier, S., Gragson, D., Jiménez-Flores, R., & Everett, D. W. (2012). β-Casein–phospholipid monolayers as model systems to understand lipid–protein interactions in the milk fat globule membrane. *International Dairy Journal, 22*, 58–65.
Gallier, S., & Singh, H. (2012). Behavior of almond oil bodies during in vitro gastric and intestinal digestion. *Food and Function, 3*, 547–555.
Gallier, S., Tate, H., & Singh, H. (2013). In vitro gastric and intestinal digestion of a Walnut oil body dispersion. *Journal of Agricultural and Food Chemistry, 61*, 410–417.
Gallier, S., Ye, A., & Singh, H. (2012). Structural changes of bovine milk fat globules during in vitro digestion. *Journal of Dairy Science, 95*, 3579–3592.
Hamosh, M., Peterson, J. A., Henderson, T. R., Scallan, C. D., Kiwan, R., Ceriani, R. L., et al. (1999). Protective function of human milk: The milk fat globule. *Seminars in Perinatology, 23*, 242–249.
Knutson, L., Koenders, D., Fridblom, H., Viberg, A., Sein, A., & Lennernas, H. (2010). Gastrointestinal metabolism of a vegetable-oil emulsion in healthy subjects. *American Journal of Clinical Nutrition, 92*, 515–524.
Le, T. T., Van de Wiele, T., Do, T. N. H., Debyser, G., Struijs, K., Devreese, B., et al. (2012). Stability of milk fat globule membrane proteins toward human enzymatic gastrointestinal digestion. *Journal of Dairy Science, 95*, 2307–2318.
Michalski, M. C. (2009). Specific molecular and colloidal structures of milk fat affecting lipolysis, absorption and postprandial lipemia. *European Journal of Lipid Science and Technology, 111*, 413–431.
Michalski, M. C., Michel, F., Sainmont, D., & Briard, V. (2002). Apparent zeta-potential as a tool to assess mechanical damages to the milk fat globule membrane. *Colloids and Surfaces B, Biointerfaces, 23*, 23–30.
Michalski, M. C., Soares, A. F., Lopez, C., Leconte, N., Briard, V., & Geloen, A. (2006). The supramolecular structure of milk fat influences plasma triacylglycerols and fatty acid profile in the rat. *European Journal of Nutrition, 45*, 215–224.
Muellertz, A., Fatouros, D. G., Smith, J. R., Vertzoni, M., & Reppas, C. (2012). Insights into intermediate phases of human intestinal fluids visualized by atomic force microscopy and cryo-transmission electron microscopy ex vivo. *Molecular Pharmaceutics, 9*, 237–247.
N'Goma, J.-C. B., Amara, S., Dridi, K., Jannin, V., & Carrière, F. (2012). Understanding the lipid-digestion processes in the GI tract before designing lipid-based drug-delivery systems. *Therapeutic Delivery, 3*, 105–124.
Pafumi, Y., Lairon, D., de la Porte, P. L., Juhel, C., Storch, J., Hamosh, M., et al. (2002). Mechanisms of inhibition of triacylglycerol hydrolysis by human gastric lipase. *Journal of Biological Chemistry, 277*, 28070–28079.
Patton, S., Borgstrom, B., Stemberger, B. H., & Welsch, U. (1986). Release of membrane from milk-fat globules by conjugated bile-salts. *Journal of Pediatric Gastroenterology and Nutrition, 5*, 262–267.
Patton, J. S., & Carey, M. C. (1979). Watching fat digestion. *Science, 204*, 145–148.
Rigler, M. W., Honkanen, R. E., & Patton, J. S. (1986). Visualization by freeze-fracture, in vitro and in vivo, of the products of fat digestion. *Journal of Lipid Research, 27*, 836–857.
Sassene, P. J., Knopp, M. M., Hesselkilde, J. Z., Koradia, V., Larsen, A., Rades, T., et al. (2010). Precipitation of a poorly soluble model drug during in vitro lipolysis: Characterization and dissolution of the precipitate. *Journal of Pharmaceutical Sciences, 99*, 4982–4991.
Vanderghem, C., Francis, F., Danthine, S., Deroanne, C., Paquot, M., De Pauw, E., et al. (2011). Study on the susceptibility of the bovine milk fat globule membrane proteins to enzymatic hydrolysis and organization of some of the proteins. *International Dairy Journal, 21*, 312–318.
Ward, R. E., German, J. B., & Corredig, M. (2006). Composition, applications, fractionation, technological and nutritional significance of milk fat globule membrane material. In P. F. Fox & P. L. H. McSweeney (Eds.), *Advanced dairy chemistry volume 2 lipids* (pp. 213–244). USA: Springer.
Wilde, P. J., & Chu, B. S. (2011). Interfacial and colloidal aspects of lipid digestion. *Advances in Colloid and Interface Science, 165*, 14–22.
Ye, A. Q., Singh, H., Taylor, M. W., & Anema, S. (2004). Interactions of whey proteins with milk fat globule membrane proteins during heat treatment of whole milk. *Lait, 84*, 269–283.
Zachariu, R. M., Zell, T. E., Morrison, J. H., & Woodlock, J. J. (1969). Glycoprotein staining following electrophoresis on acrylamide gels. *Analytical Biochemistry, 30*, 148–152.
Zhu, X., Svendsen, C., Jaepelt, K. B., Moughan, P. J., & Rutherfurd, S. M. (2011). A comparison of selected methods for determining eicosapentaenoic acid and docosahexaenoic acid in cereal-based foods. *Food Chemistry, 125*, 1320–1327.