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STUDYING THE RELATIONSHIP BETWEEN EMULSION STRUCTURE AND LIPID DIGESTIBILITY FOR INFANT MILK

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Abstract

Milk, whether maternal or formulated, provides the sole source of nutrition to infants in the early stages of life, providing critical micronutrients, support for the immune function and primary dietary macronutrients including lipids. In healthy adults, lipids are primarily digested in the small intestine. However, for infants, the neonatal small intestine is not fully developed after birth, so the gastric environment plays a more significant role in milk fat digestion. Clinical studies have shown that maternal milk fat is digested more efficiently than lipids in infant formulae in infants under infant gastric conditions. Compositional differences, the structure of the oil droplets, and especially the interfacial composition may all play a crucial role in influencing lipid digestibility in the infant's stomach. In this thesis, the simulated gastric digestion of model emulsions and commercial infant formula was studied. The model emulsions comprised either a phospholipid or complexed protein-phospholipid interface while keeping all other facets of emulsion properties equivalent. Gastric digestion of these emulsions was carried out across variable *p*H conditions using an analogue gastric lipase, alone and in combination with pepsin with findings providing insights into the role of each enzyme and their combined effect on gastric lipolysis.

The rate and extent of lipolysis were characterised, along with morphological changes to the structure of the oil droplets. Results showed that gastric lipolysis might be influenced by pH conditions in the gastric environment when lipase was present alone in the simulated gastric fluid. The inclusion of pepsin resulted in significant structural changes when emulsions were stabilised with protein, in terms of droplet aggregation, size and morphology. However, no significant differences in the extent of lipolysis were determined. Thus, while the protein interface of both model and formulated emulsions was not observed to be a barrier for gastric lipolysis. Proteolysis of protein stabilised emulsions may lead to very different structural outcomes during gastric digestion when compared to phospholipid stabilised emulsions. While the research within this thesis demonstrates how the gastric environment influences emulsion structure as a consequence of interfacial composition, any specific relationship between structure and relative rate of gastric lipolysis currently remains undetermined. This research also highlights some of the ongoing challenges in the use of *in vitro* models to provide mechanistic understanding and interpretation of findings from clinical studies.

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List of Abbreviations

AFM	Atomic force microscopy
ANOVA	Analysis of variance
AUP	Area under the peak
ARA	Arachidonic acid
BSSL	Bile salt-stimulated lipase
CLSM	Confocal laser scanning microscopy
d_{32}	Volume surface-weighted mean diameter
d_{43}	Volume moment-weighted mean
DG	Diglycerides
DHA	Docosahexaenoic acid
DIC	Differential interference contrast
DSS	3-(Trimethylsilyl) propane sulfonic acid sodium salt
EPA	Eicosapentaenoic acid
FA	Fatty acids
FAME	Fatty acid methyl ester
FFA	Free fatty acids
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GI	Gastrointestinal
GOS	Galacto-oligosaccharides
HCl	Hydrochloric acid
HGL	Human gastric lipase
^{1}H NMR	Nuclear magnetic resonance
HPL	Human pancreatic lipase
HPLC	High-performance liquid chromatography
IS	Internal standard
LPC	lysophosphatidylcholine
MFG	Milk fat globule
MFGM	Milk fat globule membrane
MG	Monoglycerides
NaOH	Sodium hydroxide
NaCl	Sodium chloride

PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
*p*I	Isoelectric point
ppm	Parts per million
PS	Phosphatidylserine
SAXS	Small-angle X-ray scattering
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SM	Sphingomyelin
TEM	Transmission electron microscopy
TG	Triglycerides
TLC	Thin-layer chromatography
WHO	World Health Organization

Chapter 1 Introduction and key objectives

Human breast milk is the only ideal food for infants during the early months of life, to achieve optimal growth rates, development and health. WHO (World Health Organization) recommends exclusive breastfeeding for the first six months (WHO, 2001), and breastfeeding can continue until two years of age or longer. Human milk provides nutritional and breastfeeding also provides non-nutritional benefits for infants. It is not only the best food for neonates to achieve optimal growth and development, but also is crucial for neonatal health and later stages for life. However, while human breast milk plays this essential role in neonatal diet and nutrition, some mothers are not able to breastfeed because of disease, discomfort or busy working life. Thus, infant formulae have been developed to provide alternative options for infant feeding.

Lipids, primarily triglycerides, in human milk represent approximately half of the energy intake for neonates (Innis, 2004; Manson & Weaver, 1997). Besides the energy provided, complex lipids such as gangliosides and phospholipids in the milk globule membrane, also play essential roles in infant brain development, immunity, growth and development.

Human breast milk and infant formulae are emulsion systems where lipid droplets are dispersed in a continuous aqueous phase comprising proteins, lactose, minerals and vitamins as soluble components. When ingested, the milk fat will keep its initial oil in water emulsion structure through to the stomach where lipid digestion is initiated. As lipid digestion is an interfacial process, the structure of the milk lipid emulsion processes.

Thus, the structure of milk lipid plays a vital role in the process of lipid digestion. The structure of milk fat globules and the dynamics of the oil/water interface are influenced by the biochemical and biophysical environment in the gastrointestinal (GI) tract, which will in turn potentially have related consequences for the rate and efficiency of lipid digestion.

Clinical studies have shown the extent of lipid digestion of human breast milk is greater than that of infant formulae (usually based on vegetable oil and bovine milk proteins in neonates (Armand, Hamosh, Mehta, & Angelus, 1994; Armand, Hamosh, et al., 1996;

Hamosh, 1996). This is a somewhat paradoxical finding given that the droplet size of formula milk is typically one order of magnitude smaller than that of maternal milk and should provide greater surface area available for the binding of digestive lipase enzymes. Thus, the main differences between human breast milk and infant formula do not only lie in composition, nutritional or immunity value, but that their structural differences are influential to the efficacy of the neonatal digestion process. Notably, while formula emulsions typically comprise protein-coated oil droplets, human breast milk is secreted from the apocrine cell of the mammary gland. Thus the milk fat globule has a unique tri-layer milk fat globule membrane (Keenan & Patton, 1995) predominantly made up of phospholipids, which provides a significantly different interfacial structure to that of formula milk.

Some studies have suggested that emulsion structure plays a fundamental part in fat digestion in the GI tract (Armand, Borel, et al., 1994a; Armand, Borel, et al., 1996a; Armand et al., 1999; Berton et al., 2012; Fave, Coste, & Armand, 2004; Golding, 2014; Golding & Wooster, 2010; Golding et al., 2011). Recently, researchers have started to realise that the milk fat globule membrane may also play a critical part in facilitating fat digestion (Gallier et al., 2013; Gallier, Ye, & Singh, 2012). However, a knowledge gap still exists in fully determining the relationship between emulsion structure and fat digestion in neonates.

Moreover, as the development of small intestine in infants is immature, milk fat could not be able to digest efficiently in by lipases in the small intestine as adults. Therefore lipase in the gastric phase may play a crucial part in the digestion of lipids for infants.

When milk emulsions are exposed to the biochemical and physical environment in the neonatal GI tract, the properties of the oil/water interface may change. This may have a decisive impact on enzyme adsorption, rate of lipid digestion and absorption, and even later growth and development for infants.

Therefore, this PhD project aims to investigate how emulsion structure dynamics in model emulsions representative of infant milk and infant formula influence the extent of lipolysis during simulated gastric digestion and exploring co-dependency of the gastric enzymes (protease and lipase).

Thus, the key research questions and the corresponding research objectives are:

- Do structural dynamics of emulsions during digestion influence the rate and extent of lipolysis during gastric digestion?
- To what extent is the rate of lipolysis influenced by the changing *p*H environment within the stomach?
- To what extent do the concerted actions of lipase and pepsin influence the rate of lipolysis on protein-stabilised emulsions?

We also present the following main hypotheses in support of the above research questions:

- The structural changes of emulsion droplets during gastric digestion are influenced by the generation of free fatty acids during lipolysis.
- Phospholipid interfaces provide a biologically more favourable environment for the adsorption of lipase.
- Lipolysis of protein stabilised emulsions is facilitated by the concerted action of both pepsin and gastric lipase.
- The dynamic *p*H environment within the stomach (as influenced by the buffering effect of consumed milk) can influence the relative activity of pepsin and lipase.
- The free fatty acids generated from gastric lipolysis can alter the interfacial composition during lipolysis, and thus may inhibit subsequent lipase adsorption.

Therefore, accordingly, the research objectives are:

- Develop a model that uses electrostatic attraction in protein-phospholipid multi-layered emulsions as substrates for *in vitro* experiments.
- Determine the relationship between emulsion structure and the extent of lipolysis for these model emulsions and investigate how gastric conditions influence this relationship.
- Compare the behaviour of model oil in water emulsions to those of infant formulae and study how different emulsion structures influence the extent of lipolysis.
- Compare the behaviour of the multi-layered emulsion with that of infant formulae.

The next chapter will introduce the literature that identifies the current gaps and questions in lipid digestion in the neonates that covers the current knowledge within the field.

Chapter 2 Literature review

2.1 Introduction

This literature review aims at understanding milk lipid digestion in the neonatal GI tract. The gastrointestinal conditions are essential for efficient lipid digestion in infants, and it is known that the processes by which infants digest fat differ from those in adults. The review will focus on understanding variables in the gastrointestinal tract that influences lipid digestion, previous studies from *in vitro* and *in vivo* approaches and recent progress in emulsion structural changes and its relations to lipid digestion. This information will assist in answering the research questions of this project.

2.2 Neonatal gastrointestinal (GI) tract

The neonatal diet shifts from predominantly carbohydrates during fetal development to include higher amounts of fat after birth. At this stage, milk is the only source of food for newborns to provide energy and nutrients for growth and development. Among the necessary macronutrients, lipids provide energy and facilitate brain development (Hamosh, Iverson, Kirk, & Hamosh, 1994; Martin, Ling, & Blackburn, 2016; Uauy & Castillo, 2003). Maternal milk, in particular, contains all necessary macro- and micro-nutrients, bioactive components and immunological factors that are critical for the survival and growth of infants.

In adults, gastric lipolysis of the triglycerides in foods accounts for about 10-30% of the total lipolysis (Armand, Borel, et al., 1994b; Armand, Borel, et al., 1996b; Armand et al., 1999; Fave et al., 2004) while duodenal lipolysis accounts for 42-45% (Armand, Borel, et al., 1996a). However, in infants, lipid hydrolysis in the gastric phase can reach up to 40% (Armand, Hamosh, et al., 1994; Armand, Hamosh, et al., 1996; Hamosh, 1979a; Roman et al., 2007). Due to the immaturity of the neonatal GI tract, secretion of pancreatic digestive enzymes (lipase, trypsin and amylase) and bile salts are generally much lower than that of adults (Figure 2.1). Therefore, alternative pathways take on increased relevance for milk digestion in infants, which may result in greater reliance on gastric lipolysis to achieve effective lipid digestion.

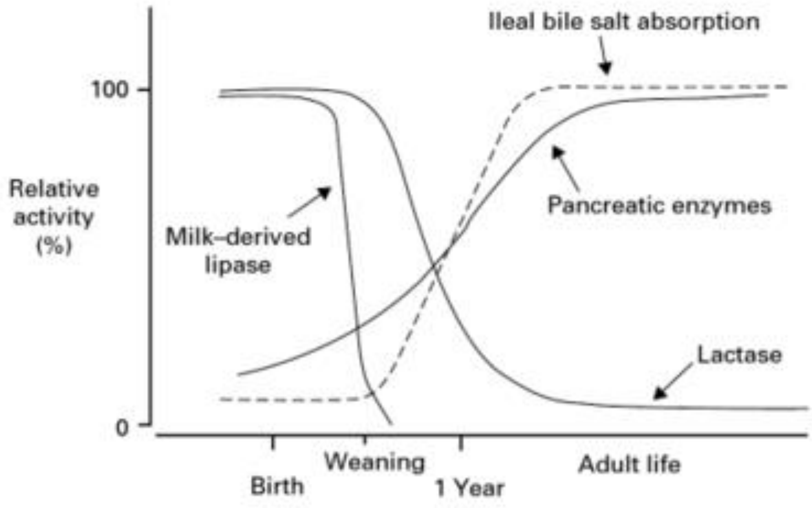


Figure 2.1 Diagrammatic representation the main changes in the digestive function in fetus, newborn and child, graph adapted from Thureen (Fordtran & Sleisenger, 1989; Thureen, 2006). Reproduce with permission from Cambridge University Press.

The milk emulsions ingested by infants are first mixed with a small amount of saliva. Whether digestion of milk lipids is initiated by lingual lipase is still controversial, so far there was no evidence showing the existence human lingual lipase (Hamosh, 1979b; Nguyen, Bhandari, Cichero, & Prakash, 2015). However, the mixture passes through the oral cavity quickly and then enters the stomach. The stomach plays an indispensable role for neonatal lipid digestion, where it provides digestive enzymes, an acidic environment that with gastric mixing contribute to the process of lipid digestion. Therefore, the following part will focus on the gastric digestive phase in the neonates as well the small intestinal phase which might be of further interest in this project–while the *in vitro* research component has a specific focus on the gastric digestion.

2.2.1 Gastric phase

2.2.1.1 Human gastric lipase

Human gastric lipase (HGL, EC 3.1.1.3) is a hydrophobic enzyme secreted from the chief cells from glands in the gastric fundus (K. E. Barrett, Barman, & Boitano, 2010; Carriere, Barrowman, Verger, & Laugier, 1993; Hamosh, 1990; Roussel et al., 1999). Human gastric lipase contributes to about 10-30% of lipolysis in the GI tract of healthy adults, converts triglycerides to diglycerides and liberates long-chain fatty acids (Carriere et al., 1993). Although lipid digestion by gastric lipase may be less effective than lipolysis in the small intestine for adults—where the majority of lipid digestion takes place—it may be more critical in neonatal lipid digestion and in patients with pancreatitis where the secretion of lipase is compromised (Muller, McCollum, Trompeter, & Harries, 1975). HGL is stable in the acidic gastric environment and does not depend on the action of bile salts and other specific protein cofactors for its action (Hamosh et al., 1981; Roussel et al., 1999), and it is not vulnerable to pepsin proteolysis (Bernback & Blackberg, 1989).

Purified HGL from human gastric aspirates had a molecular weight of approximately 50,000 Da (Bodmer et al., 1987). The molecular weight was predicted to be 43,162 Da by molecular cloning of a *c*DNA coding for human gastric lipase (Bodmer et al., 1987). Like many other lipases, HGL has a α/β-hydrolase fold core domain and a Ser-His-Asp catalytic triad in the active site and an oxyanion hole (Roussel et al., 1999). Notably, the N-terminal tetrapeptide (Leu-Phe-Gly-Lys-) is critical for HGL binding to lipid emulsion substrates. When trypsin cleaves the N-terminal tetrapeptide end, HGL is not able to bind at the oil/water interface and hydrolyse the emulsified lipid substrate (Bernback & Blackberg, 1989). The crystal structure of recombinant HGL (*r*HGL) showed three structurally independent elements the “lid”, “cap” and “core” domains (Figure 2.2). The active site (shown as red) lies beneath the lid and cap domain. The lid and cap domains may play an essential role in the interaction between HGL and lipid substrates. When the lid is removed from rHGL, the active site is accessible to the oil/water interface on the lipid substrate, and the hydrophobic residues of the cap domain form a ring around the active site (Figure 2.3). This hydrophobic surface may align with the oil/water interface of the lipid substrate, ensuring the *r*HGL bind and interact with the substrate (Miled et al., 2003; Roussel et al., 1999). Hence the lid domain combined with the cap domain may facilitate the catalysis of lipid substrates (Miled et al., 2003).

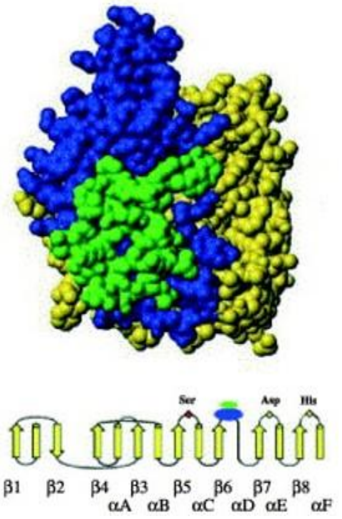


Figure 2.2 Corey-Pauling-Koltun view of the recombinant HGL (*r*HGL) with core domain (yellow), cap domain (blue) and lid domain (green). Graph adapted from Miled *et al*. (Miled et al., 2003). Reproduce with permission from Elsevier.

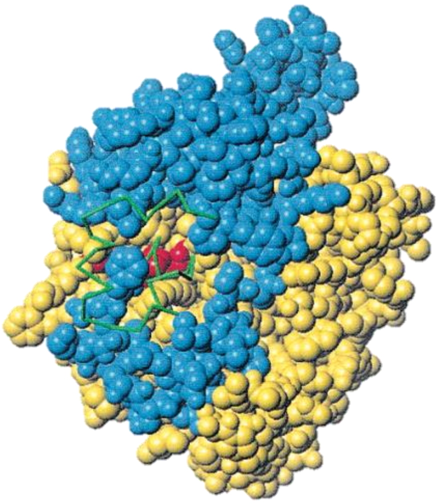


Figure 2.3 Corey-Pauling-Koltun view of the *r*HGL with catalytic triad (red), core domain (yellow), cap domain (blue), and the putative lid (given by a C_α trace in green). Graph adapted from Roussel *et al*. (Roussel et al., 1999). Reproduce with permission from American Soc for Biochemistry & Molecular Biology.

Poulsen *et al*. (2005) used cutinase to show that the environment around the active site might be more important than the ambient *p*H in the bulk solution. They proposed that binding of lipase to the oil/water interface could form a compartment around the active site separate from the bulk solution, so blocking the chemical communication between the site and solution. Thus, the enzymatic reaction may operate in a different environment than in the bulk solution so that the local pK_a of the active site could be more important than the optimal ambient *p*H in describing the kinetics of lipolysis.

Since a considerable amount of proteolysis by pepsin also takes place in the stomach, it is useful to note that the *p*H in the physiological environment of the stomach generally allows both lipase and pepsin to act together, with the possible exception of the *p*H occurring in the fasted state, or at post-prandially elevated levels. In these cases, the *p*H (very acidic) may be outside the optimum *p*H range of the lipase. HGL is stable at a *p*H 2.0 to 7.0 (Ville, Carriere, Renou, & Laugier, 2002), while its optimal range is from 3.0

to 6.0 (Carriere et al., 1991; Gargouri, Sarda, et al., 1986; Roussel et al., 1999; Ville et al., 2002). It may lose its activity at a very low *p*H level, such as in the fasting state (Carriere et al., 1993; Hamosh et al., 1981; Roussel et al., 1999; Ville et al., 2002). The local chemical environment near the active site of gastric lipase may affect the lipolytic products during gastric lipolysis. When gastric *p*H is within the optimal range for HGL, the enzyme may carry positive charges as its isoelectric point lies between *p*H 6.8 and 7.3 (Moreau et al., 1992). Depending on the pK_a values of the fatty acids, if they are in the optimal *p*H range of HGL, fatty acids liberated from lipolysis may carry negative charges. If the positive charges are on the active site of HGL, free negative fatty acids liberated from lipolysis may be trapped in the active site by electrostatic attraction affecting the local environment around the enzyme. In this case, the speed of reaction may slow down as the active site would not be able to access the substrate. However, since little evidence exists on where the positive charges are located on HGL, its isoelectric point may not be critical in understanding its impact on lipolysis. Even though a maximum reaction rate can be obtained over the optimum *p*H range, the final digestion rate is dependent on several other factors.

HGL has a stereoselective preference for the sn-3 position of triglycerides (Rogalska, Ransac, & Verger, 1990), thereby releasing a single fatty acid through hydrolysis (as opposed to pancreatic lipase that generates two fatty acids and a monoglyceride). The main difference between lipases and other enzymes which act on soluble substrates is that they work at the oil/water interface. The presence of emulsion droplets in the stomach can facilitate the catalysis process for lipase adsorption, thanks to the large surface area provided by the droplets. This process is much less effective when liquid oil is layered in the stomach, though gastric lipase has been found to act on soluble short-chain fat like vinyl butyrate in solution form (Chahinian et al., 2006). HGL has also been found to release both long- and short-chain fatty acids from the triglycerides (Gargouri, Pieroni, et al., 1986; Rogalska et al., 1990). The rate of lipolysis by HGL has been measured as 1160 U/mg for tributyrin and 620 U/mg for Intralipid (Gargouri, Sarda, et al., 1986) at their optimal conditions—tributyrin (short-chain fatty acid) at *p*H 6.0 and Intralipid (mainly long-chain fatty acid) at *p*H 5.0. The kinetic properties of lipase differ from those enzymes which act on soluble substrates since the activity of HGL is related to the relative emulsion surface area—with smaller droplets providing an increasing number of available binding sites than larger droplets. Accordingly, the rate of lipolysis for fine emulsions has been found to be faster than that for coarse emulsions (Armand et al., 1999; Borel et al., 1994). Thus the kinetic properties of lipolysis can be influenced by the adsorption of lipase at the oil/water interface as well as the interactions between the active site of lipase and its substrates.

2.2.1.2 Pepsin

Digestion of protein starts within the stomach. The acidic environment in the stomach partially denatures some dietary proteins, unfolding the structure and providing easier access for pepsin to hydrolyse the peptide bonds (Stipanuk & Caudill, 2013; Wildman & Medeiros, 1999). Similar to the small intestinal trypsin, pepsin is also an endopeptidase, it cleaves the internal rather than the terminal peptide bonds of proteins. Pepsin is the only protease in the stomach, which hydrolyses peptide bonds, breaking proteins down into oligopeptides and amino acids before further hydrolysis by the small intestinal proteases. Pepsin has broad specificity, preferably cleaving proteins at tyrosine, leucine and phenylalanine residues (Oka & Morihara, 1970; Trout & Fruton, 1969; Untersmayr & Jensen-Jarolim, 2008). The zymogen form of pepsin‒pepsinogen is secreted from the gastric chief cells. The acidic environment within the stomach activates the pepsinogen. It happens spontaneously by cleavage of a peptide at the N-terminal of pepsinogen (A. J. Barrett, Woessner, & Rawlings, 2012). The activated pepsin also further converts pepsinogen into pepsin by autocatalysis. Gastric proteolysis does not make a significant contribution to protein digestion in the stomach especially for newborns (Mason, 1962). An *in vivo* study showed only 15% of gastric proteolysis of total ingested protein from human milk or infant formula in preterm infants (Henderson, Hamosh, Armand, Mehta, & Hamosh, 1998).

Pepsins are stable under an acidic environment with maximum activity being achieved at *p*H 2. At *p*H > 5, pepsins gradually lose their activity (Johnston, Dettmar, Bishwokarma, Lively, & Koufman, 2007). The pK_a values of the active sites are 1.57 (pK_{a1}) and 5.02 (pK_{a2}) (Lin et al., 1992; Rawlings & Salvesen, 2013). These kinetic parameters can be used to calculate the maximum velocity of proteolysis at certain *p*H levels. During the digestion of protein stabilised emulsions, pepsin may work in concert with gastric lipase for lipolysis, by removing the protein from the oil/water interface thus facilitating lipase adsorption and lipolysis (Lueamsaisuk, Lentle, MacGibbon, Matia-Merino, & Golding, 2015). Accordingly, pepsin may also play an important role in infants when they are fed with infant formula since the oil droplets are mainly coated with milk proteins. This consideration does not necessarily apply when dealing with the digestion of maternal milk, which is predominantly stabilised by phospholipids. The pepsin output in full-term infants has been found to range from 0.19 to 0.32 mg/mL/hr/kg, being lower than that in adults ranging from 0.47 to 0.73 mg/mL/hr/kg (Agunod, Yamaguchi, Lopez, Luhby, & Glass, 1969). The fasting activity of pepsin measured on haemoglobin substrate was found to be 63 U/mL/kg in premature infants. The value in adults has been found to be much higher ranging from 942 to 1333 U/mL (Armand et al., 1995). Gastric enzymatic secretions, enzyme activity, and other variables during both fasting and postprandial states regarding lipid digestion (Table 2.1) show differences between full-term infants and adults.

Table 2.1 A selection of parameters in the gastric phase compared full-term infants to adults. Reproduce with permission from Taylor & Francis.

Parameters	**Full-term infants**	**Adults**
Total volume (mL)	F:2 - 2.65 (1 day)	F: 24-45
Secretions (mL/h/kg)	F:1 (1 week)	F: 79.7 ± 37.9 mL/h S: 210 ± 51.3 mL/h
Ionic strength (mM)	72 - 152	F: 100 - 150
*p*H	Depending on the buffering capacity of milk and frequency of feeding 2.0 - 7.5 $pH = -0.015 T + 6.53$	Depending on the buffering capacity of the meal 1.0 - 2.5 $pH = -0.0001 T^2 - 0.0004 T + 2.59$
HGL activity (U/mL)	F:38.9 ± 22.8 PP: N.A.	F: 5.7 - 9.9 S: 5.2 - 7.5
Pepsin activity (U or mg/mL)	S: 0.16 - 0.18 mg/mL (10-110 days)	F: 942 - 1333 U/mL PP: 718 - 1042 U/mL
Gastric emptying (T½, min)	Infant formulae 76 - 87 Human milk 48 - 61	Liquid meal: zero-order kinetics T ½ = *10 - 60* Solid meal: first-order kinetics T ½ = *60 - 277*
(Rate of emptying to the duodenum, Kcal/min)	0.63 - 0.43	2 Emptying dependent on the volume, osmotic pressure and caloric content of the meal

F, Fasting; T, Time after feeding (min); PP, Postprandial; S, Stimulated; N.A., not available; $T_{1/2}$, gastric emptying half time. Content in the table was adapted from Bourlieu *et al*. (2014).

2.2.1.3 The secretion of hydrochloric acid

Studies have shown that immediately after birth, the gastric *p*H of neonates may be alkaline due to ingestion of uterus amniotic fluid. Gastric *p*H can be influenced by the frequency and volume of feeding and the buffering effect of milk. In healthy adults, the fasting gastric *p*H is from 1 to 2, and postprandial gastric *p*H is usually from 4 to 6. However in the neonates, the gastric *p*H level is relatively higher than that in adults (Dressman et al., 1990; Russell et al., 1993) and the secretion of hydrochloric acid (HCl) is also much lower, rising to adult level after one year of age (Bourlieu et al., 2014).

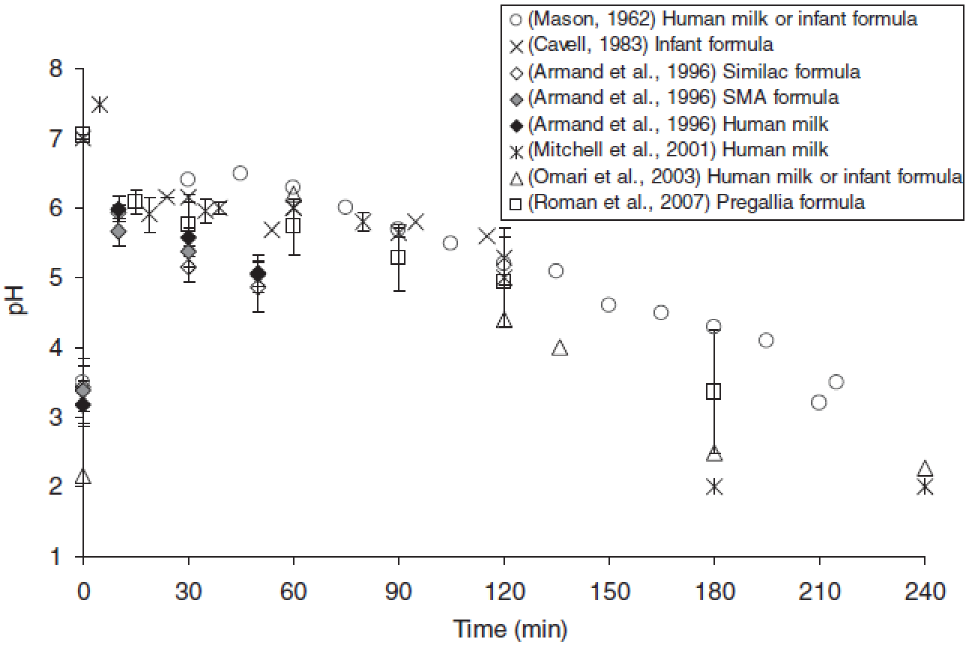


Figure 2.4 The fasting and postprandial gastric pH in preterm infants (Armand, Hamosh, et al., 1996; Cavell, 1983; Mason, 1962; Mitchell, McClure, & Tubman, 2001; Omari & Davidson, 2003; Roman et al., 2007). The figure was adapted from Bourlieu *et al.* (Bourlieu et al., 2014). Reproduce with permission from Taylor & Francis.

The changing gastric *p*H after feeding in preterm infants shows a similar trend as with full-term infants (Figures 2.4 and 2.5). For healthy newborns, the *p*H level can range from 2.0 to 7.1. During the fasting state, the mean gastric *p*H value usually stays around 3.5. However, after feeding, milk diluted with HCl acts as a buffer, so the *p*H increases and stays at a range between 7.1 and 5.2 for up to 120 min. A *p*H peak value (6.0-7.0) can be detected for about 60 mins after which the *p*H gradually starts to fall, with the postprandial gastric *p*H dropping back below 3.0 after four hours of digestion. Another study has shown similar gastric *p*H values in infants ranging from 2.5 to 5.5 (Agunod et al., 1969).

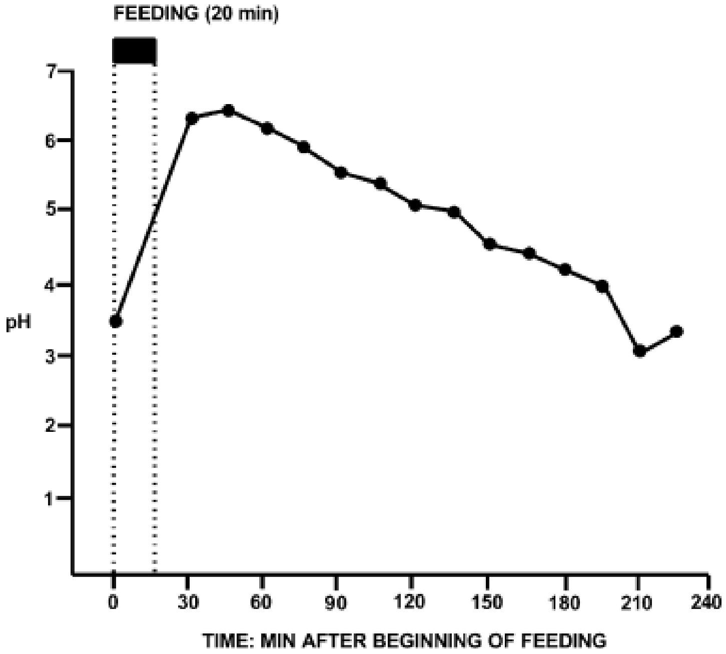


Figure 2.5 The fasting and postprandial *p*H of the stomach content from 25 healthy full-term infants (mean value) (Chatterton, Rasmussen, Heegaard, Sørensen, & Petersen, 2004; Mason, 1962). Reproduce with permission from Elsevier.

Consequently, due to the relatively high level of *p*H in the fasting and postprandial phase, the *p*H in the neonatal stomach seems to always fall outside the optimal *p*H (2.0) for pepsin activity, as opposed to the *p*H range (2.0-6.0) observed in the adult stomach which is more appropriate for human gastric lipase activity. Thus, only minor proteolysis could be expected to take place in the neonatal stomach, whereas triglycerides are partially digested in the stomach. Thus, gastric lipolysis may play a more critical role in initiating lipid digestion and facilitate lipolysis in the later stage in the small intestine.

2.2.2 Intestinal phase

1.1.1.1 Pancreatic lipase and bile salts

After the chyme passes through the stomach and enters into the small intestine, gastric lipase may still retain some activity at the duodenal phase (Carriere et al., 1993; Miled et al., 2000), thus lipid digestion may take place under the action of both, gastric lipase and pancreatic lipase, in the presence of bile salts. In the intestinal phase, pancreatic lipase is the primary lipase, preferentially hydrolysing triglycerides and diglycerides in the digesta at the sn-1 and sn-3 positions, producing 2-monoglycerides and free fatty acids. Since the digestive tract has been fully developed in children and adults, pancreatic enzymatic secretions and biliary secretions are sufficient for full lipid digestion and absorption. Human pancreatic lipase (HPL) is active at neutral *p*H, more precisely in the range of 4.5 to 7.5 with an optimal activity at around 6.5 (Carriere et al., 1993). During intestinal lipolysis, HPL may be inhibited by small-molecule surfactants such as Tween 80 (Gargouri, Julien, Bois, Verger, & Sarda, 1983).

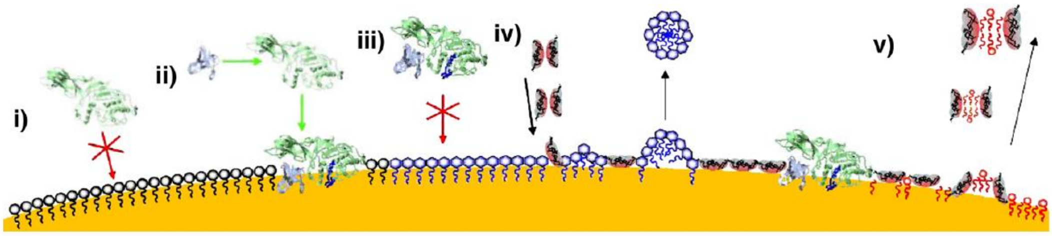


Figure 2.6 i) Pancreatic lipase could not bind to the emulsion interface by its own. The activation of this enzyme needs co-lipase, bile salts and calcium. ii) Binding of co-lipase binds to pancreatic lipase. This complex facilitates the exposure of the active sites of pancreatic lipase and its adsorption on the oil/water interface. iii) Adsorption of the complex of co-lipase and pancreatic lipase can be inhibited by some surfactants. iv)Those surfactants can be removed from the interface via an orogenic displacement mechanism by bile salts. v)Micelles of bile salt and phospholipid can also remove surface-active lipolysis product from the interface for further absorption in the intestinal phase. Diagram was adapted from Golding *et al*. (2010). Reproduce with permission from Elsevier.

The process of lipid digestion and absorption is shown in Figure 2.6. Bile salts and phospholipids secreted by the liver are particularly crucial for lipid digestion in the intestine (Bauer, Jakob, & Mosenthin, 2005), as bile salts have amphiphilic properties and act as a detergent. They have a planar structure with a steroidal nucleus for hydrophobic side, and a few hydroxyl groups and an ionic head forming the hydrophilic side (Bauer et al., 2005; McMurry, Hoeger, Ballantine, & Peterson, 2012). Bile salts can facilitate lipid digestion through two different mechanisms. Firstly, when the concentration of bile salts is above their critical micellar concentration (CMC, 2-4 mM), bile salts can displace some surfactants such as Tween 80 which is an inhibitor to HPL, and allow the complex of HPL and co-lipase binding on the oil/water interface (Gargouri et al., 1983). Secondly, when products of lipolysis such as monoglycerides and free fatty acids accumulate at the interface, those products can prevent the complex of HPL and co-lipase from binding onto the oil/water interface (Pafumi et al., 2002). Bile salts can also emulsify and incorporate these lipolysis products from the interface into mixed micelles and the aqueous phase (Golding & Wooster, 2010; Lentle & Janssen, 2011; Mu & Hoy, 2004; Porter, Trevaskis, & Charman, 2007).

As stated earlier, the concentration of digestive enzymes, especially pancreatic lipase, is significantly lower in preterm and full-term infants compared to children and adults (Lebenthal & Lee, 1980). Also, the secretion of bile salt phospholipids postprandially in both neonates and full-term infants is lower than that in adults. Overall the concentrations may be insufficient for efficient lipolysis (Table 2.1). Alternative pathways, as mentioned in the previous section, such as gastric lipase, and the additional contribution of lipases present in maternal milk, such as bile salt stimulated lipase (BSSL), may support lipid digestion and absorption. However, the relative value of these contributions is still not fully determined.

1.1.1.1 Bile salt stimulated lipase

Bile salt stimulated lipase (BSSL) is an external lipase which is considered as one of the most important lipolytic enzymes for infant lipid digestion. It is secreted with human breast milk from the mammary gland (Hernell & Olivecrona, 1974a, 1974b). BSSL is also secreted from the acinar cells of the pancreas in the neonates and the expression in fetal tissues is as early as the 6 weeks of gestation (Roudani, Miralles, Margotat, Escribano, & Lombardo, 1995). BSSL is a highly glycosylated enzyme with a molecular weight of 107,000 Da (Hansson et al., 1993; Landberg et al., 2000). It is a nonspecific lipolytic enzyme which hydrolyses not only triglycerides, diglycerides and monoglycerides but also cholesteryl esters, phospholipids, lysophospholipids, and ceramide (Hui & Howles, 2002). Similar as human gastric lipase, lipolysis by BSSL in human milk is considered to have an important contribution to the total lipid digestion in the neonates (Bernback, Blackberg, & Hernell, 1990; Roman et al., 2007). BSSL cannot hydrolyse lipids from human milk fat globule alone, and it remains stable (cannot be hydrolysed by pepsin) and inactive within the *p*H range of the neonatal gastric environment (Armand, Hamosh, et al., 1996; Bourlieu et al., 2014). It does not hydrolyse lipids in human milk until reaching the duodenum, being activated by bile salts (Hernell & Bläckberg, 1994). The mechanism of this process is not well understood. In an *in vitro* test, hydrolysis of more than 90% of human milk triglycerides could be achieved with the combined work of pancreatic lipase, colipase and BSSL (Bernback et al., 1990; Hernell & Bläckberg, 1994). The optimum *p*H range of BSSL is from 7.4 to 8.5 (Hamosh, 2001). Its activity in preterm and term human milk ranges from 25 to 30 U/mL and remains constant within the length of lactation (Mehta, Jones, & Hamosh, 1982).

2.3 Human breast milk and infant formula

2.3.1 History of infant formula

In the early ages, before artificial infant foods appeared, wet nurses who fed other mother's children were very common (Stevens, Patrick, & Pickler, 2009). Starting from the 16th century, liquid substitutes such as pap (bread soaked with water or milk) and panada (cereals cooked in broths) appeared in the cities in Europe (Radbill, 1981; Wickes, 1953). Animal milk such as cow and sheep milk were also alternatives for human breast milk. However, many of the alternatives raised hygiene and health problems, which increased malnutrition and mortality rates among the newborns (Kleinman, Barness, & Finberg, 2003; Wegman, 2001). In the mid-19th century, German chemist Justus von Liebig produced the first liquid infant formula based on bovine milk, with wheat with addition of malt flour and potassium bicarbonate. Nestlé formulated the first powder infant formula (Schuman, 2003), and many brands of infant formulae became commercially available afterwards (Bracken, 1953).

From the beginning of the 20th century, researchers started to understand the critical components of mammal-originated milk, and manufacturers used different approaches to improve infant formula. In the early 20th century, skim milk (without fat) was used to avoid short-chain fatty acids found in bovine milk, which were believed not to be tolerated by infants (Fomon, 2001; Gerstenberger, Ruh, Brickman, Leslie, & Ochsner, 1919). The manufacturers increased protein level as people believed that the protein content in the human breast milk was much higher than the actual value (Fomon, 1967, 2001). In general, formula milk at that stage were either evaporated milk with added vitamins or low protein milk with added oleic oils, vitamins and minerals (Wargo, 2016). In the late 20th century, formula with similar casein/whey ratio to human milk was developed in the United States, and this added-whey formula became a predominant formula type in the 1990s (Fomon, 2001). Fortified infant formula was developed at a later stage, with added polyunsaturated fatty acids, probiotics, taurine and nucleotides to support healthy growth and enhanced immune system (Schuman, 2003; Wargo, 2016).

From the 1960s and onwards, infant formulae have gradually become what they are today, by using ingredients that can improve growth and development of the neonates such as galacto-oligosaccharides (GOS), unsaturated fatty acids, probiotics. With further developments in the contemporary days, companies in this industry have improved their formulae by adding compounds such as docosahexaenoic acid (DHA) to improve the fat composition and facilitate cognitive and brain development and membrane fragments of the bovine milk fat globules to improve growth and development for infants at early ages.

2.3.2 The droplet structure of human breast milk and infant formula

2.3.2.1 Human milk

Lipids in human milk are packed as fat globules, secreted directly from the epithelial cells of the mammary glands. Hence, these triglyceride cores are surrounded by a tri-layered membrane structure composed of polar lipids, proteins, enzymes, cholesterol and glycoproteins (Jensen et al., 1995a). The tri-layered structure results from the combination of an inner mono-layer derived from the endoplasmic reticulum and an outer bilayer derived from the apical plasma membrane of the mammary epithelial cells (Keenan & Patton, 1995; Lopez, 2011; Lopez & Menard, 2011). The tri-layer membrane, known as the milk fat globule membrane (MFGM) that envelopes the lipid core.

A structural model (Figure 2.7) of the human milk fat globule membrane (MFGM) proposed by Lopez & Menard (2011) postulated that two lipid phases coexist in the outer bi-layer of the MFGM – the liquid-ordered (L_o) phase and the liquid-disordered (L_d) phase where the tri-layer of polar lipids acts as the backbone. These two lipid phases have the same fluid symmetry but a different degree of acyl chain order (Ipsen, Karlström, Mourtisen, Wennerström, & Zuckermann, 1987; Mouritsen, 2010). The liquid-disordered (L_d) phase is a fluid (liquid-crystalline) phase (Ipsen, Mouritsen, & Bloom, 1990) whereas the liquid-ordered (L_o) phase exists between a gel and a fluid phase with a tight packing and relatively extended acyl chains (Mouritsen, 2010). The L_d phase domain consists of glycerophospholipids—phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) and it is surrounded by L_o phase domains enriched of sphingomyelin (SM) and cholesterol. In the (SM-rich) L_0 domain, this phase could be symmetrically orientated across the two leaflets of the MFGM bi-layer, or asymmetrically placed in the outer layer of the bi-layer. Cholesterol could fill the space between the polar head groups (phosphocholine and ceramide, or phosphoethanolamine) of SM locally increasing the thickness of MFGM. The glycoproteins and glycolipids are randomly distributed in the external surface of MFGM.

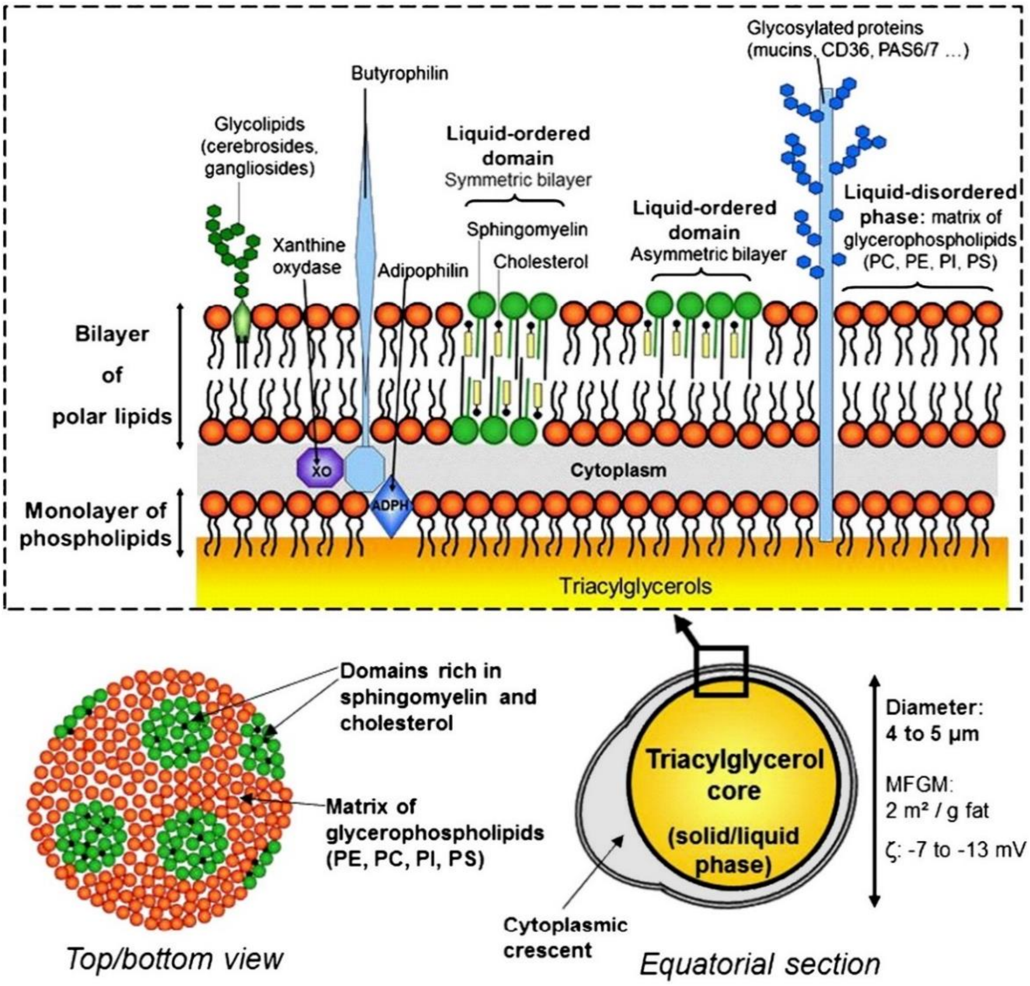


Figure 2.7 A model structure of the human MFGM. The schematic presentation showed the tri-layer of the MFGM, with the polar lipid backbone and the lateral view of the plane of bi-layer organisation consisted of phase-separated L_o domain (rich in sphingomyelin) and L_d domain (glycerophospholipids) with proteins bound to the periphery of the L_d domain. Adapted from Lopez *et al*. (2011).

This particular structure of MFGM at the interface stabilises the milk fat during the short period of expression to digestion. The structure composed of phospholipids and a group of glycoprotein filaments in the outer layer of MFGM may facilitate sufficient digestive lipase binding and adsorption to achieve hydrolysis of the triglycerides core and lipid absorption (Hamosh et al., 1999; Jensen, Ferris, & Lammi-Keefe, 1992; Lopez & Menard, 2011).

2.3.2.2 Infant formulae

The structure of the oil droplets in infant formulae depends on the ingredients in milk, i.e. proteins, fat and carbohydrate content, and the composition of the interface and the processing methods. Also, the interface relies on the presence of surface-active ingredients in the formulation, mainly proteins and phospholipids, which will partition across the oil/water interface depending upon the degree of hydrophobicity and the distribution of polar groups. A schematic presentation of the oil droplet in infant formula based on this description is shown in Figure 2.8.

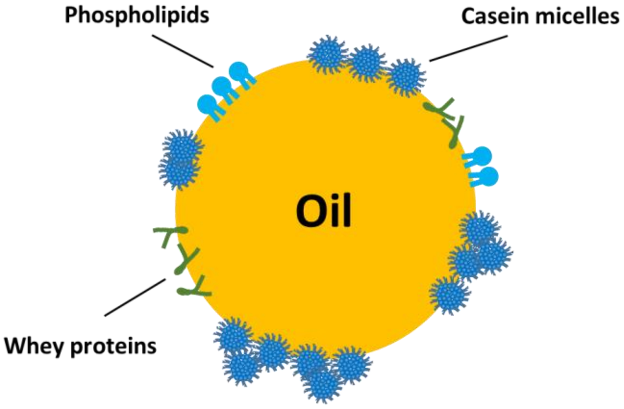


Figure 2.8 An assumed structure of infant formula oil droplet depends on ingredients and process.

Adsorption of proteins and emulsifiers provides a physical barrier for oil droplets and particularly proteins prevent them from coalescence, still providing enough interface for lipase binding and action. However, the interfacial composition may vary depending on the competition between milk proteins and the more surface-active emulsifiers like monoglycerides and phospholipids at the interface of fat droplets in infant formula (McSweeney, Healy, & Mulvihill, 2008; Wilde, Mackie, Husband, Gunning, & Morris, 2004). Lipase has a different affinity for different interfacial layers. Thus the interfacial composition and characteristics of the emulsion droplet are very likely to influence the adsorption and activity of digestive lipase (Hur, Decker, & McClements, 2009).

For both human milk and infant formulae, the stability and properties of the interface of the fat globules are influenced by the physiochemical environment of the GI tract. The conditions may change when digestion processes, and therefore, the rate of lipolysis may change accordingly.

2.3.2.3 Droplet sizes in human breast milk and infant formulae

The average size of human breast milk fat globule is shown in Figure 2.9. The volume surface-weighted mean diameter (d_{32}) ranges from 1.5 to 2.1 μm where the volume moment-weighted mean (d_{43}) ranges from 2.5 to 4.6 µm depending on the milk source (Huppertz & Kelly, 2006). The oil droplet size in infant formulae is much smaller, always below 1 µm for d_{32} values (Table 2.5). The size distribution of two infant formulas is shown in Figure 2.9.

Table 2.2 A comparison of the droplet properties of fat globules from different infant milk sources.

Parameters	Mature human milk*	Infant formula
d_{32} (μm)	3.5 ± 0.1[a]; 4.0 ± 0.3[b]	0.3 ± 0.0[a]; 0.8 - 1.1[c]
d_{43} (μm)	4.4 ± 0.2[a]; 5.2 ± 0.4[b]	2.0 ± 0.9[a]
Surface (dm^2/mL)	6.7 ± 0.3[d]	64 ± 9[a]; 20[c]
Specific surface area (m^2/g)	1.9±0.1[a]; 1.4±0.1[a]	22.1±1.7[a]; 5.2~7.2[c]
Natural surface charge (mV)	-20[e]	-33 - -38[e]

This table was adapted from Michalski *et al*. (Michalski, Briard, Michel, Tasson, & Poulain, 2005). Reproduce with permission from Elsevier.

*Sampled from 5 mothers (Lueamsaisuk, 2015).

[a] Data collected from Michalski (Michalski et al., 2005).

[b] Data collected from Rüegg (Rüegg & Blanc, 1981).

[c] Data collected from Simonin (Simonin, Rüegg, & Sidiropoulos, 1984).

[d] The fat content in mature human milk was considered as 35 g/L.

[e] Data from Lueamsaisuk (Lueamsaisuk, 2015)

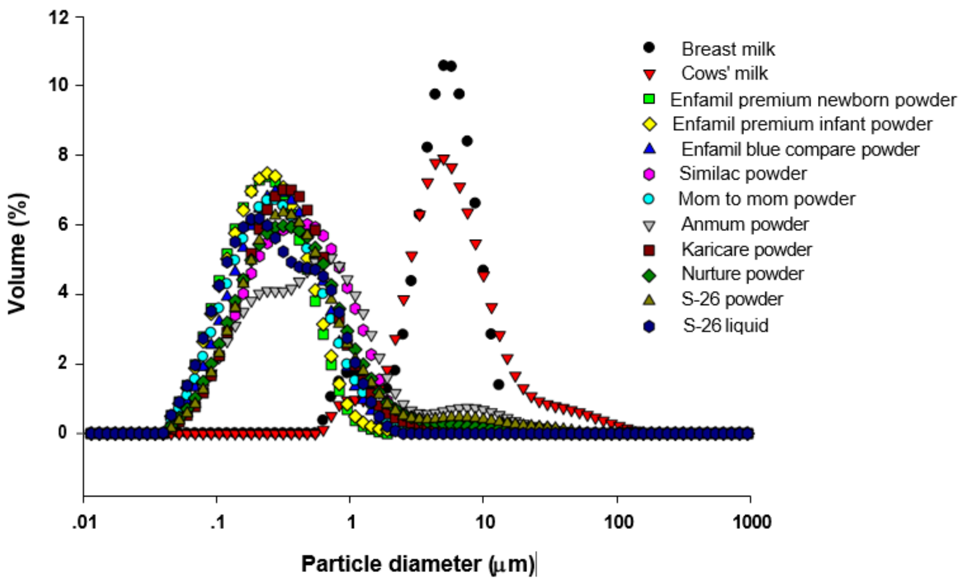


Figure 2.9 Droplet size distribution of several milk samples, including maternal milk and some infant formulae (Lueamsaisuk, 2015). Reproduce with permission from Lueamsaisuk.

Due to the high-pressure homogenization during the production of infant formula, the oil droplets always present a smaller droplet size and consequently larger surface area (64±9 dm^2/mL) than in human milk (6.7±0.3 dm^2/mL). Hypothetically, lipid components in infant formulae would be more efficiently digested than in human milk if digestion was only dependent upon the surface area available for lipase binding. However, it has been shown that the oil droplets in human milk are digested more rapidly than in infant formula (Armand, Hamosh, et al., 1996). This is most likely due to the different membrane structure in the fat globule of human milk compared to infant formulae, with the latter presenting a protein layer at the oil/water interface which may be bound to affect the action of lipase.

2.3.3 The composition of human milk and infant formulae

Human milk provides essential nutrients and growth factors for infants. Infant formula as a supplement or replacement for human milk, therefore, needs to provide equivalent nutritional value to that of human milk. Production of milk, either via secretion from the mammary glands or through the processing of the infant formula invariably leads to differences between the two types of milk, not only in composition, nutritional value and immune functions but also in oil droplet size and globule structure. These differences are believed to play an essential part in lipid digestion, growth and development of infants.

A comparison between the main nutrient components of human milk and infant formula is shown in Table 2.3.

Table 2.3 The Composition of human milk and infant formula in the percentage of the total components. Reproduce with permission from Elsevier.

Components	Human milk (%)	Infant formula (%)
Protein	1.0	1.5
Whey/Casein	70/30	60/40
Fat	3.8	3.8
Lactose	7.0	7.2
Total solids	12.0	13.0
Ash	0.2	0.3

This table was adapted from Guo *et al*. (2013).

Lipids contribute about 4% of the human milk volume (Jensen, 1996). Of these, triglycerides make up for about 98%, with phospholipids accounting for 0.7%, cholesterol for 0.5% and with the presentation of a small amount of lipolytic products (Koletzko et al., 2001). The lipid content of human milk varies to some extent between individuals and with diet, as does protein and lactose, though to a lesser extent. Other carbohydrates in human milk such as oligosaccharides are significant micronutrients that usually consist of approximately 1 g/100 mL depending on the stage of lactation and other factors (Ballard & Morrow, 2013). Lipid composition in infant formula is relatively invariant although there are some differences between manufactures. The lipids in infant formula also consist mainly of triglycerides, some phospholipids, lecithins and small quantities of monoglycerides as emulsifiers to stabilise the emulsion and as wetting agents for power formulations.

The profile of the primary fatty acids in triglycerides in human milk differs from that of infant formula (Table 2.4). In human milk, saturated fatty acids account for a significant part of the total fatty acids in the triglycerides, with palmitic acid accounting for a large part of the saturated fatty acids. The mono-unsaturated fatty acids, such as oleic acid (18:1) and polyunsaturated fatty acids such as linoleic acid (18:2) constitute the highest proportion of the unsaturated fatty acids. The amount of long-chain polyunsaturated fatty acids such as docosahexaenoic (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) have significant nutritional value and vary with the maternal diet (Innis, 1991; Innis & Kuhnlein, 1988). Hence, deficient intake of DHA and ARA may lead to decreased visual and brain development of the new-borns (Innis, 2004; Innis, 2007; Uauy, Hoffman, Mena, Llanos, & Birch, 2003).

Table 2.4 The fatty acid content from human milk compared to that from infant formulae (% of the total fatty acids).

Fatty acids	Abbreviations	Human milk	Infant formula
Saturated			
Caprylic	$C_{8:0}$	-	1.3 (0.7-1.5)
Capric	$C_{10:0}$	1.4 (1.2-1.6)	1.1 (0.9-1.3)
Laurie	$C_{12:0}$	5.4 (4.4-6.0)	9.4 (6.6-12.1)
Myristic	$C_{14:0}$	7.3 (6.9-7.9)	4.9 (4.3-6.6)
Palmitic	$C_{16:0}$	26.5 (26.0-27.0)	20.0 (14.0-26.3)
Stearic	$C_{18:0}$	9.5 (8.5-10.5)	4.9 (3.6-7.5)
Unsaturated			
Palmitoleic	$C_{16:1}$	4.0 (3.7-4.2)	0.4 (0.1-1.1)
Oleic	$C_{18:1}$	35.5 (34.5-36.0)	40.6 (28.7-53.0)
Eicosaenoic	$C_{20:1}$	0.5 (0.5-0.6)	0.3 (0.2-0.5)
Linoleic	$C_{18:2\ (n\text{-}6)}$	7.2 (6.4-7.7)	12.4 (8.8-18.9)
α-linolenic	$C_{18:3\ (n\text{-}3)}$	0.8 (0.7-0.9)	1.5 (0.7-2.2)

This table was adapted from Wells (1996). Reproduce with permission from Elsevier.

About 60% of the palmitic acid (16:0) is located on the sn-2 position in human milk fat, with the major portion of lauric (12:0) and oleic acids (18:0) located at either the sn-3 or sn-1 positions. Similarly, the bulk of linoleic (18:1) and α-linolenic (18:2) acid are located at the sn-1 and sn-3 position (Jensen et al., 1995b; Koletzko et al., 2001; Thureen, 2006). The molecular structure of triglycerides is important as the digestive lipases only hydrolyse the sn-3 or sn-1 and sn-3 positions. If palmitic acid is positioned at the sn-3 or sn-1 position, the released palmitic acid by digestive lipases could interact with cations such as calcium, which forms insoluble soaps that are not available for absorption (López-López et al., 2001). Palmitic acid located at the sn-2 position could form monoglycerides that are better suited for the formation of mixed micelles and increase the availability of palmitic acid, facilitating absorption through the intestinal wall. Compare to infant formulae where most of the palmitic acids are located at the sn-1 or sn-3 positions, palmitic acids in human milk have shown better absorption when they are esterified at the sn-2 positions as proved by clinical trials (Carnielli et al., 1995; Carnielli et al., 1996).

Triglycerides in infant formulae usually come from a blend of two or three vegetable oils such as sunflower oil, palm, coconut, soy and canola oils (Lueamsaisuk, 2015; Talbot, 2015). The use of different proportions of vegetable oils can mimic some aspects of the fatty acid profile found in human milk fat. While formulae can be improved in this way, it is challenging to imitate the proportion of all 160 kinds of fatty acids (Jensen, Hagerty, & McMahon, 1978; Thureen, 2006) that are known to be present in human milk. Coconut oil provides a rich source of short-chain saturated fatty acids such as lauric (12:0) and myristic acids (14:0), while palm oil is a rich source of the palmitic acid (16:0) (Packard, 2012; Prosser, Svetashev, Vyssotski, & Lowry, 2010; Talbot, 2015). However, as stated above, palmitic acids in vegetable oils are usually located at sn-1 and sn-3 positions, unlike in human milk they are located at the sn-2 positions. Also, different from vegetable oils, palmitic acids in human milk account for 20-25% of the total fatty acids (Xu & Akoh, 2002) with most of them positioned at the sn-2 positions. Palmitic acids from human milk can be absorbed better than from infant formulae in infants, as already mentioned. In order to change the palmitic acid position in the vegetable oils, some speciality fat such as Betapol that are developed by enzyme technology to better mimic human milk fat and increase the intake of palmitic acids (Talbot, 2015; Xu & Akoh, 2002). Safflower, sunflower, soybean oil and corn oil are excellent sources for mono and polyunsaturated fatty acids (Thureen, 2006). Soybean oil and linseed oil are good sources of α-linolenic acid and fish oil a good source of docosahexaenoic acid (DHA) and EPA (eicosapentaenoic acid) (Talbot, 2015). As DHA and arachidonic acid (ARA) presented in maternal milk are essential for the development of cognitive, vision and immune function for infants, the European Food Safety Authority has recommended providing adequate supply for DHA and ARA in infant formulae (EFSA Panel on Dietetic Products & Allergies, 2013; Lien, Richard, & Hoffman, 2018).

The membrane of fat droplets in human milk consists of phosphatidylcholine, phosphatidylserine, phosphatidylinositol, sphingomyelin and glycosphingolipids such as gangliosides (Jensen et al., 1995a). Though these complex lipids are not the main energy source, they are important for infant growth, cognitive and neuro-development

(Dewettinck et al., 2008; Gurnida, Rowan, Idjradinata, Muchtadi, & Sekarwana, 2012; Motouri et al., 2003; Timby, Domellöf, Hernell, Lönnerdal, & Domellöf, 2014). These lipids also protect infants against pathogen infections (Dewettinck et al., 2008; German, Dillard, & Ward, 2002; Hamosh et al., 1999; Lopez & Menard, 2011). Infant formulae usually contain only trace quantities of membrane fractions that come from ingredients such as milk powder. The lack of MFGM in infant formulae is an important difference that distinguishes infant formulae from maternal milk. Accordingly, the functional roles and benefits of complex lipids are areas of increasing interest to the formula industry.

Human milk protein comprises approximately 1% of the total milk volume, decreasing slightly between different lactation periods (Hendricks & Guo, 2014). The whey/casein ratio in human milk changes from 90/10 in early lactation to 60/40 in mature milk and 50/50 in late lactation (Kunz & Lönnerdal, 1992). The main whey proteins consist of α-lactalbumin, lactoferrin and secretory IgA (SIgA). In human breast milk, no β-lactoglobulin is present, α-lactalbumin which accounts for about 40% of the whey and 17 - 28% of the total protein dominates in human milk, whereas in bovine milk the level of this protein is only 3-3.5% (Heine, Klein, & Reeds, 1991; Thao, Bhesh, Cichero, & Sangeeta, 2015). Casein in human milk is in a micellar form comprising largely of β-casein, κ-casein and a small amount of α_{S1}-casein (Lönnerdal, 1985, 2016a). Other important proteins including lysozyme, folate-binding protein, bifidus factor, lipase, amylase, and haptocorrin (Hendricks & Guo, 2014) provide good sources of amino acids for infant growth and development. Many enzymes in human milk facilitate the digestion of lipids and starch. Human breast milk also contains proteins such as immunoglobulins, lactoferrin, lysozyme and κ-casein that provide a barrier and defence for infants from being infected by pathogenic bacteria, viruses and yeasts (Hendricks & Guo, 2014; Lönnerdal, 2003).

The proteins in infant formulae may come from various animal sources such as bovine or goat milk, or vegetable sources such as soy or rice. Those proteins have an appropriate balance between lactalbumin and casein and provide a source of amino acids (Packard, 2012), but do not adequately simulate human milk proteins. In order to meet the requirements of essential amino acids, Skim milk powder, whey protein concentrate powder and demineralised whey protein powder are commonly used instead of milk powder. Moreover, in order to match the whey/casein ratio in maternal milk, infant formulae usually use whey-based milk, consisting of demineralised whey and skim milk to achieve a whey/casein ratio of 60/40 (Hileti-Telfer, 2003). Finally, human milk also provides bioactive proteins such as lactoferrin that acts as intact proteins can prevent infants from infection. Lactoferrin from bovine has also been added as a supplementary component in infant formulae (Tomita, Wakabayashi, Yamauchi, Teraguchi, & Hayasawa, 2002). Some other bioactive proteins such as α-lactalbumin and osteopontin which form bioactive peptides during digestion may lead to an enhancement of the immune system and provide some other health benefits (Lönnerdal, 2016a, 2016b).

2.4 *In vitro* and *in vivo* approaches for digestion studies

In order to understand the digestion process, scientific studies usually use *in vitro* and *in vivo* approaches to collect data. Compared to *in vitro* studies, the *in vivo* approach—which is based on human and aminal trials—is usually a more representative way to understand digestion. However, studying this process is technically challenging and costly in human or aminal subjects, as this requires sufficient numbers of participants to ensure statistical relevance. For ethical reasons, it is even more difficult to conduct human trials in infants. Therefore, scientists in the past few decades have developed many *in vitro* models to roughly imitate the process of digestion. However, there are several problems with these simulations. Firstly, the digestive process is highly complex in nature and researchers still have not gained a comprehensive understanding of the physiology of digestion. This means that the *in vitro* models cannot properly simulate this process, and therefore, these models may not lead to physiologically relevant outcomes from lipid digestion in human subjects. Thus studies through an *in vivo* approach are ultimately needed correspondingly in order to verify findings through the *in vitro* approach. Secondly, the enzymes used in the *in vitro* models are not usually from human subjects because human digestive enzymes are not always available, and the cost is usually very high. Alternatives from other sources like animals and fungi may not have the same affinity, optimal *p*H range and activity to the substrates compared to those from human origin. Hence the results obtained from these *in vitro* studies could hardly explain what happens *in vivo*. Thirdly, the physiological forces in the human GI tract are complex and challenging to simulate. Many *in vitro* models have failed to apply the appropriate mechanical forces to simulated digestion with physiological relevance. Furthermore, most of the *in vitro* models are adult models and not designed specifically for infant studies. The digestive conditions of infants differ from that of adults and especially for lipid digestion, the lack of pancreatic lipase and bile salts in the neonates suggests that lipid digestion in the gastric phase may be more critical than in the small intestine. However, only a few *in vitro* models have considered gastric lipid digestion (Ménard et al., 2014; Ménard, Picque, & Dupont, 2015). Besides, the BSSL in human milk may also play an essential role in neonatal lipid digestion. However, the role of BSSL in the neonatal lipid digestion has only been included in several *in vitro* models (Bernback et al., 1990; Chen, Bläckberg, Nilsson, Sternby, & Hernell, 1994) and those models hardly have adequate physiological relevance. Although *in vitro* models always have limitations as such, studies still need the *in vitro* approach to understand digestion in the GI tract for their lower cost compared to human trials and convenience to conduct experiments.

In considering gastric digestion, food introduced to the stomach is influenced by several biophysical and biochemical conditions, such as the mechanical forces, the dynamic *p*H levels, and the production of digestive enzymes and salts. For *in vitro* analysis, any parameters are usually chosen for the model and can additionally be tailored to answer specific digestive questions. The following parts will give more details on the simulation of gastric digestion, and the appropriate selection and application of the varying parameters.

2.4.1 *p*H level

The *p*H profile for infant gastric digestion is markedly different from that in adults (mentioned in Chapter 2.2.1). Though it is possible to simulate the dynamic profile of the changing *p*H during gastric digestion, the results are challenging to interpret for that it is difficult to know what stage it stands for the entire process of digestion. Therefore most of the static *in vitro* models have selected fixed *p*H levels which are representative of the gastric environment at different phases, for example, at fasting or postprandial phase (Gallier et al., 2012; Golding et al., 2011; Lueamsaisuk, Lentle, MacGibbon, Matia-Merino, & Golding, 2014). The advantage of a fixed *p*H level for *in vitro* tests is that it is relatively easy to study the influences from other parameters. However, it only provides physiological relevance for a specific window of digestion. The main problem arises from the fact that most of the studies use their own *in vitro* models, making the results very difficult to compare with each other.

Some semi-dynamic or dynamic models have been developed to simulate the secretion of gastric content and the dynamic *p*H profile by gradually adding hydrochloric acid into the system which follows a predetermined curve relevant to physiological conditions (Guerra et al., 2012; Kong & Singh, 2010; Minekus, 2015). A dynamic model that simulates infant digestion has been recently developed with adjustment of parameters considering physiological aspects of the GI tract in infant (Ménard et al., 2014). The volume adjustment in this study through acid addition can dilute the mixture, and therefore influencing sampling. For the development of infant gastric *in vitro* models, the *p*H profile is largely affected by the buffering effect of the ingested milk and the frequency of feeding. This, in return, influences the concentration and activity of the digestive enzymes.

2.4.2 Digestive enzymes used in *in vitro* digestive systems

Enzymes used in *in vitro* models for human digestion are usually selected from animals or microbial sources, rather than using the extracts from the stomach or small intestine from human subjects due to their costs, accessibility and the challenges of standardising the extracted materials. Human gastric pepsin and lipase are the main digestive enzymes that would need to be used in gastric *in vitro* studies. Human gastric pepsin shows optimal activity at *p*H 2. It is stable in the acidic gastric environment until *p*H reaches 6, where the activity starts to drop off. *In vitro* studies usually use porcine pepsin because of its low cost and commercial availability. The optimal *p*H range for the activity of this porcine pepsin is from 1.5 to 2.5, which is close to that of human gastric pepsin. Porcine pepsin is widely used as a substitute for human gastric pepsin. They are commercially available and relatively more stable than human pepsin with known activity. A variety of pepsin concentrations from 0.4 mg/mL to 5 mg/mL with activity from 800 to 2500 U/mg have been used in previous studies (Gallier et al., 2012; Golding et al., 2011; Minekus et al., 2014; Ruby et al., 1993; Sarkar, Goh, & Singh, 2009; C. Versantvoort & Rompelberg, 2004; C. H. Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005).

The use of gastric lipase in the *in vitro* models is very challenging. Some early *in vitro* studies omitted gastric lipase entirely, while others usually applied lipases from uncommonly-used sources rather than from porcine or human subjects (Golding et al., 2011; Hur, Lim, Decker, & McClements, 2011; Lueamsaisuk et al., 2014). In terms of finding appropriate analogues for human gastric lipase with similar optimal *p*H range and activity, while a few studies have indeed used human gastric extracts (Carrière et al., 1997; Garcia, Antona, Robert, Lopez, & Armand, 2014) as part of *in vitro* analysis, it is still very challenging to obtain extracts from human subjects because of their ethical restrictions, commercial availability and variability. Alternatives such as fungal lipase (Golding et al., 2011; Lueamsaisuk et al., 2014, 2015; Ménard et al., 2014), rabbit gastric lipase (Bourlieu et al., 2015; Capolino et al., 2011), dog gastric lipase (Vié et al., 2015) and calf pre-gastric lipase (Lueamsaisuk et al., 2014, 2015) have already been used for *in vitro* studies for lipid digestion. Some of these lipases from mammal origins (Table 2.5) with a relatively low optimal *p*H range could be treated as more appropriate analogues to HGL.

Table 2.5. Stable and optimal pH ranges of selected lipases.

lipase	Stable *p*H range	Optimal *p*H range
Human gastric lipase	2 – 7[a]	3-6[b]
Dog gastric lipase	1 – 2[c]	3.5-6.5[c]
Rabbit gastric lipase	3 – 6[c]	4[c]
Calf pre-gastric lipase	6.0 - 8.0[d]	6.5[d]
Fungal lipase	4.5 - 7.5[e]	7.5[e]

[a] Data from Ville *et al*. (Ville et al., 2002).

[b] Data from Roussel *et al*., Gargouri *et al*., Ville *et al*., and Carriere *et al*. (Carriere et al., 1991; Gargouri, Sarda, et al., 1986; Roussel et al., 1999; Ville et al., 2002).

[c] Data from Carriere *et al*. (Carriere et al., 1991).

[d] Data from (Manuel, 1999).

[e] Data from (Hiol et al., 2000).

Lipases from fungal or mammal origins usually have different stereo-specificity for triglycerides from human gastric lipase. For example, fungal lipases cleave the sn-1 and sn-3 positions, and lipases from mammal origins only cleave sn-1 or sn-3 position (Ménard et al., 2014; Rogalska, Cudrey, Ferrato, & Verger, 1993; Rogalska et al., 1990). Among those alternatives, some *in vitro* studies prefer to use fungal lipases from a selected strain *Rhizopus oryzae* (ATCC 1996) because of its stability under gastric conditions compared to lipases from mammal origins. Fungal lipases are widely used in the food industry. They are commercially available with a known amount of activity. The activity of fungal lipase from this selected strain has optimal activity at *p*H 7.5. It is stable at a *p*H 4.5-7.5 and hydrolyses triglycerides at their sn-1 and sn-3 positions (Hiol et al., 2000). Its optimum deviates from that of human gastric lipase (*p*H 3.0-6.0) (Hamosh et al., 1981; Roussel et al., 1999; Ville et al., 2002). Since after ingestion, gastric *p*H in infants is relative higher (5.2 to 7.1) than that in adults (where *p*H stays from 3.5 to 5.0), both fungal lipase and porcine pepsin may not achieve their optimal performance for *in vitro* tests under such *p*H range. Some studies have proposed that the optimal *p*H range may not reflect the local pK_a in the vicinity of the active site of lipases with lid domains (Poulsen et al., 2005). For human gastric lipase, the lid domain of lipase needs to be shifted to expose its active site to the oil/water interface (Miled et al., 2003). Therefore, the *p*H at which the lid is activated between human gastric lipase and its alternatives also needs to be considered in simulated gastric digestive models.

Table 2.6 A comparison between adult and infant simulated gastric systems.

Parameters	**Infant**	**Adult**
Meal : Simulated Gastric Fluid (v:v)	63:37	1:1
Pepsin	1250 U/mL Flow: 0.25 mL/min	2,000 U/mL
Lipase from rabbit or dog gastric extract	60 U/mL Flow: 0.25 mL/min	60 U/mL
Incubation time (min)	$t_{1/2}{}^{a}$ = 70	120
pH	-0.011*t^{b} + 5.4	3
Temperature (ºC)	37	37

Data in this table was from Ménard *et al*. (2018), Ménard *et al*. (2014), Minekus *et al*. (2014), Brodkorb *et al*. (2019).
[a] *t* is the time after ingestion, min.
[b] $t_{1/2}$ is the half time of delivery.

A comparison of adult and infant simulated gastric model is shown in Table 2.6. The infant model refers to an *in vitro* dynamic system proposed by Ménard *et al*. (2014). The adult model was a static system based on a standard method that uses enzymes from the same sources and at fixed concentrations. The gastric *p*H of the infant model has a dynamic *p*H profile and was much higher than that of the adult in general. In the digestion protocol proposed by the INFOGEST network (http://www.cost-infogest.eu) involving multidisciplinary experts from more than 35 countries was proposed in 2014 (Minekus et al., 2014), lipid digestion in the gastric phase was considered to have a minor influence on adult lipid digestion (as opposed to the greater role in infant digestion), therefore usage of gastric lipase in this static *in vitro* model has not been recommended. An update of this protocol was published in 2019 by Brodkorb *et al*. (2019) has added a recommendation for involving gastric lipase in the static model together with the limitations of using gastric lipase. This method has also proposed one constant *p*H level (*p*H 3) for digestion as a basic suggestion. Since the gastric phase has a changing *p*H profile from fasting to the postprandial stage, it may only provide information for gastric digestion at a certain stage. Furthermore, for both infant and adult model, there was not a recommended shear rate for *in vitro* digestion tests in the gastric phase, the deficiency of information on the shearing force may reduce physiological relevance of the model and add difficulties in understanding the effect of mechanical forces on the structure of lipid emulsions.

2.4.3 Physical forces

Replicating the mechanical environment in the stomach is also a critical consideration in developing physiologically relevant *in vitro* models. A series of contractions in the stomach assists in breaking down food components, mixing food with gastric juice and emptying gastric content (Johnson, 2014; D. J. McClements, Decker, & Park, 2009; Schulze, 2006; Schwizer, Steingoetter, & Fox, 2006). Contractions from the antrum and pylorus generate shearing forces to assist in the process of food mixing and breaking down. Studies by Lentle *et al*. (Lentle & Janssen, 2011; Lentle, Janssen, Goh, Chambers, & Hulls, 2010) have observed a maximum shear rate of approximately 0.7 s^{-1} in the gastric phase, as estimated from an *ex vivo* rat stomach, while the velocity of the gastric retropulsive jet has been calculated to be approximately 7.5 mm/s, based on the observations from human gastric models (Pal et al., 2004; Schulze, 2006). However, many *in vitro* models use a stirring rate of 100 rpm (or even higher) which generates much higher shear rates than 0.7 s^{-1}. While this may ensure good mixing of samples, these shear rates are significantly higher than those was present in the physiological conditions of the human stomach. The application of elevated shear rates may be particularly problematic as it induces structural changes to ingested food that would not be expected to occur with low shearing forces applied for the digestive environment. This is particularly an issue for static models, where the common use of magnetic stirrers always provides a shear rate far above the physiological low shear rate due to their speed limitations.

2.4.4 Use of *in vitro* models

The growing interest in the digestion process of foods has led to the development of many *in vitro* models capable of simulating digestion in the human GI tract. Static models are the most widely used approach not only for food materials but also for drug development. Static models usually have fixed parameters such as enzyme concentration, *p*H level, and ionic strength, and reactions take place in a thermostatically controlled vessel. Static models have been shown as a convenient means of investigating the influences of gastric conditions on lipid digestion separately (Golding et al., 2011; Lueamsaisuk et al., 2014; Minekus et al., 2014). However, the variability between model design and development of methods used to analyse the digestate has presented challenges in providing consistent information to prove their relevance to human physiology. An example of this particular concern is the determination of the extent of lipolysis during simulated lipid digestion, which is one of the most important links in understanding lipid digestion. Over the past few decades, the *p*H-stat method has been frequently used as an assay for lipolysis (Helbig, Silletti, Timmerman, Hamer, & Gruppen, 2012; Li, Hu, & McClements, 2011; D. J. McClements & Li, 2010; Mun, Decker, Park, Weiss, & McClements, 2006). The *p*H-stat method measures the amount of lipolysis product by titrating the liberated FFA with NaOH while keeping *p*H at a constant level (usually at *p*H 7). However, this titration method is more suitable for determining the degree of lipolysis under neutral small intestinal conditions rather than under acidic gastric conditions, as the *p*H endpoint of the digestate needs to be higher than the pK_a of liberated fatty acids. Detection of the protons released from lipolysis requires ionisation of the liberated free fatty acids. Since pK_a of these free fatty acids varies from 4.7 to 4.9 (D. J. McClements & Li, 2010), they are only in the ionised form at the neutral intestinal *p*H (5.8-6.8), where sodium hydroxide (NaOH) can neutralise the released protons and keep the *p*H level constant to the initial one.

Sometimes digestion of lipids also involves breaking down of proteins. If proteolysis is taking place simultaneously, the generation of amino acids could also influence the accuracy of the method. This method also does not allow measurement of other lipolytic products such as mono- and di-glycerides. These surface-active compounds may change the properties of oil/water interface and in consequence, have an impact on the rate of lipolysis. Other methods such as gas chromatography (GC) and thin-layer chromatography (TLC) can be applied in combination with static models to determine the types and quantities of the lipolytic products more precisely. Such measurements need to be made over the course of the digestion period. Thus, the combined method using *p*H-stat and GC/TLC, or comparison between different methods may provide more powerful results than using the *p*H-stat alone.

While static models are most commonly used to simulate digestion, many dynamic models have also been developed to monitor digestion in a simulated way, with considerations of stomach emptying, dynamic gastric *p*H profile and constant enzyme secretion (Ménard et al., 2014; Minekus, 2015).

2.4.5 Validation of *in vitro* models

Studies based on human clinical trials have allowed correlations to link between properties of emulsions and gastric function, plasma triglycerides concentration, hormone respond for satiety and food intake (Keogh et al., 2011; Marciani et al., 2009; Marciani et al., 2007; Oosting et al., 2014; Steingoetter et al., 2015), which validate the *in vitro* models used in many studies. Studying digestion through human models has always brought ethical issues and increased costs. This is particularly true for studies associated with infant digestion. Experiments conducted among premature infants may provide a pathway for *in vivo* investigation since they fed on naso- or orogastric tube, which facilitates data collecting (Armand, Hamosh, et al., 1996). However, *in vivo* studies conducted in healthy infants can be more challenging (Abrahamse et al., 2012). This is not only from an ethical perspective, other considerations, such as the rapidly changing digestive physiology of infants during the early stages of growth, means that human studies using infants can be challenging to implement. Therefore, animals are also commonly used in lipid digestion studies, particularly when considering neonatal digestion, with several options available, such as mini-pigs, rats, dogs and rabbits (Capolino et al., 2011; Gallier & Singh, 2012; Goncharova et al., 2014; Iverson, Kirk, Hamosh, & Newsome, 1991). However, digestion in the animals differs significantly from that of human, which means the results obtained and conclusions drawn from the animal models may not be valid in humans. Developing proper methods for *in vivo* and *in vitro* correlations will provide robust findings for us to understand the real physiology of digestion in the GI tract.

2.5 Factors influencing the stability of infant milk emulsions during gastric digestion

Many factors in the gastric environment influence the stability of milk emulsions such as *p*H level, digestive enzymes, ionic force, and physical forces as well as the physical characteristics of the milk emulsions. The following sections will discuss some of the most important features that influence the colloidal status of milk emulsions in the gastric phase.

2.5.1 The impact of gastric *p*H on interfacial properties

Gastric *p*H can influence the stability of emulsions as it changes from fasting to postprandial states. The *p*H profile of the fasting states in the adult human is 1.7-2.9 (Armand, Borel, et al., 1996a; Armand et al., 1999; Dressman et al., 1990; Kenmogne-Domguia, Meynier, Viau, Llamas, & Genot, 2012; Lindahl, Ungell, Knutson, & Lennernäs, 1997) which differs very much from that of the neonates (*p*H 3.5) (Chatterton et al., 2004; Mason, 1962). In the gastric environment, the postprandial *p*H level in healthy infants usually varies from 3.5 to 6.7 (Mason, 1962; Mitchell et al., 2001), whereas in adults, it remains at 4.0-6.7 (Armand, Borel, et al., 1996a; Armand et al., 1999; Dressman et al., 1990; Kenmogne-Domguia et al., 2012; Lindahl et al., 1997).

After ingestion, the milder acidic neonatal gastric environment in infants plus the buffering effect of infant milk (breast milk or infant formulae) will bring gastric *p*H close to neutral. As digestion proceeds, the dropping *p*H level transits through the isoelectric point of proteins at the interface (usually between 4 and 5) (Law & Leaver, 2000; Walstra, 1999). At that *p*H range, the electrostatic repulsion is reduced— the absolute ζ-potential values much lower than -20 mV (towards 0 mV) (Lentle & Janssen, 2011), which is not enough to stabilise milk emulsions by electrostatic repulsive forces, so the aggregation of proteins and the emulsion droplets happens.

Maldonado *et al*. (2009) have used native β-lactoglobulin to form a film at the air/water interface to study the effect of physiological conditions on interfacial properties in the gastric phase. They found that under acid *p*H conditions, the dilatational and surface shear modulus had reduced. When the temperature increased to body temperature (37 °C), there was also a decrease in dilatational response and increase in surface pressure (Mellema & Isenbart, 2004). Therefore, the combined effect of low *p*H and body temperature could weaken the network of interfacial proteins. However, if *β*-lactoglobulin was heated to above 60-80 ºC (as it would happen when processing protein-stabilised emulsions), the unfolding of its native form occurs, leading to the denaturation of the protein molecules (Eric Dickinson, 2010). The denaturation exposes more hydrophobic domains to the oil/water interface, thus increasing protein-protein interactions and therefore increasing the interfacial shear elasticity and viscosity of the interfacial protein layer at *p*H 5.5 and 7. This added resistance to the interfacial protein network at the oil/water interface might decrease the propensity for the coalescence in the emulsions to some degree (Kim, Cornec, & Narsimhan, 2005). Clearly, the interfacial properties of *β*-lactoglobulin were sensitive to heat treatment before or after emulsification (Eric Dickinson, 2010; Raikos, 2010). On the other hand, interfacial layers made up of caseins such as *β*-casein at the oil/water interface, where the hydrophobic domains attached to the oil/water interface and the N-terminal hydrophilic tail pocks into the aqueous phase (Eric Dickinson, 2001, 2010), are resistant to heating process but highly sensitive to *p*H as well as ionic strength (Eric Dickinson, 2010; E. Dickinson, Valerie, David, & Frans, 1997).

Raw milk can aggregate at acidic gastric *p*H because of changes in the electrostatic repulsion of milk proteins as well as milk fat globules in the aqueous phase (Garcia et al., 2014). An *in vitro* study has shown that after mixing, the surface-area mean diameter (d_{32}) of fat globules in raw bovine milk with simulated gastric juice (*p*H 1.2), increased by a factor of 25 and the volume mean diameter (d_{43}) increased by a factor of 10 because the milk fat globules were trapped into the aggregates formed with whey proteins and caseins (Gallier et al., 2012). Gallier *et al.* (2013) also confirmed an increase in the size of the fat globules under gastric conditions in a rat study. However, a study by Lueamsaisuik *et al.* (2015) observed no significant changes in droplet size distribution for human milk fat globules at *p*H 3.5, 4.5 and 5.5 under gastric conditions in a simulated neonatal model.

The colloidal stability of native milk fat globules depends on three forces: Van der Waals forces, electrostatic interactions and repulsive steric forces (Huppertz & Kelly, 2006). The electrostatic repulsion is provided by the charges on the milk fat globule membrane, and the steric repulsion provided by glycoproteins on the membrane surface give counter forces to the Van der Waals attractions—which depend on droplet distance, size and other physical properties of the fat globules (Bergenståhl & Claesson, 1997; Friberg, 1997; Huppertz & Kelly, 2006; David Julian McClements, 2015). When Van der Waals attraction is stronger than the two other repulsive forces, aggregation occurs. The electrostatic repulsion is mostly affected by the changing *p*H in the gastric phase as well as the presence of salts. In the gastric environment, the dropping *p*H decreases the charges of the milk fat globule membrane (Garcia et al., 2014) promoting aggregation. This destabilisation process reduces the effective interfacial area for lipase adsorption and therefore, may indirectly affect the rate and extent of gastric lipolysis.

2.5.2 Proteolysis

Proteolysis of the interface in protein-stabilised emulsions can change the interfacial properties and reduce the protein layer thickness, thereby inducing emulsion instability. The major forms of aggregation in protein-stabilised emulsions are flocculation, coalescence and partial coalescence (Huppertz & Kelly, 2006). When proteins adsorb at the oil/water interface, the electrostatic and steric repulsions provided by this polymer layer, protect droplets against flocculation and coalescence (Golding, 2013). Gastric proteolysis can cleave the hydrophilic domains and leave hydrophobic domains still adsorbed to the oil/water interface, impacting emulsion stability as a result of disrupting the interfacial layer (David Julian McClements, 2015; Singh & Sarkar, 2011), increasing the attractive hydrophobic forces between droplets and therefore the propensity for flocculation or coalescence (Golding, 2013).

In the human GI tract, the polymeric protein layer at the oil/water interface in protein-stabilised emulsions will encounter hydrolysis by proteases, losing the steric hindrance (repulsion) that usually prevents aggregation. The main whey protein in human breast milk is α-lactalbumin, whereas β-lactoglobulin, which exists in bovine milk, is not present in human breast milk (Heine et al., 1991). The specific structural differences in proteins determine their susceptibility to pepsin. Several studies have shown that β-lactoglobulin and α-lactalbumin were resistant or partially resistant to pepsinolysis at different *p*H levels (1.5-4.0), as structurally casein is more susceptible to pepsin proteolysis than β-lactoglobulin (Astwood, Leach, & Fuchs, 1996; Chatterton et al., 2004; Dupont et al., 2010; Inglingstad et al., 2010; Sakai et al., 2000; Thao et al., 2015). Emulsification can also alter the conformation of milk proteins upon adsorption on the oil/water interface, as indicated by Macierzanka and co-researchers (2009). These authors showed that β-casein and β-lactoglobulin, adsorbed on interfaces were more susceptible to pepsin than in the aqueous phase being consistent with two more recent studies (Maldonado-Valderrama, Wilde, Mulholland, & Morris, 2012; Sarkar, Goh, & Singh, 2010). However, for α-

lactalbumin, the adsorbed protein showed more resistance to proteolysis at the interface than in the aqueous phase (Malaki Nik, Wright, & Corredig, 2010).

Processing methods, such as heating or applying high hydrostatic pressure, can unfold proteins and increase the susceptibility of the proteins to pepsinolysis. This phenomenon is especially important in the case of β-lactoglobulin, where heating can partially unfold the protein structure and expose more cleavage sites for pepsin to hydrolyse (Reddy, Kella, & Kinsella, 1988; Sakai, Ogawa, Takashima, Mizunuma, & Manabe, 1997; Sitohy, Chobert, Dalgalarrondo, & Haertlé, 2001).

Proteolysis, apart from reducing the thickness and mechanical strength of protein films at the oil/water interface(Golding, 2013), can also cleave the charged groups from the adsorbed interfacial proteins, reducing the electrostatic repulsion which may induce emulsion flocculation. Partial hydrolysis by pepsin on pre-formed β-lactoglobulin adsorbed at air/water interface did not impair the protein network (Gunning & Morris, 2018) whereas, at a pure oil/water interface, pepsin hydrolysis could weaken the protein network, reducing its coverage and the interfacial elasticity (Maldonado-Valderrama, Holgado Terriza, Torcello-Gomez, & Cabrerizo-Vilchez, 2013; Scheuble, Geue, Windhab, & Fischer, 2014). This process can increase the propensity of film rupture and induce droplet coalescence. However, for β-casein, pepsinolysis has been shown to increase the elasticity of the protein network resulting in a more cohesive network at a pure oil/water interface (Maldonado-Valderrama et al., 2013). In this case, pepsin hydrolysis of the protein network increased the resistance of the β-casein emulsion to coalescence.

For raw bovine milk and human milk, proteins exist in the serum phase (caseins and whey proteins) and as part of the membrane in the milk fat globules. The main proteins in the milk fat globule membrane, such as MUC 1, PAS III and butyrophilin, are largely glycosylated (Martini, Salari, & Altomonte, 2016; Singh, 2006). Studies have found that some of the membrane glycoproteins are less susceptible to pepsinolysis under gastric conditions compared to serum proteins in bovine milk (Hamosh et al., 1999; Le et al., 2012). However, in a study by Ye *et al*. (2011) using washed cream (without aqueous proteins), peptides became dominant in the digestate after simulated gastric digestion (in the absence of gastric lipase) which indicated that most of the membrane proteins had been hydrolysed. By using high concentrations of pepsin, it was found that the membrane proteins disappeared gradually on SDS-page gels, and peptides dominated on the bands. Since the study by Ye *et al*. (2011) did not provide enough information for the conditions of experiments, it is difficult to compare with the results obtained by Le *et al*. (2012) who found that glycoproteins were quite resistant to pepsinolysis. However, in the study by Ye *et al*. (2011), a variety of enzyme concentrations showed that at a higher enzyme level, the degradation of proteins in the milk fat globule membrane was substantial. In general, and as mentioned above, caseins in the milk serum are more susceptible to pepsin hydrolysis than β-lactoglobulin in whey protein.

Some *in vitro* studies have observed aggregation or flocculation for both human and bovine milk in simulated gastric models for protein digestion (with or without the presence of gastric lipase) (Bourlieu et al., 2015; de Oliveira, Bourlieu, et al., 2016; de Oliveira, Deglaire, et al., 2016; Gallier et al., 2012; Ye et al., 2011). An *in vivo* study in rats showed an increased size in raw bovine milk fat globules during gastric digestion (Gallier et al., 2013). In this study, gastric lipase was present (and hence lipolysis was presumably occurring), whereas the previous *in vitro* studies cited, were carried out in the absence of lipase (to study protease activity) with the milk fat globules remained intact during digestion with no coalescence being observed (Bourlieu et al., 2015; de Oliveira, Bourlieu, et al., 2016; de Oliveira, Deglaire, et al., 2016; Gallier et al., 2012; Lueamsaisuk, 2015; Ye et al., 2011). Therefore, the network formed by serum proteins and their hydrolysates could be the reason for the aggregation observed.

During simulated gastric digestion (in the absence of gastric lipase), the ζ-potential of the milk fat globule did not change significantly, suggesting that pepsinolysis had not changed much the charge of the membrane (Gallier et al., 2012; Ye et al., 2011). The electrostatic repulsion provided by charges of proteins and peptides at the interface and the steric repulsion from intact undigested glycoproteins may provide protection and barrier from the coalescence of the fat globules (Gallier et al., 2012; Le et al., 2012).

2.5.3 Lipolysis

When dealing with gastric digestion, lipolysis is not usually discussed in isolation but combined with proteolysis by pepsin. Gastric lipolysis may change the stability of the emulsion as well as its specific surface area. During gastric lipolysis, the generation of free fatty acids and other lipolysis products accumulate on the oil/water interface and change the interfacial tension and film properties. The interface becomes more hydrophobic compared to the initial state where proteins occupied the interface. Instability may occur when lipolysis products create more attractive forces between the droplets. Golding *et al*. (2011) have found that a whey protein-stabilised emulsion showed a steady increase in average particle size and flocculation, under a confocal microscope. The rate of gastric lipolysis was significantly affected by the changes in the droplet surface area in both *in vitro* and *in vivo* studies. Moreover, proteins were proven not to act as a barrier for gastric lipolysis as shown by a study using pure oil/water interface covered by β-lactoglobulin and β-casein (Maldonado-Valderrama et al., 2013), where the authors found that lipolysis still occurred.

A study by Luo *et al*. (2019) has investigated the oil droplets coated by either the lipids from the milk fat globule membrane or by casein in a simulated *in vitro* infant gastric system. It was found that the droplets covered by the lipids from the milk fat globule membrane were generally more stable during digestion than the casein-coated ones. The absolute ζ-potential of casein-coated droplets dropped during gastric digestion showing instability, due to a decrease in the electrostatic repulsion. They observed an increase in the droplet size and large aggregations formed at the end of the simulated gastric digestion.

Because of the unique tri-layer membrane interface with integrated proteins in the membrane, the interfacial properties of the milk fat globule membrane are very different from other emulsions stabilised by surfactants or biopolymers. Gastric lipase has been proven to have an affinity for the membrane, and so it can adsorb on the mono phospholipid layer on the oil/water interface (Pafumi et al., 2002). Thus the milk fat globule membrane naturally provides favourable conditions for gastric lipase to adsorb and hydrolyse triglycerides in the lipid core.

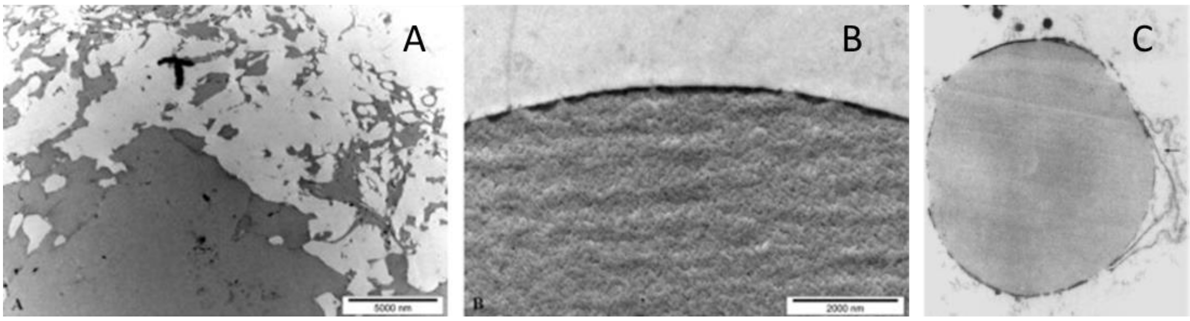


Figure 2.10 A and B transmission electron microscope images of fat globules in the chyme collected from rats 180 min after gavaging raw bovine cream, scale bar = 2,000 nm (A) and 10,000 nm (B), images adapted from Gallier *et al*. (2013). Reproduce with permission from Elsevier. C, a phase-contract image of milk fat globule in gastric chyme of a 10-day-old suckling rat, magnification: ×10,000, image adapted from Berendsen & Blanchette-Mackie (1979). Reproduce with permission from John Wiley and Sons.

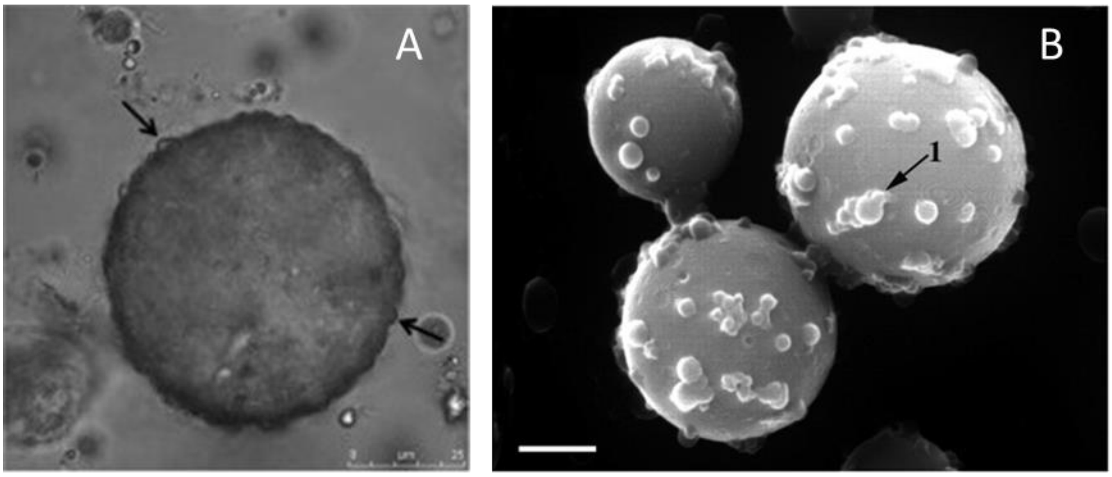


Figure 2.11 A Confocal laser scanning microscope image under DIC (differential interference contrast) mode of milk fat globules in the chyme collected from rats 180 min after gavaging raw bovine cream, scare bar = 25 μm, image adapted from Gallier *et al*. (2013). Reproduce with permission from Elsevier. B, Scanning electron image of artificial emulsion droplets hydrolysed by human gastric lipase, magnification: × 5,400, image adapted from Pafumi *et al*. (2002). Arrows indicate protrusions on the membrane surface. Reproduce with permission from American Soc for Biochemistry & Molecular Biology.

As fat is majorly digested in the small intestine, studies on gastric lipolysis are very limited. As found for protein-stabilised globules, the gastric lipolysis of the MFGM stabilised globules strongly related to proteolysis in both *in vitro* and *in vivo* studies.

During gastric digestion, proteins aggregate due to *p*H and proteolysis in the stomach. Researchers have observed fat globules trapped in the aggregated network of proteins and peptides with membrane structure remaining intact in some *in vitro* studies (Bourlieu et al., 2015; de Oliveira, Bourlieu, et al., 2016; de Oliveira, Deglaire, et al., 2016). Another study also reported that the droplet size of fat globules from raw bovine milk increased in a simulated gastric system (Garcia et al., 2014). Similarly, an *in vivo* study (Gallier et al., 2013) in rats and an *in vivo* preterm infant study (Bourlieu et al., 2017) also found an increase in droplet size similar to an *in vitro* study (Garcia et al., 2014) when digesting milk fat globules in the gastric phase. The irregular surface of the fat globules with protrusions (Gallier et al., 2013) in Figure 2.11, and lamellar phase at the interface (Figure 2.10) were observed after gastric digestion in rats, which was also observed in another *in vivo* study in suckling rats (Berendsen & Blanchette-Mackie, 1979). In an *in vitro* study using phospholipid-emulsified artificial emulsions by Pafumi *et al.* (2002), trapped gastric lipase was observed in the accumulated lipolytic products on the surface (Figure 2.11). Thus, it is reasonable to presume that the protrusions in the rat study were accumulated lipolytic products from gastric lipolysis of triglycerides.

Gastric lipolysis has been proven to be a fundamental step in the lipid digestion process, which provide a favourable interface for pancreatic lipase, co-lipase and bile salts in the intestinal phase for further hydrolysis of triglycerides in the lipid core (Gargouri, Pieroni, et al., 1986; Hamosh, 1979b; Hamosh, Bitman, Wood, Hamosh, & Mehta, 1985).

2.5.4 Temperature and mechanical forces

The body temperature can also influence the interfacial properties of protein-stabilised emulsions in the gastric phase. Scheuble *et al.* (2014) observed the weakening of the interfacial layer of *β*-lactoglobulin-emulsified medium-chain triglyceride oil when the temperature increased to body temperature in an acidic environment. In addition, the shearing forces in the stomach can also increase the collision rate of the oil droplets, thinning or impairing the interface of the oil droplet, which may further induce instability.

2.5.5 Droplet size and the surface area of milk emulsions

It can be argued that the emulsion structure of human breast milk has evolved to provide the most effective design for digestion of fat within the neonatal digestive tract. However, the structural factors of infant formulae, such as droplet size, interfacial composition, changing interfacial properties and inhibition effect of additional compounds, may adversely influence gastric and small intestinal lipid digestion.

When milk emulsions enter in the neonatal stomach—because of the low shear rate in the oral environment and very limited oral process—the size of the oil droplets is expected to remain unchanged to the initial size upon ingestion. However, gastric lipase, in acting to hydrolyse the triglyceride component of fat droplets, has a pronounced effect on the interfacial and emulsion size and structure.

Research with some *in vitro* systems has demonstrated that the rate of lipolysis was inversely correlated to the droplet size. With the changing *p*H in the stomach, no correlation was found between emulsion droplet size and gastric *p*H. The fat droplets with smaller size are likely to induce more extensive gastric lipolysis as they have a larger surface area for lipase adsorption. A study which collected gastric juice from human adults and obtained rabbit gastric lipase and examined the gastric lipolysis of four different emulsions with droplet sizes of 0.19 μm, 0.43 μm, 0.46 μm, and 3.18 μm (Borel et al., 1994) has observed greater fatty acid generation for the fine emulsions compared to the coarse ones (Figure 2.12). to be used in the experiment.

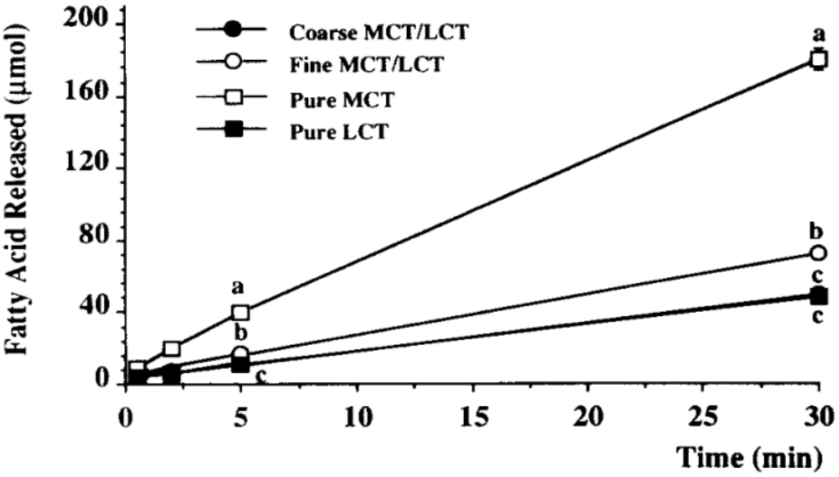


Figure 2.12 The release of free fatty acids by human gastric lipase as a function of incubation time. Different letters (a, b or c) indicated a significant difference ($p<0.05$) at given times (Borel et al., 1994). Reproduce with permission from Elsevier.

Another study has shown similar findings that emulsions with a lower surface area could be correlated with a lower rate of lipolysis (Keogh et al., 2011). Similar results have been found with pancreatic lipase in an *in vitro* study (Berton et al., 2012), where the rate of lipolysis by this lipase was found to be 4.6-fold higher in small native milk fat globules (1.8 μm) than in large milk fat globules (6.7 μm). However, it should be noted that the latter study conducted the experiments at 20 °C a lower temperature than physiological temperature (37 °C).

Some *in vivo* studies with human trials have also confirmed that the rate of lipolysis by gastric or pancreatic lipase can be related to droplet size (notably under highly controlled conditions). Armand *et al*. (1999) used a fine emulsion (0.7 μm) and a coarse emulsion (10.1 μm) for digestion and absorption tests performed in healthy human subjects. They found that during four hours of digestion in the human GI tract, the coarse emulsion resulted in no much change to droplet size. However, the fine emulsion had a four- to nine-fold of increase in median diameter of the emulsion droplets. Gastric lipolysis rate was found to be 1.7 to 3.3 times higher in the fine emulsion than in the coarse emulsion (Figure 2.13).

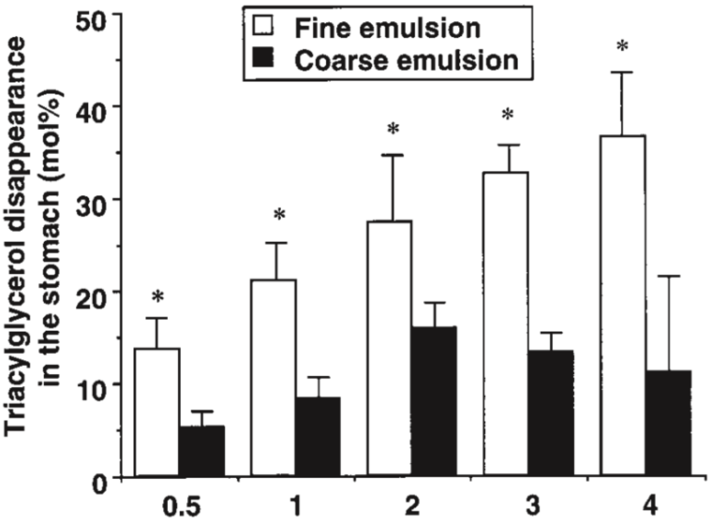


Figure 2.13 The percentage of four hours of gastric lipolysis of fine and coarse emulsions (Armand et al., 1999). Reproduce with permission from Oxford University Press.

Concerning the digestibility of human and formula milk, a study in premature infants showed that gastric hydrolysis of human milk is 1.7 to 2.5-fold greater than that of infant formula after ingestion (Armand, Hamosh, et al., 1996) although the diameter of human milk fat globules (4 μm) was much larger than that of the infant formula (0.6 μm). Again, the composition of the fat differs between human breast milk and infant formula probably influencing the digestion. Hence, chemical differences at the oil/water interfaces of the two milk types could concurrently influence the affinity of lipase. The unique phospholipid tri-layer stabilising human milk fat (Lopez & Menard, 2011) which differs markedly from that of milk-protein-stabilised infant formulae is considered to facilitate the binding of lipase (Bourlieu-Lacanal et al., 2015; Jensen et al., 1992). Although it is difficult to compare studies conducted *in vitro* and *in vivo* with adult human subjects as the emulsion may be affected by oral processing (D. J. McClements et al., 2009), this may have an insignificant impact on infants as they swallow milk with a minimal oral process.

A considerable amount of studies has investigated gastric lipid digestion from both *in vitro* and *in vivo* approaches. However, the relationship between structural changes in the emulsions and the degree of gastric lipolysis is still not clear, especially in the neonates. The *in vitro* approaches have been used over the past two decades, allowing increasingly sophisticated models of digestion to be built. However, the gastric phase of lipid digestion, which is especially crucial for neonates, has been largely neglected because of its minor contribution to adult digestion. This project aimed to understand the relations between the structural changes in lipid digestion and the degree of lipolysis in a neonatal gastric model. In this project, we used an *in vitro* model with a low shear rate that could physiologically represent the low shearing forces in neonatal gastric phase. In this way, we were able to investigate the effects of different reactants and substrates on gastric lipolysis in a separate or combined manner.

2.6 Development of *in vitro* gastric digestion and analysis of FFA

In vitro models are widely used to mimic digestion in the GI tract. Different types of models have been introduced in the previous sections (Chapter 2.4.4). Researchers have adjusted various parameters used in the *in vitro* models through the years, to answer specific research questions. For lipid digestion, the simulation of the processes in the mouth, stomach and small intestine are usually studied separately.

2.6.1 Determination of the degree and rate of lipolysis

Quantification of lipid digestion has been investigated for decades. Titration is the most commonly used method. As it is an assay for determining lipase activity, an auto-titration method — the *p*H-stat method has been introduced to analyse the rate of lipolysis in lipid digestion conducted in the *in vitro* studies. The *p*H-stat method determines the amount of free fatty acids released by continuous titration with a base to a constant *p*H level. It is usually used under small intestinal conditions (*p*H 6.8-8.0) (Helbig et al., 2012) which is quite limited. The pK_a of many fatty acid esters from dietary fats is at 4.7-4.9 (de Maria, Fernandez-Alvaro, ten Kate, & Bargeman, 2009; D. J. McClements & Li, 2010). Thus, this method is not suitable for measuring the rate of lipolysis under gastric conditions where the *p*H would be 2-6. Besides, apart from the fatty acids released from simulated digestive lipolysis, diglycerols and monoglycerols are also generated from the process. This method only gives a rough estimation of the released fatty acid from lipolysis, and therefore cannot accurately quantify all of the lipolytic products. Other than the *p*H-stat method, more accurate methods such as GC, TLC and mass spectrometry have been used in many *in vitro* and *in vivo* studies (Bourlieu et al., 2015; Brenna, 2013; Helbig et al., 2012; Zhu, Ye, Verrier, & Singh, 2013). For instance, the reversed-phase high-performance liquid chromatography (HPLC) was used to analyse the released fatty acids from lipolysis of triglycerides with the advantage of quick sample preparation. However, due to limitation of the detectors, this method is more suitable for a pure substrate—such as defined triolein, rather than a mixture of substrates (Ergan & André, 1989; Thomson, Delaquis, & Mazza, 1999; Veeraragavan, 1990). Compared to HPLC, GC with a flame ionisation detector (FID) have shown to perform better in determining the released fatty acids by analysing the fatty acid methyl esters (FAMEs) (Gallier et al., 2012; Helbig et al., 2012; Zhu et al., 2013). However, it is difficult to differentiate other lipolytic products such as diglycerides and monoglycerides. Some researchers have combined TLC with flame ionisation detector that could be able to analyse and quantify all of the lipolytic products (free fatty acids, mono- and diglycerides and residual triglycerides) more precisely (Bourlieu et al., 2015; Bourlieu, Rousseau, Briard-Bion, Madec, & Bouhallab, 2012).

2.6.2 Determination of the changes in the structural properties of emulsions during gastric digestion

Changes in structural and interfacial properties of emulsions can be quantified by measuring the emulsion droplet size (particle size, via light scattering or microscopy) and the droplet surface charge (ς-potential analysis). Microscopy techniques such as confocal microscopy, cryo-transmission electron microscopy, scanning electron microscopy and transmission electron microscopy have been widely used to observe the morphological changes of the oil droplets during digestion (Gallier, 2010; Gallier et al., 2013; Singh & Gallier, 2017). These tools can not only observe the transitions during the digestion process but also provide insights into the mechanism of lipid digestion and absorption. In some of the most recent studies, scattering techniques such as small-angle X-ray scattering (SAXS) have been introduced in the research of lipid digestion to observe lipid assemblies during emulsion digestion (Marze, Gaillard, & Roblin, 2015; Salentinig, Phan, Hawley, & Boyd, 2015).

2.7 Summary

In conclusion, lipid digestion is vital for infants, and the structural properties of infant milk can play an essential role in the digestive process. Understanding of the GI tract environment in infants can help us building physiologically valid *in vitro* models to study lipid digestion in the neonates. Nowadays, research is increasingly focusing on structural properties and their relationship to lipid digestion in adult human subjects from both *in vitro* and *in vivo* perspectives. However, limited attention has been placed on understanding the relationship between the structural changes and the degree of lipid digestion in infants, and how digestive variables in the GI tract could influence the process. Many findings are built on the experimental data using different *in vitro* systems to simulate neonatal GI environment. However, it is still difficult to compare those studies with each other. Therefore, this project aimed to focus on understanding the relationship between structural changes and the extent of lipolysis in a neonatal gastric model, and the understanding the influence of gastric conditions on the lipolysis rate.

Chapter 3 Materials and methods

3.1 Materials

Bovine blood haemoglobin H2500 was purchased from Sigma-Aldrich Pty Ltd., St. Louis, MO, USA.

Bovine lactoferrin powder was provided by the Fonterra Research and Development Centre, Palmerston North, New Zealand (made in December 2015). This powder contained 93.98% of lactoferrin, with 0.65% of ash, 1.09% of moisture and 0.014 % of iron content (14 mg Fe/100g powder, 10.9% of iron saturation).

Fungal lipase derived from a selected strain of *Rhizopus oryzae* was chosen as an alternative for human gastric lipase. Fungal lipase 8000 is purchased from Connell Brothers Company Australia Pty Ltd., Australia. This fungal lipase was derived from a selected strain of *Rhizopus oryzae* (ATCC 1996) with an activity of 80 LU/mg. It was a food-grade lipase and was classified as triacylglycerol acylhydrolase (EC 3.1.1.3). Fungal lipase 8000 primarily hydrolysed triglycerides at sn-1 and sn-3 positions, producing short, medium and long-chain fatty acids, di- and mono-glycerides. It was active and stable over a wide *p*H range from 4.5 to 8.5 and with optimal performance at *p*H 7. This fungal lipase was active at a temperature up to 45 ºC with an optimal temperature at 35 to 40 ºC.

Intralipid® was a commercial soya bean oil in water emulsion stabilised by phospholipids from egg lecithin intended for intravenous use (purchased from Fresenius Kabi Australia Pty Limited, NSW Australia). This product contained 20% of soybean oil, 0.12% egg lecithin and 0.22% glycerol. The main phospholipid components in egg lecithin are phosphatidylcholine (PC, 80.5%) and phosphatidylethanolamine (PE, 11.7%). Minor components also include lysophosphatidylcholine (LPC), sphingomyelin (SM), and neutral lipids (Palacios & Wang, 2005). The *p*H has been adjusted to 6.0-9.0 by hydrochloric acid (HCl). The osmolality was 350 mosmol/L. Oil in the diluted Intralipid emulsion was stabilised by egg lecithin containing phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM). The head groups of these phospholipids show no charge at *p*H 3.5. Thus, the negative surface charge is reflected in the phosphate group.

Myristic acid was purchased from BDH Ltd, Poole, England.

S-26® Gold Newborn [Wyeth Nutrition (Singapore) Pte. Limited, Singapore] was purchased from a local supermarket in August 2018.

Palmitic acid and **Pentadecanoic acid** (~ 99% capillary GC) were purchased from Sigma-Aldrich Pty Ltd., St. Louis, MO, USA.

Porcine gastric pepsin P7000 (EC 3.4.23.1) powder with an activity ≥ 250 units/mg solid was purchased from Sigma-Aldrich Pty Ltd., St. Louis, MO, USA.

Deionised water (Milli-Q water) was purchased from Millipore, Bedford, MA, USA.

All other chemicals were purchased from Sigma-Aldrich Pty Ltd., St. Louis, MO, USA.

3.2 Methods

3.2.1 *In vitro* model for neonatal gastric digestion

The set-up for the *in vitro* neonatal gastric digestion based on the work by Lueamsaisuk *et al*. (2014). In this *in vitro* set-up, a 400 mL glass beaker with a 7.5 cm diameter was placed in a water bath at 37 °C. Mixing within the stomach was simulated by a magnetic stirring bar with a diameter of 7.0 cm rotating at 10 rpm using a submersible device. The shear rate at this speed was calculated to be 16.25 s^{-1}. It should be noted that the value is still higher than 0.7 s^{-1} which has been calculated as the maximum shear rate occurring in the human stomach (Lentle & Janssen, 2011; Lentle et al., 2010). In fact, due to the immature development of stomach for neonates, the actual shear rate in the neonatal stomach should be even lower than that of the adult. However, most of the stirring devices used in *in vitro* studies cannot achieve such a low shear rate. Therefore, the lowest rotating speed in the current device was chosen, as the stirring rate throughout this entire study (10 rpm).

Simulated gastric fluid (SGF) was prepared by dissolving 200 mg of sodium chloride (NaCl) in Milli-Q water. The *p*H of the solution was adjusted to 2, 3.5, 4.5 or 5.5 with 0.1 M HCl solution, and the total volume made up to 100 mL with Milli-Q water. This *p*H range was aiming at simulating the postprandial variation of *p*H in infants' stomach, as reported in Lueamsaisuk *et al*. (Lueamsaisuk, 2015; Lueamsaisuk et al., 2014). An amount of 450 mg of dry powdered porcine pepsin (800 - 2500 units/mg protein) and 20 mg of dry powdered *Rhizopus oryzea* lipase (80 U/mg) was first hydrated with a small amount of Milli-Q water (usually 5 to 10 times of the enzymes own weight) and added to the SGF (details in the following section).

A schematic of this process is shown in Figure 3.1.

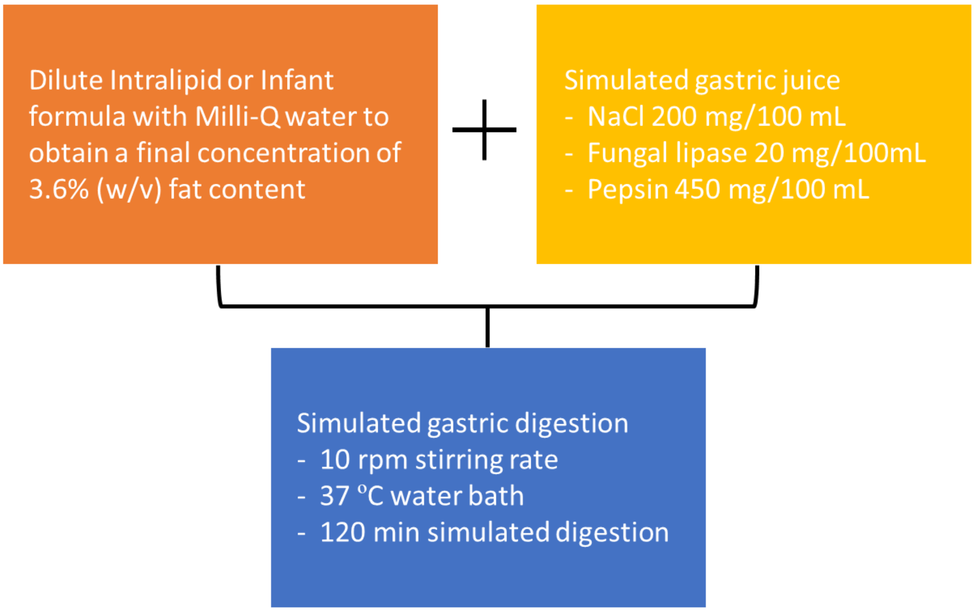


Figure 3.1 A scheme of *in vitro* digestion set-up.

3.2.2 Pepsin assay

The pepsin assay method was modified from Minekus *et al*. (2014). Pepsin powder was brought to room temperature and 100 mg of pepsin were weighed accurately into a tube. A pepsin stock solution 10 mg/mL was made by adding a 0.15 M NaCl solution (prepared with Milli-Q water), and the *p*H was adjusted to 6.5 with 0.1 M NaOH. An aliquot of 50 μL pepsin stock solution was diluted (1:20) by adding 950 μL of Milli-Q water and stored on ice. A haemoglobin stock solution was made by adding 0.5 g bovine blood haemoglobin to 20 mL Milli-Q water followed by 5 mL 0.3 M HCl in order to achieve *p*H 2.

In order to generate the reaction curve, an amount of 12×2 mL Eppendorf tubes with 500 μL haemoglobin stock solution in each tube were prepared. A 100 μL of diluted pepsin was added to each six of them and were incubated in a shaking water bath set at 37 °C for 0, 2, 4, 6, 8, 10 min. An amount of 1 mL of 5% w/v TCA (tricyclic antidepressant) was added in each tube to stop the reaction. The other six tubes were marked as blank tubes where 1 mL of 5% w/v TCA was added in each tube in advanced before incubation. The same amount of diluted pepsin (100 μL) was added to each of the six blank tubes and was also incubated in the shaking water bath for 0, 2, 4, 6, 8, 10 min. After incubation, the 12 tubes were centrifuged at 12,000 × g for 6 min and warmed up to room temperature. The supernatant was diluted (1:4), by adding 250 μL supernatant to 750 μL Milli-Q water.

As a result of this reaction, haemoglobin was hydrolysed by pepsin into TCA-soluble peptides with absorbance at A_{280}. The absorbance of water at 280 nm was used as background reading, and the absorbance of each sample was read at this wavelength in quartz cuvettes. Results were reported in units defined as below (one unit of pepsin will produce ΔA_{280} of 0.001 per minute at *p*H 2.0 at 37 ˚C measured as TCA-soluble products using haemoglobin as the substrate). The unit was calculated as:

$$\text{Units/mg enzyme} = \frac{\frac{\left(\frac{\Delta A_{280}\,test}{\Delta t} - \frac{\Delta A_{280} blank}{\Delta t}\right)*1000}{X_{pepsin}}}{10\,mg/mL} = \frac{\frac{(k_{test} - k_{blank})*1000}{X_{pepsin}}}{10\,mg/mL}$$

Where,

$\Delta A_{280}\ test/\Delta t = k_{test}$ is the slope of the test curve (linear line)

$\Delta A_{280}\ blank/\Delta t = k_{blank}$ is the slope of the blank curve (linear line)

X_{pepsin} is the concentration of pepsin powder in the final reaction mixture (quartz cuvette) [mg/mL]

3.2.3 Lipase assay

The assay method carried out on the fungal lipase used here was modified from Minekus *et al*. (2014) and was conducted by *p*H-stat titration using Intralipid (phospholipid-emulsified soybean oil) as the substrate. The free fatty acids released from lipolysis were titrated at a constant *p*H of 7.0 with 0.05 M NaOH for 20 min. The rate of lipolysis was calculated based on the first 5 min of titration. Results were reported in units defined as 1 U = 1 μmol carboxyl group (i.e.,-COOH) released per minute at 37 ºC and *p*H 7.0.

The Intralipid substrate solution was prepared as described above in the simulated digestion section with the only difference being that a higher enzyme concentration was used (16 times greater). The *p*H of the digestate was adjusted to 7.0 at the starting point of lipolysis, before titration. The units were calculated as:

$$U_{fungal\ lipase} = \frac{k_{FFA} * 10^6}{1\ \mu mol/min}$$

Where,

$$k_{FFA} = \frac{\Delta V_{NaOH}}{\Delta t} * \frac{1}{1000} * C_{NaOH}$$

k_{FFA} is the amount of fatty acids released per minute (in moles),

V_{NaOH}/t is the initial rate of lipolysis (mL/min)

1 mole/min are the units of $U_{fungal\ lipase}$.

3.2.4 Analysis of lipolysis products by gas chromatography (GC)

3.2.4.1 Validation of the GC instrument

Validation of the GC instrument was done using six equivalent GC vials with 1 mL mixture of solvents and 30 μL internal standard (IS) Pentadecanoic acid (C15:0) stock solution (20 g/L) with or without the initial digestate extractions in the absence of enzymes. The coefficient of variation of this validation test is shown in Table 3.1 and indicating the reproducibility of the GC instrument.

Table 3.1 The standard deviation and coefficient of variation of the area under the curve (mV*min) using an internal standard.

IS C15:0	**Multiple injections**	**Multiple vials**
Standard deviation	2.71	5.17
Coefficient of variation (%)	4.86	10.35
IS C15:0 with digestate	**Multiple injections**	**Multiple vials**
Standard deviation	6.50	5.24
Coefficient of variation (%)	9.47	6.98

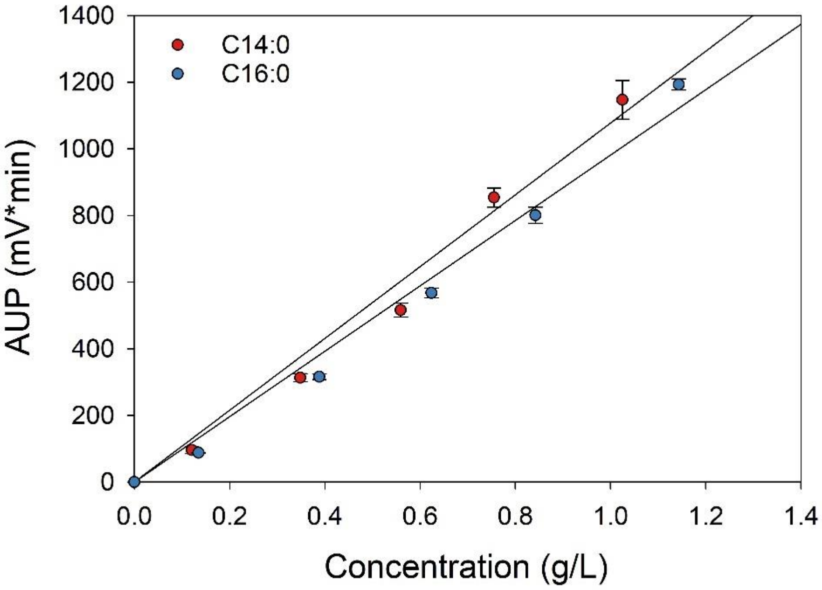


Figure 3.2 Calibration curve using myristic acid (C14:0) and palmitic acid (C16:0).

Table 3.2 Parameters of the linear regression of the calibration curve.

	C14:0	**C16:0**
b	1077.550	981.696
SEM (b)	39.286	32.918
df	1;5	1;5
F	752.321	889.368
Sig.	<0.001	<0.001
R^2	0.993	0.994

b, slope value.

SEM (b), standard error of the mean of *b*.

df, degrees of freedom and residual degrees of freedom.

F, F value.

Sig., *p*-value, α=0.05.

R^2, coefficient of determination.

3.2.4.2 GC analysis

The samples were analysed in a ThermoQuest Trace GC 2000 (Thermo Fisher Scientific, Milan, Italy). An amount of 0.6 μL of the sample was injected into a guard column (Fused silica capillary tubing, deactivated, 0.50 m × 0.53 mm ID, Alltech Associates Inc., Deerfield, IL) by an autosampler Thermo Scientific AS 2000 (Thermo Fisher Scientific, Milan, Italy). The sample passed through a guard column and then into an Agilent capillary non-polar column [DB-5ht, 8 m (length) × 0.32 mm (ID), 0.10 μm (film), -60 - 400 °C (the temperature limit), Santa Clara, California, United States]. The carrier gas was helium, and it was running at a constant flow rate of 2 mL/min. Temperature ramped from 60 °C (for 1 min) to 380 °C (for 8 min) at a heating rate of 10 °C/min. The total run time for one sample was 41 min. Signals were detected by an FID detector. Calibration curves were produced using a series of known concentrations of mixed fatty acids solutions (similar amount of myristic acid and palmitic acid in hexane) as in Figure 3.2. The parameters of the calibration curves showed that the area under the peak (AUP) was proportional to the sample concentration.

The identification of peaks was performed by comparing the retention time of myristic and palmitic acids used to produce the calibration curve, to those from the analysed samples. Their retention time and area under the peak were recorded and integrated by using Chromeleon™ 7.2 Chromatography Data System Software (Thermo Fisher Scientific, Milan, Italy).

3.2.5 Scanning electron microscopy (SEM)

The digesta samples (1 mL) were first mixed with 3% agarose at a ratio of 1:1 (v/v) and then cut into cubes (approximately 10×5×5 mm). The cubes were placed in a primary fixative, which consisted of 3% glutaraldehyde and 0.1M sodium cacodylate buffer at *p*H 7.2 for at least 8 hours at room temperature. Then the cubes were washed three times, 10-15 min each with 0.1 M *p*H 7.2 sodium cacodylate buffer. Then the cubes were dipped into 1% osmium tetroxide sodium cacodylate buffer for an hour at room temperature and washed again with 0.1 M *p*H 7.2 sodium cacodylate buffer three times. Each sample cubes were dehydrated in graded ethanol series with a volume percentage of 25%, 50%, 75%, 95% and 100% for 10-15 min and washed in 100% ethanol for an hour.

The sample cubes were critical point dried using liquid CO_2 and 100% ethanol was used as the intermediary by Polaron E3000 series II critical point drying apparatus (Quorum Technologies Ltd, Laughton, United Kingdom). The sample cubes were then torn to expose the oil droplets needed to be observed. They were mounted on to aluminium stubs, sputter-coated with approximately 100nm of gold (Bal-Tec SCD 050 sputter coater) and viewed in the FEI Quanta 200 Environmental Scanning Electron Microscope at an accelerating voltage of 25 kV. This method was a standardised method for analysing milk-like samples for SEM analysis in Manawatu Microscopy & Imaging Centre, Palmerston North, New Zealand.

3.2.6 Transmission electron microscopy (TEM)

A 1.5 mL of digestate was sampled at time 0 min and after 120 min. The liquid sample was injected into 3% agarose tubes and sealed with agarose to form an enclosed capsule. The tubes were placed into 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (*p*H 7.2) for at least 24 hours. The buffer was then washed in 0.1 M sodium cacodylate (*p*H 7.2) three times for 45 min per wash. The samples were post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for an hour at room temperature and overnight at 4 °C and finally brought up to room temperature for an hour. The buffer was rewashed three times in 0.1 M sodium cacodylate as described above. The samples were then dehydrated through a graded acetone series (25%, 50%, 75%, 95%, 100%, 100%, and 100%) for 45 min for each grade. The samples were then put into resin: acetone (50:50) and placed on a stirrer overnight then replaced by fresh 100% resin and placed on the stirrer for another 8 hours. This step was repeated four more times. Then the samples were embedded in moulds with fresh resin and cured at 60 °C oven for 48 hours. Light microscope sections were cut at 1 μm using a glass knife on the ultramicrotome (Leica EM UC7, Germany) and heat-fixed onto glass slides. These sections were stained with 0.05% toluidine blue for approximately 12 seconds and viewed under the light microscope. Those blocks were then trimmed down to the selected areas and cut using a diamond knife (Diatome, Switzerland) at 100 nm and then stretched with chloroform and mounted on a grid using a Quick Coat G pen (Daido Sangyo, Japan). The grids were stained in saturated uranyl acetate in 50% ethanol for 6.5 min and washed with 50% ethanol, and Milli-Q water then stained in lead citrate (Venable & Coggeshall, 1965) for another 6.5 min and followed by a wash in Milli-Q water. Then the samples were viewed and imaged with a transmitted electron microscope FEI Tecnai G^2 Spirit BioTWIN (FEI Company, Czech Republic) with a camera Veleta (Olympus SIS, Germany). Samples were analysed in duplicate. This method was a standardised method for analysing milk-like samples for TEM analysis in Manawatu Microscopy & Imaging Centre, Palmerston North, New Zealand.

3.2.7 Statistical analysis

Every experiment was triplicated. The results from GC were analysed using IBM SPSS Statistics 25.0 (IBM, Armonk, New York, United States) and SigmaPlot 14.0 (Systat Software Inc, San Jose, CA, United States).

Chapter 4 Using ^{1}H NMR for lipolysis analysis

4.1 Introduction

Monitoring the process of lipolysis is of great importance in studying the dynamics of lipid digestion. Studies in the past few decades have used a variety of different methods to quantify lipolytic products from lipid digestion. As discussed in the literature review, chromatographic methods, such as HPLC and GC, and titration methods such as the *p*H-stat method are widely used for direct or indirect quantification of free fatty acids generated from lipolysis. However, the disadvantages of those methods are also evident. For GC method, the standard FAME analysis requires chemical modification of lipolytic products and is always time-consuming (Brenna, 2013; Helbig et al., 2012; Rueda et al., 2014; Zhu et al., 2013). For the *p*H-stat method, this tends to be more suitable for digestion under neutral *p*H, and is, therefore, more widely used in studying lipid digestion in the small intestine rather than in the gastric phase (Helbig et al., 2012). Additionally, the titration method may not give reliable results when conducting digestion in a complex simulated digestive system rather than simple ones (Hur et al., 2009), particularly due to buffering effects from compositions containing appreciable amounts of protein. Some more recent studies have shown that ^{1}H NMR (Proton Nuclear Magnetic Resonance) spectroscopy may be a promising new method for analysing lipolysis in the complex lipid matrix or the lipid digestate (Nieva-Echevarría, Goicoechea, Manzanos, & Guillén, 2014; Nieva-Echevarría, Goicoechea, Manzanos, & Guillén, 2015). The ^{1}H NMR method was reported to be less time consuming and did not require chemical alteration of the lipolytic products generated from lipid digestion. However, it still required sampling at specific time points and lipid extraction before conducting ^{1}H NMR analysis.

In order to find a methodology to quantify the generation of free fatty acids during gastric lipolysis either *in-situ* or dynamically, and without terminating the reaction, we explored the possibility of adapting ^{1}H NMR spectroscopy to monitor the dynamics of lipid digestion in a simulated gastric environment. This chapter provides an overview of methodology development using this approach and some initial findings, along with a discussion of the challenges of applying this approach and some recommendations for future development.

4.2 Materials and Methods

4.2.1 ^{1}H NMR spectroscopy

^{1}H NMR (Nuclear magnetic resonance) is an analytical chemistry technique used to determine the content and molecular structure of a sample. ^{1}H NMR spectroscopy operates through the application of a magnetic field to nuclei and then measuring the amount of energy required to put various nuclei in resonance. Nuclei in different electronic environments require different amounts of energy to bring them into resonance. A typical ^{1}H NMR spectrum provides a peak representing the energy required to bring each nucleus into resonance. The technique can also provide information for the dynamics, reaction state, and chemical environment of the molecules.

In the ^{1}H NMR spectra, molecules usually present themselves in a well-resolved and often predictable way. In the simulated gastric lipolysis process of our study, one molecule of triglycerides is hydrolysed by fungal lipase, liberating two free fatty acids and one monoglyceride. The release of fatty acid creates a unique proton environment around its carboxyl group, which produce a unique magnetic field. This magnetic field makes the proton generated from the reaction resonance in a unique frequency. Since the resonance frequency is depended on the applied magnetic field in the ^{1}H NMR, this will generate a unique peak on the spectra, which represents the released free fatty acids from the reaction. The peak will show at a specific position on the spectrum as $\delta -$ ppm (parts per million).

The δ unit is defined as $\delta = \frac{Distance\ downfield\ from\ the\ reference\ signal\ (Hz)}{Operating\ requency\ of\ NMR\ (MHz)}$. Since δ is a ratio not unit, it is independent of the applied magnetic field of NMR.

A 500 MHz ^{1}H NMR (Bruker BioSpin GmbH, Karlsruhe, Germany) was used for all experiments. A 1-dimensional proton spectrum was acquired. The water signal was suppressed using a standard pulse sequence from pulsed-field gradient (Zgesgp, Bruker, Germany). The free induction decay was acquired with 16 k data points for 1.5 seconds with a recycle delay of 2 seconds. Spectra were processed using Mnova MestreNova NMR processing software (MestreNova, Spain).

A D_2O matched symmetrical microtube (Shigemi 5 mm Symmetrical NMR microtube assembly matched with D_2O, bottom length 8 mm, Merck KGaA, Darmstadt, Germany) was used for simulated gastric digestion (Figure 4.1).

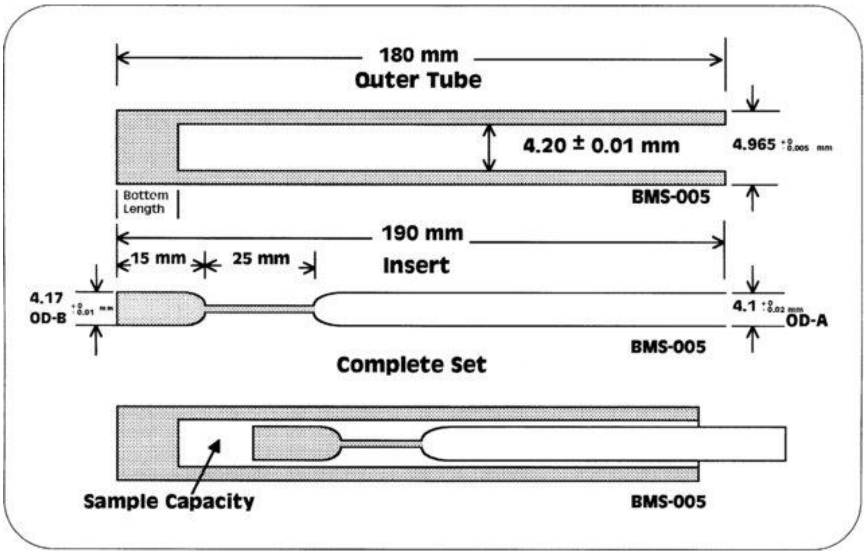


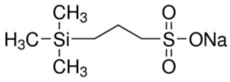
Figure 4.1 Shigemi 5 mm Symmetrical NMR microtube assembly matched with D_2O, bottom length 8 mm (Merck KGaA, 2020).

A sample of initial digestate was prepared by mixing a diluted Intralipid emulsion containing 3.6% w/v fat, and SGF at a ratio of 20:50 v/v. In this experiment, the volumes of the diluted Intralipid and SGF were 40 ml and 100 ml, respectively. An amount of 20 mg of dry powdered *Rhizopus oryzea* lipase (80 U/mg) was first hydrated with a small amount of Milli-Q water (usually 5 to 10 times of the enzymes own weight) and then added to the initial digestate. The ^1H NMR analysis of each sample was done by filling the sample up to 17 mm in the D_2O matched symmetrical microtube from the bottom. The microtube was rotated at 10 rpm during lipolysis in ^1H NMR. A spectrum was acquired every 5- minute intervals during the length of reaction. Two lipolysis tests of the digestate were conducted at *p*H 3.5, 37 °C for 85 min.

4.2.1 Tests of adding fatty acid as an internal standard

Oleic acid was added as an internal standard in the initial digestate without any addition of digestive enzymes to test the feasibility of adding fatty acid as an internal standard. Addition of oleic acid in the Intralipid emulsion (3.6% w/v) digestate and an artificial emulsion made by 3.6% w/v soybean oil and 1% Tween 20 w/v was tested respectively at neutral pH, 37 ºC. The final concentration of oleic acid in the digestate was 10 mM. The artificial emulsion was blended by an ULTRA-TURRAX T 25 digital dispenser (IKA, Germany) at 6000 rpm for 3 min.

4.2.2 Calculation of FFA concentration

In order to calculate the concentration of liberated fatty acids from lipolysis, 3-(trimethylsilyl) Propane Sulfonic Acid Sodium Salt (DSS, 

) was used as an external reference.

A known amount of DSS was added into the initial digestate without lipase existing as a reference digestate, concentration of the DSS in the reference digestate was calculated accordingly. The reference digestate was filled in the microtube and analysed by ^{1}H NMR. Since this DSS was referred to as external reference without adding lipase, this reference digestate was different from test digestate for lipolysis.

Since in one molecule of DSS, there are three methyl groups around the silicon atom where the 9 protons (hydrion) in the methyl groups are in the same electronic environment (chemical environment), whereas in one molecule of fatty acid there is only one proton in the carboxyl group which presents a unique chemical environment. Therefore the two different proton environments show two separate peaks on the spectrum. Since the ratio of area under the two peaks and the ratio of the proton number of the two peaks for which responsible are the same, to get the same area under the peak, the number of fatty acid molecules needs to be nine times of the number of DSS molecules. It means in the same digestate, the concentration of free fatty acids should be nine times the concentration of DSS to achieve an equal area under the peak. Analysis of the signal intensity is done by calculating the area under the curve by integration. Then the initial concentration of free fatty acids in the digestate can be estimated as shown in the equation below,

$$\frac{9C_{t0(FFA)}}{C_{r(DSS)}} = \frac{I_{t0(FFA)}}{I_{r(DSS)}}$$

$$C_{t0(FFA)} = \frac{1}{9} \cdot C_{r(DSS)} \cdot \frac{I_{t0(FFA)}}{I_{r(DSS)}}$$

where $C_{t0\ (\mathrm{FFA})}$ is the initial concentration of FFA in the test sample at the starting time when the sample was placed into the ^{1}H NMR, $C_{r\ (\mathrm{DSS})}$ is the concentration of DSS in the reference digestate, $I_{t0\ (\mathrm{FFA})}$ is the integral (area under the peak) of free fatty acids at starting time when the sample was placed into the ^{1}H NMR, and $I_{r\ (\mathrm{DSS})}$ is the integral (area under the peak) of DSS in the reference solution.

Because DSS is used as an external reference which was different from the digestate samples during tests, the signal from DSS was integrated and cross-referenced to the test ones. When the concentration of free fatty acids is estimated from the reference, the changing of the concentration is proportional to that of the peak area. As lipolysis progresses, the concentration of the generated free fatty acids at different time points could be estimated accordingly.

Data from ^{1}H NMR was processed by MestReNova Version 9.1 (Mestrelab Research S.L., Spain).

4.3 Results

Before using the modified microtube with a flat bottom, as described in the methods, a conventional ^{1}H NMR tube with a round bottom was initially tested. Since during digestion, the destabilised emulsion droplets creamed to the top of the digestate in the tube, the liquid/air interface, in this case, stayed out of the detection coil. This phenomenon can affect the quality of the spectra, leading to a signal loss of the free fatty acid, for that the density difference between liquid and air can influence the magnetic field. Then the D_2O matched symmetrical microtube with flat bottom was used then to keep the creaming-up oil layer within the NMR coil to prevent the signal loss.

In order to involve an internal standard fatty acid in the simulated dynamic digestion, oleic acid (10 mM) was used in the digestate at neutral pH, 37 ºC for testing. The addition in neither Intralipid nor artificial emulsion digestate showed a significant difference in the fatty acid peak on the spectrum at 3.18 ppm. Phase separation could be observed in the digestate before and after the tests.

The ^{1}H NMR analysis of samples was repeated twice and showed good repeatability. A unique proton peak of fatty acids appeared on the spectrum at 3.18 ppm when the initial *p*H of the digestate was 3.5 with the water signal being suppressed (Figure 4.2). A magnified view of the proton peaks of the liberated fatty acid during 85 min of digestion showed that the signal intensity (area under the peak) of free fatty acids increased along with time, indicating that the concentration of FFA liberated during lipolysis was progressively increasing (Figure 4.2).

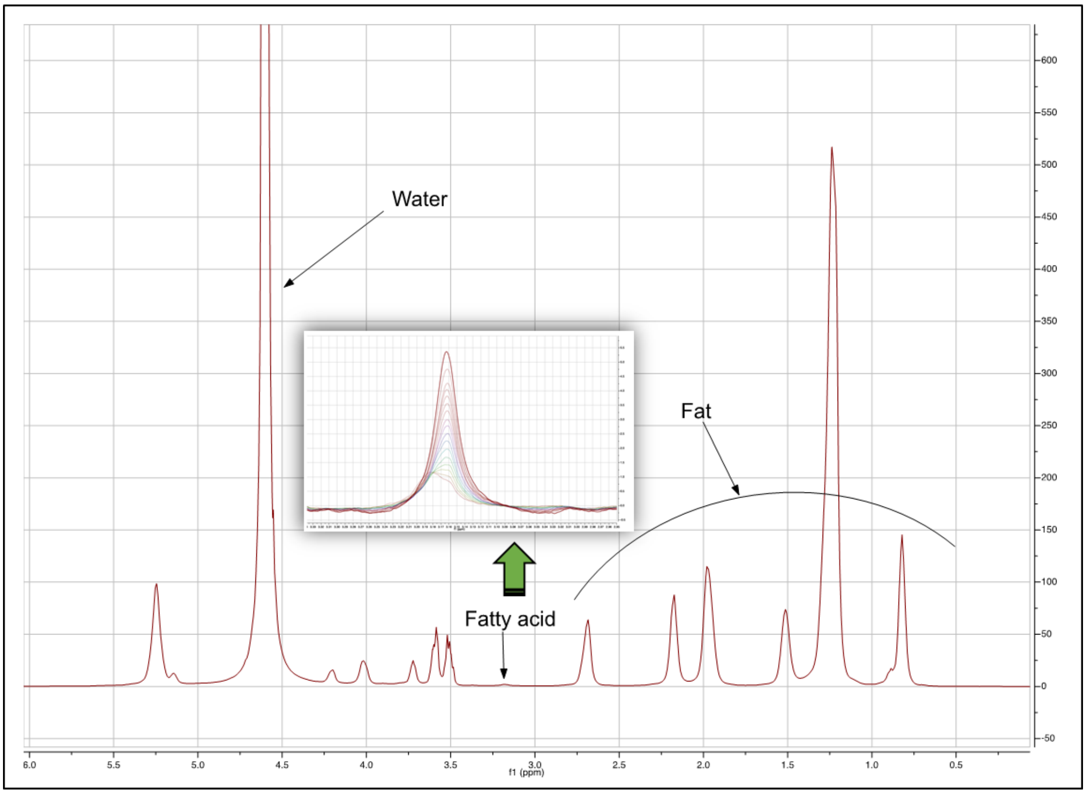


Figure 4.2 A ^{1}H NMR spectrum for Intralipid digested with lipase alone at *p*H 3.5 with a magnified view of free fatty acids liberated in 85 min.

In both repeated ^{1}H NMR tests, there was found to be a linear relationship between the concentration of total fatty acids in the digestate and reaction time (Figure 4.3). Linear regression applied to the data showed that the values of the slope were 1.337×10^{-5} and 1.456×10^{-5} respectively, reflecting that the reaction rates of the two tests were close. The values of the coefficient of determination (R^2) of the two regression lines were both close to 1, which showed excellent linearity of time to FFA concentration (Table 4.1). Tailing in the first 10 min was due to sample preparation in the flat tube. However, the intercept values of the two regression lines were different, although both tests were repeated at the same digestion conditions. This may due to two reasons, 1) there were already an amount of free fatty acids in the digestate originated from the diluted Intralipid before the initiation of digestion; 2) due to experimental operation, those two tests were not able to start simultaneously. Thus, the lipolytic reaction already commenced before the recording of the analysis.

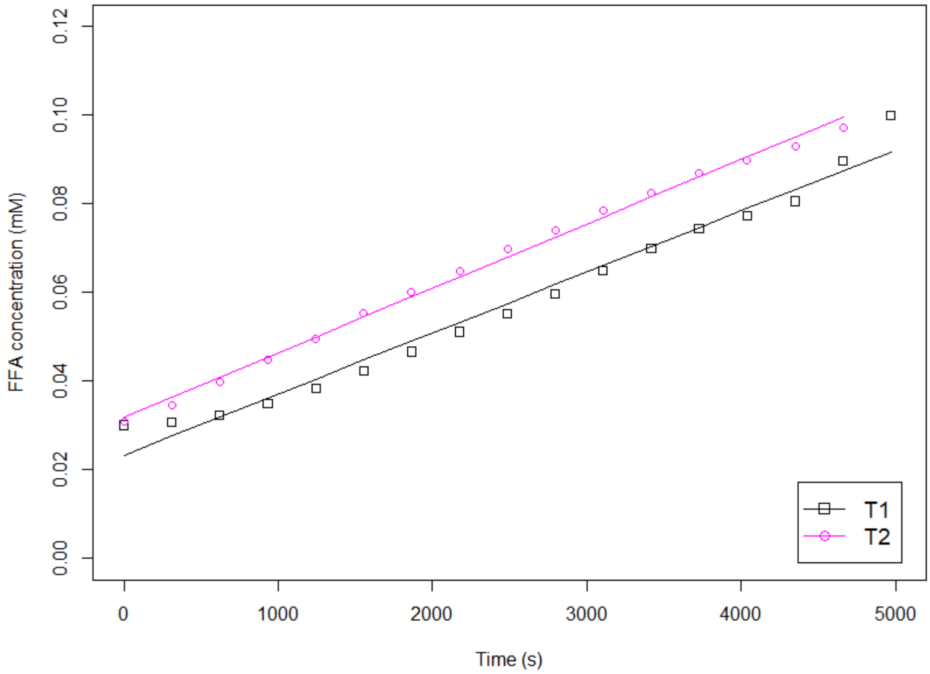


Figure 4.3 Two repeated lipolysis tests in ^{1}H NMR using a D_2O matched symmetrical microtube.

The total fatty acid esters in triglycerides in the initial digestate (not including non-hydrolysed fatty acids presented in the initial digestate) were estimated to be 33.6 to 34.2 mM. After 85 min of digestion, the concentration of the total free fatty acids released from lipolysis in both tests was close to 0.1 mM. Thus, the extent of lipolysis was approximate to 0.3% for lipid digestion at *p*H 3.5 after 85 min. Clearly, the extent of lipolysis was quite low, which may suggest that the amount of triglyceride substrate may become far excess to that of fungal lipase. Therefore, repeated analysis is still needed in future statistical analysis.

Table 4.1 Coefficients of the linear regression of two ^{1}H NMR tests using a D2O matched symmetrical microtube.

Test No.	***b***	***SEM (b)***	***a***	***SEM (a)***	***R^2***
T1	1.377×10^{-5}	5.343×10^{-7}	2.338×10^{-2}	1.557×10^{-3}	0.978
T2	1.456×10^{-5}	2.603×10^{-7}	30187×10^{-2}	7.117×10^{-4}	0.996

a, intercept of the regression lines.

SEM (a), standard error of the mean of *a*.

b, the slope of regression lines, unit mM/s.

SEM (b), standard error of the mean of *b*.

R^2, coefficient of determination.

The dynamic measurement of FFA yield by ^{1}H NMR is a promising method in determining the rate of gastric lipolysis in an *in vitro* system, as well as enabling analysis in small volumes where availability of enzymes may be limited. However, the volume limitation of the NMR analysis may itself skew digestion behaviour. Digestion tests for the determination of lipolytic rate conducted in NMR were different from the simulated gastric system in the previous studies. Due to the difference in the volume range of the two *in vitro* systems, the rate of the reaction determined by ^{1}H NMR analysis may not represent the rate of lipolysis in the simulated gastric system. Therefore, future work will need to combine GC method with ^{1}H NMR to find the correlation of the two *in vitro* systems.

4.4 Discussion

^{1}H NMR has previously been reported to be an appropriate analytical method for measuring the rate of lipolysis. Madden *et al*. (1993) used ^{1}H NMR to relate methylene peak area to the triglycerides combined with a biochemical assay to quantify triglyceride lipolysis in triacylglycerol metabolism in the rat heart. Nieva-Echevarría *et al*. (2014)proposed using ^{1}H-NMR spectra to quantify triglycerides, partially glycerol and free fatty acids from the matrix of the complex lipid system. More recently, Lolli *et al*. (2018) used similar ^{1}H NMR spectra method as in the study from Nieva-Echevarria *et al*. (2014) and combined with gas chromatography-mass spectrometry (GC-MS) to quantify cyclopropane fatty acids in Grana Padano cheese. ^{13}C and ^{31}P NMR have also been used to study mono-, di-glycerides and fatty acids in some studies (Gusntone, 1991; Ng, 2000; Spyros & Dais, 2000; Vlahov, 1996). Those studies provide valuable information for quantification of lipolysis in the gastric digestion. However, these tests were all conducted in the isolated oil phase or using extractions in the organic solvents. None of the studies reviewed has determined emulsion lipolysis dynamically as part of an *in vitro* system, nor has research been undertaken to analyse digesta from human subjects.

In this study, we wanted to obtain the signal change of the -COOH group from when free fatty acids were generated from the hydrolysis of triglycerides under dynamic conditions. The proton changes of the -OH group of glycerol in the aqueous phase was considered to be able to be minimised at the beginning of the study. However, the results later proved that this proton exchange was significant and could not be neglected.

The use of oleic acid as an internal standard for the design of dynamic gastric digestion tests was proven to be inappropriate as the addition of oleic acid could not blend well with the initial digestate. The phase separation made it difficult for the detective coil in 1H NMR to capture the correct signal and it was assumed the major cause of this issue. In addition, the stickiness of oleic acid has made the installation of the plunger impossible to avoid involving air bubbles in the sample. This as well deteriorated the accuracy of data gathering.

As suggested by some of the studies (Nieva-Echevarría et al., 2014; Nieva-Echevarría et al., 2015), monitoring the signal changes of the -CH_2 group next to the glycerol backbone of the triglycerides might give better quantification of the lipolytic products as well as the extent of lipolysis. However, the use of appropriate standard compounds and the development of a calibration curve would still need to investigate in the future.

One of the major hurdles in our study was the accurate quantification of the lipolytic products while enzymes were still reacting in the system. The objective of the study was to measure the dynamics of simulated gastric digestion in the ^{1}H NMR unit in real-time. This required the lipolytic products to be analysed in the mixture of digestate rather than in single phase (pure oil, aqueous solutions, or extractions in proper solvents). As samples in single phase would get the best of the signals than in multiple phases for ^{1}H NMR unit, the accuracy of the signal would be compromised under conditions in this simulated digestion. Additionally, as discussed earlier, as lipolysis proceeded, the mixture of mono-, di-, and triglycerides and fatty acids showed a tendency to cream to the top of the sample and exceed the detectable range of the coil in the ^{1}H NMR unit. Signals from the mixed compounds might partially be lost due to this reason. Considering the inherent challenges in using ^{1}H NMR as a tool for dynamic analysis of lipolysis under *in vitro* gastric conditions, coupled with the time taken to develop the approach, and the acknowledgement that without appropriate validation, any results generated may be an inaccurate reflection of the actual lipolysis. It was decided to halt the development of this methodology and focus on more widely established techniques, namely the GC method.

4.5 Conclusions and recommendations

In conclusion, the ^{1}H NMR spectroscopy that has been used in this chapter may be a promising innovative method to dynamically analysis the changes of lipid digestion in the simulated gastric environment. However, still, several issues need to be addressed before further application.

To recommend possible solutions, as mentioned earlier, the use of soluble short-chain fatty acids in order to generate a suitable calibration curve may be applied. Additionally, more work needs to be done to calculate the correspondent shear rate within the reaction tube with a spinning stirrer. Another issue that needs notice is that the tube used for 1H NMR spectroscopy has a plunger on the top space of the sample to expel air. It was very challenging to appropriately insert the plunge due to the adhesive properties of the digestive mixture. Moreover, in order to minimise the influence of phase separation occurred in the process of lipolysis, a selection of specific 1H NMR tubes needs to be considered in the future as well.

Chapter 5 Digestion of protein emulsions using a simulated neonatal gastric system

5.1 Introduction

The immaturity of the GI tract environment in newborns can be consequential in compromising the effectiveness of lipid digestion in the small intestine, leading to a greater reliance on gastric digestion relative to adults. Arguably, infant digestion is biologically predisposed towards digestion of a singular food, i.e. milk. However, the availability of infant formula as an alternative to maternal milk may lead to differences in the manner and effectiveness by which these are digested, noting that infant formulae have distinct differences in structure and composition to breast milk.

Certainly, in the case of lipid digestion, human trials have shown that maternal milk appears more efficiently digested than infant formulae. Compositional differences in lipid and protein types and pronounced variations in droplet interfacial layer composition and structure are considered to have a significant impact on the dynamics of gastric lipid digestion of maternal milk and infant formulae. Such differences are likely a consequence of processing methods such as pasteurisation and homogenisation. In particular, the subsequent stabilisation of formulated emulsion droplets with a milk protein adsorbed layer is speculated as influencing the ability of droplets to be hydrolysed by endogenous lipase enzymes (de Oliveira, Bourlieu, et al., 2016; de Oliveira, Deglaire, et al., 2016; Gallier et al., 2013). In response to such hypotheses, recent studies have explored the use of membrane fractions extracted from milk fat globules, which were found to have an impact on lipase adsorption (Bourlieu-Lacanal et al., 2015).

However, it is still not fully established whether, or how, the structural differences of the maternal milk and infant formula emulsions influence the lipid digestion process. Previous *in vitro* studies have shown different structural outcomes during the gastric digestion of maternal, formulated and model milk emulsions, but these were not correlated with the dynamics of lipolysis. This study attempts to determine whether lipolysis during gastric digestion is influenced by the emulsion structure using a static *in vitro* gastric model. In order to study how the interfacial compositions and properties could influence the digestibility of infant milk, a model emulsion system was developed comprising a phospholipid stabilised emulsion that could be protein-coated using lactoferrin. This would allow the digestion of emulsions of equivalent lipid composition and droplet size, but with variable interfacial compositions to be studied. To further explore the role of protein adsorption on the kinetics of lipolysis, gastric digestion was carried out with both fungal lipase and pepsin present, as well as with the individual protease and lipase. In this way, any co-dependency of enzyme action on hydrolysis of either interfacial layer or lipid substrate could be determined. Analysis of lipolysis rates of the reaction was conducted using GC, while SEM and TEM were used to determine the impact of gastric digestion conditions on the structure of emulsion droplets during gastric incubation.

5.2 Materials and Methods

5.2.1 Materials

A scheme of model protein emulsions is shown below.

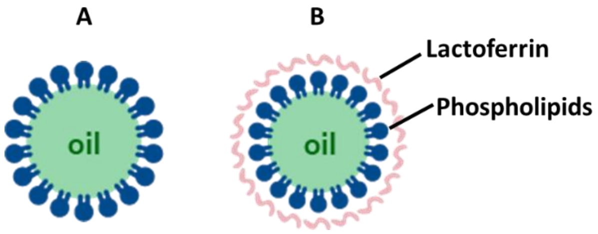


Figure 5.1 A, Intralipid, a phospholipid coated soybean oil emulsion; B, lactoferrin coated Intralipid.

All the other materials were described in Chapter 3.1.

5.2.2 *In vitro* digestion using Intralipid model emulsions

Intralipid was used as a model emulsion system to establish the appropriateness of the *in vitro* model and the associated characterisation of the simulated digestion. This is a commercial product that shows very stable emulsion properties and consistency where soybean oil droplets are stabilised by a monolayer of phospholipids from egg lecithin. A final 3.6 % (v/v) oil/water emulsion was achieved by diluting Intralipid emulsion with Milli-Q water, to mimic the fat content encountered in standard infant formulae.

The initial digestate was prepared by mixing a diluted Intralipid emulsion containing 3.6% (w/v) fat or Intralipid emulsion containing 3.6% (w/v) fat and 1% (w/v) of lactoferrin as the substrate, and SGF at a ratio of 20:50 v/v. In this experiment, the volumes of diluted Intralipid and SGF (without the enzymes) were 40 ml and 100 ml, respectively.

In this experiment, the volumes of diluted Intralipid and SGF were 40 mL and 100 mL, respectively. The mixtures were then kept in a water bath for around 10 min until they reached a temperature of 37 ^{0}C.

An amount of 450 mg of dry powdered porcine pepsin (800-2500 units/mg protein) and 20 mg of dry powdered *Rhizopus oryzea* lipase (80 U/mg) was first hydrated with a small amount of Milli-Q water (usually 5 to 10 times of the enzymes own weight) and then added to the mixture. The mixture was incubated at 37 ^{0}C till 120 min with stirring rate of 10 rpm. Starting from 0 min and in every 15 min, an amount of 50 μL, 1.5 mL or 1 mL of two aliquots was sampled for measurements such as ζ-potential, droplet size measurement or confocal microscopy observation.

5.2.3 Analysis of lipolysis products by GC

Lipid extraction

The extraction process referred to the method in Helbig *et al.* (2012) with small modifications. An aliquot of 0.5 mL sample was taken during the *in vitro* digestion at 0, 15, 30, 45, 60, 90, 105, and 120 min. Each sample was poured into a Kimax tube containing a mix of solvents (1 mL ethanol, 1.5 mL of 1:1 (v/v) diethyl ether and heptane, and 0.1 mL of sulphuric acid (2.5 M)). Each tube was mixed by vortex for about 1 min and centrifuged for another 5 min at 1000 × g at room temperature. The supernatant from each tube was carefully removed and transferred to another Kimax tube. The residual lipidic fraction in the first Kimax tube was extracted with 1 mL of 1:1 (v/v) diethyl ether and heptane again following the previous procedure. The collected supernatant of the two extractions was brought up to 3 mL with 1:1 (v/v) diethyl ether and heptane. Then 300 mg of anhydrous sodium sulphate was added into each tube to dry any water residue. About 1 mL of the dried supernatant was carefully transferred to a GC sample vial for further analysis.

Validation of the GC instrument

See Chapter 3.2.4.

GC analysis

See Chapter 3.2.4.

5.2.4 Scanning electron microscopy (SEM)

See Chapter 3.2.5.

5.2.5 Transmission electron microscopy (TEM)

See Chapter 3.2.6.

5.2.6 Statistical Analysis

In this study, free fatty acids have been used as markers of the degree of lipid digestion. On the GC chromatograms, the area under the peaks of total FFA was measured throughout digestion. In this study, as there was no internal standard involved, calibration curves of pure myristic and palmitic acid (FA C14:0 and C16:0) were used to determine the concentration of FFA (See calibration curves in 3.2.4). As the carbon number of the primary FFA released from soybean is in the range 8 to 18, and the theoretical response correction factors of fatty acids from C8 to C24 are very similar (0.96-1.19) (Christie & Han, 2012). Therefore, we treated the response factor of each type of fatty acid released from our model emulsion as 1. This showed that the area under the peak (AUP, mV*min) was in proportion to the sample concentration (g/L). Thus, we used AUP instead of concentration to provide an empirical representation of the dynamics of the lipolysis process.

In order to compare the rate of lipolysis from different treatment without internal standard involved, we normalised the original data of AUP and results were shown in Figure 4.4. The z-score for each AUP value is

$$z = \frac{x - \bar{x}}{S}$$

Where z-score is the normalised AUP value; x is the AUP value of the sample; $\bar{x}$ is the sample mean, and S is the standard deviation of the mean.

5.3 Results

5.3.1 Increase of free fatty acids (FFA) from a model emulsion in a simulated gastric digestion

The d_{43} of diluted Intralipid at pH 7 was 0.32 μm (before digestion). The value for lactoferrin coated Intralipid was 0.4 μm. The ζ-potential of the Intralipid emulsion (3.6%, v/v) was -44.06 mV at pH 7 whereas for the lactoferrin coated Intralipid emulsion the value was +16.6 mV at neutral pH. The droplet size and surface charge data were done in a previous study (Lueamsaisuk, 2015). The extent of lipolysis for Intralipid model emulsions digested in different *p*H levels and enzyme treatments was around 20% (w/w) of total triglycerides (Figure 5.2) estimated using the standard curve in Chapter 3.2.4. This figure was within the range of degree of lipolysis (10-30%) observed in the previous *in vivo* study (Carriere et al., 1993)

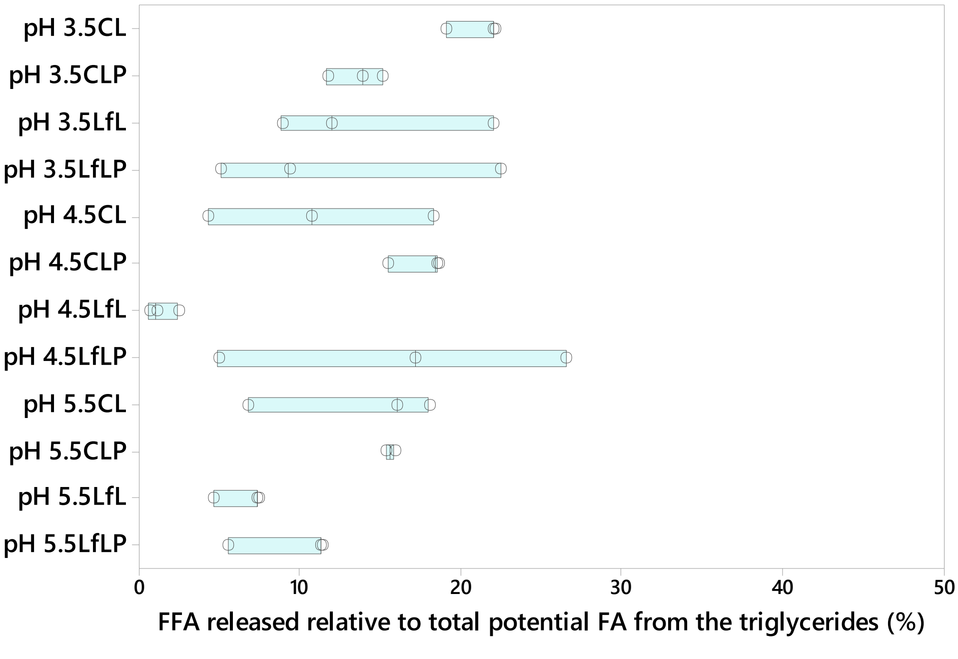


Figure 5.2 GC analysis on FFA released relative to total potential FA from the triglycerides from Intralipid model emulsion in a simulated gastric system. CL, Intralipid digested with lipase alone; CLP, Intralipid digested with lipase and pepsin; LfL, lactoferrin coated Intralipid digested with lipase alone; LfLP, lactoferrin coated Intralipid digested with lipase and pepsin. The *p*H values were all initial *p*H levels.

Figure 5.3 showed the original AUP for each treatment. From observation, the digestate of Intralipid itself with lipase or lipase and pepsin did not have visible phase separation during 120 min of simulated digestion. However, when Intralipid was coated with lactoferrin, the digestate showed aggregations from 30 min and onwards. This made sampling problematic as the digestate became increasingly inhomogeneous. It also confirmed that in this study, without an internal standard in each experiment, the high degree of variation was most likely due to issues with sampling.

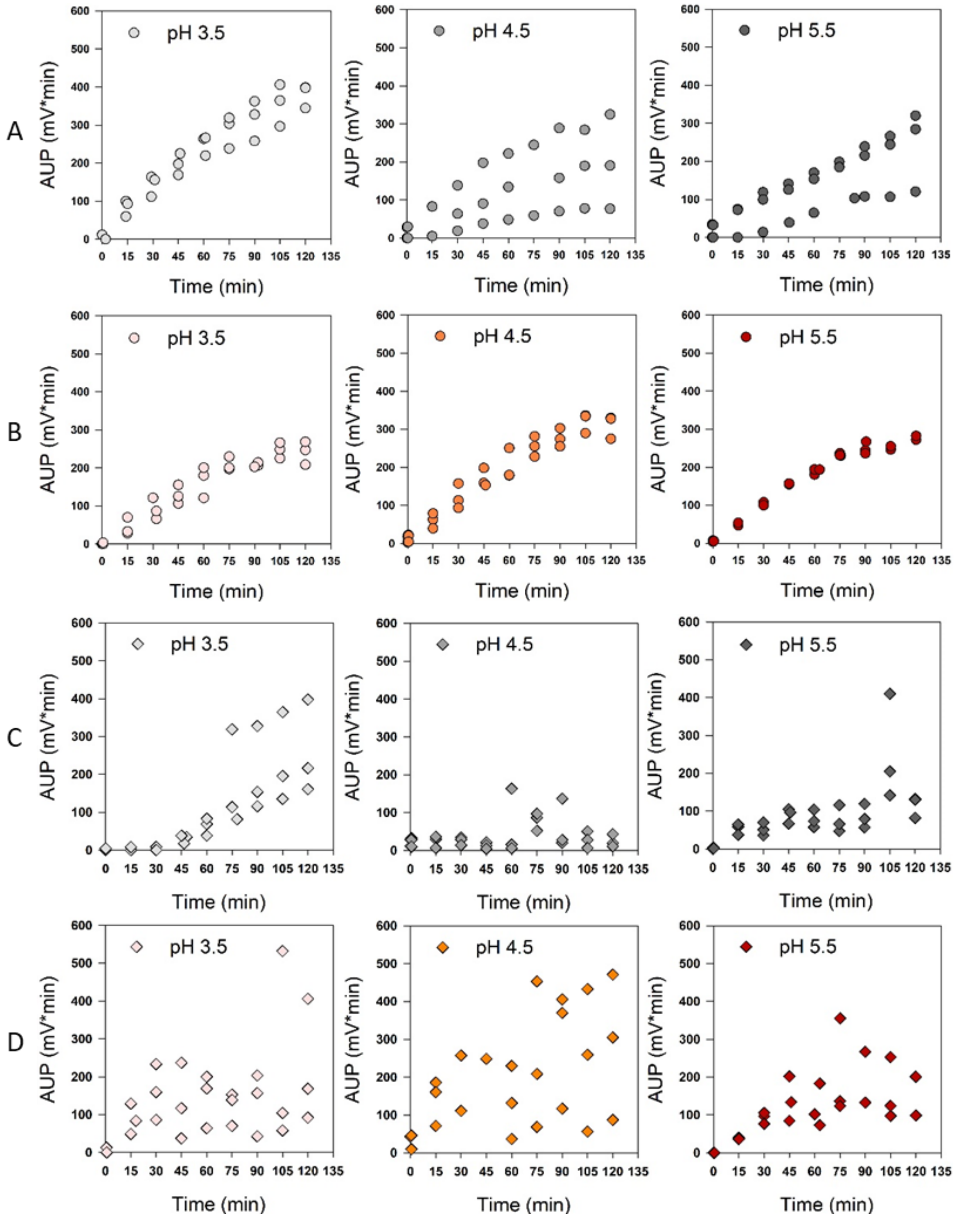


Figure 5.3 FFA increase of original AUP values of each *in vitro* digestion treatment. A, Intralipid digested with lipase; B, Intralipid digested with lipase and pepsin; C, lactoferrin coated Intralipid digested with lipase; D, lactoferrin coated Intralipid digested with lipase and pepsin.

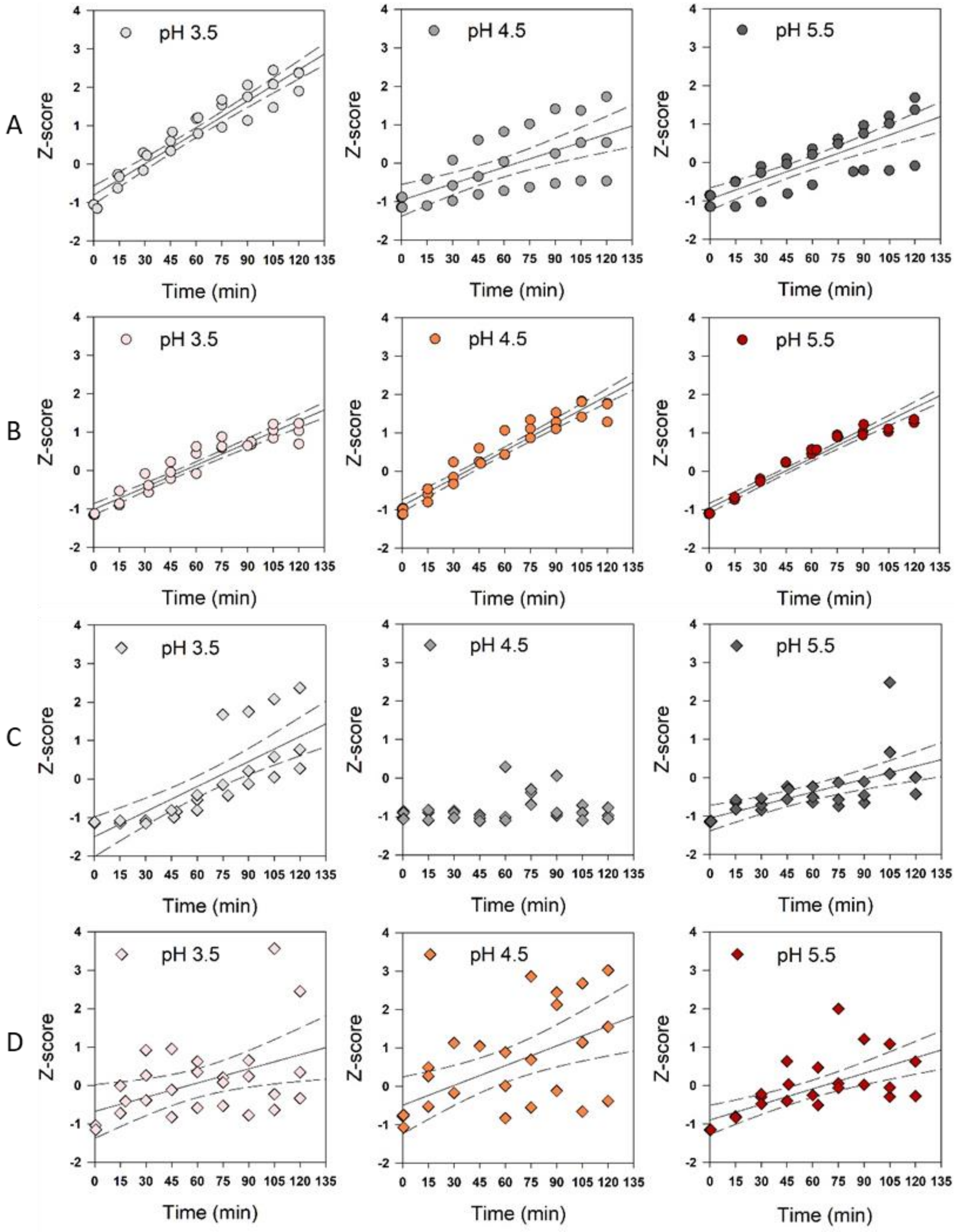


Figure 5.4 Normalised FFA increase from original AUP values for each *in vitro* digestion treatment. A, Intralipid digested with lipase; B, Intralipid digested with lipase and pepsin; C, lactoferrin coated Intralipid digested with lipase; D, lactoferrin coated Intralipid digested with lipase and pepsin. Solid line, linear regression; dash line, 95% confidence interval.

Table 5.1 Parameters of linear regression from normalised FFA increase (α=0.05).

	***p*H3.5 CL**	***p*H4.5 CL**	***p*H5.5 CL**	***p*H3.5 CLP**	***p*H4.5 CLP**	***p*H5.5 CLP**	***p*H3.5 LfL**	***p*H4.5 LfL**	***p*H5.5 LfL**	***p*H3.5 LfLP**	***p*H4.5 LfLP**	***p*H5.5 LfLP**
a	-0.801	-0.968	-0.955	-1.011	-0.899	-0.968	-1.500	-	-1.059	-0.679	-0.492	-0.894
SEM (a)	0.117	0.202	0.143	0.074	0.079	0.062	0.250	-	0.165	0.339	0.359	0.185
b	0.027	0.014	0.016	0.019	0.024	0.022	0.022	-	0.012	0.012	0.017	0.013
SEM (b)	0.002	0.003	0.002	0.001	0.001	0.001	0.003	-	0.003	0.005	0.005	0.003
df	1;25	1;26	1;28	1;28	1;28	1;25	1;24	1;27	1;27	1;25	1;24	1;26
F	277.625	23.849	57.494	310.299	414.907	514.781	39.833	1.150	21.350	6.726	11.744	24.717
Sig.	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.293	<0.001	0.016	0.002	<0.001
R^2	0.918	0.478	0.672	0.917	0.937	0.954	0.624	-	0.442	0.212	0.329	0.487

CL, Intralipid digested with lipase alone.

CLP, Intralipid digested with lipase and pepsin.

LfL, lactoferrin coated Intralipid digested with lipase alone.

LfLP, lactoferrin coated Intralipid digested with lipase and pepsin.

a, intercept value.

SEM (a), standard error of the mean of *a*.

b, slope value.

SEM (b), standard error of the mean of *b*.

df, degrees of freedom and residual degrees of freedom.

F, F value.

R^2, coefficient of determination.

The normalised data of increase of FFA was shown in Figure 5.4. As the normalised data for each treatment shown in Figure 5.4 were very noisy. Several statistical methods on the same data set were applied to attempt to resolve the large variation of the data in each treatment.

5.3.1.1 Linear regression

Linear regression was first applied to the normalised data in Figure 5.4 and Table 5.1. The data confirmed that there were significant increases with time for all of the treatments except for lactoferrin coated Intralipid digested with lipase alone at *p*H 4.5. There was also no significant difference between the slope values of the relative rate for each treatment when linear regression was applied to data for the first 30 min (see Appendix A2). However, linear regression was not proper since the normal probability plot of the residue for each treatment deviated from the diagonal line (graphs not shown). As the data was very noisy for most of the treatments, it was also challenging to apply models or curves to describe the trends of the data.

Except for the treatment of Intralipd digested with lipase alone (Figure 5.4 A), the other treatments (Figure 5.4 B, C, and D) showed a curvilinear trend. Because of the large variations of the data, it was also very challenging to apply a non-linear curve model on each treatment (data not shown).

5.3.1.2 Repeated-measures ANOVA for digestion time

One-way repeated-measures ANOVA was also applied to the same set of data to check whether there was any significant difference for the within-subject—time. The probability of the repeat-measures ANOVA was shown in Table 5.2. The results showed that for the digestion of Intralipid emulsion, except for *p*H 4.5 digested with lipase alone, FFA showed a significant increase for all the other treatments. However, for the digestion of lactoferrin coated Intralipid emulsion, there was no significant increase for FFA during 120 min of simulated digestion.

As noted, the repeatability of some of the treatments shown in Figure 5.4 was not good. For example, in treatment *p*H 4.5 Intralipid digested with lipase alone, and all treatments for lactoferrin coated Intralipid digestion, the replicates in each treatment showed different trends which made statistical method difficult to interpret. This indicated that our experimental method was not repeatable due to difficulties in sampling from the phase-separating digestate.

Table 5.2 Probability table of repeated-measures ANOVA for normalised FFA increase.

	Repeated Measures ANOVA
***p*H 3.5 CL**	0.006
***p*H 4.5 CL**	NS
***p*H 5.5 CL**	0.038
***p*H 3.5 CLP**	0.001
***p*H 4.5 CLP**	0.001
***p*H 5.5 CLP**	0.030
***p*H 3.5 LfL**	NS
***p*H 4.5 LfL**	NS
***p*H 5.5 LfL**	NS
***p*H 3.5 LfLP**	NS
***p*H 4.5 LfLP**	NS
***p*H 5.5 LfLP**	NS

CL, Intralipid digested with lipase alone.

CLP, Intralipid digested with lipase and pepsin.

LfL, lactoferrin coated Intralipid digested with lipase alone.

LfLP, lactoferrin coated Intralipid digested with lipase and pepsin.

The grade shade showed no significant difference in the data after specific time points.

NS, no significant difference.

5.3.1.3 Repeated-measures ANOVA for a two-way interaction between *p*H and time, and a three-way the interaction between of enzyme treatment, *p*H and time

As repeated-measures ANOVA showed, there was a significant increase with time for treatment of Intralipid digested with lipase alone or with lipase and pepsin. A mixed two-way ANOVA was applied to explore whether there was a significant difference between the interaction term "time**p*H level". There was a significant interaction [$F(4.273, 10.682) = 5.734$, $p = 0.010$, partial $\eta^2 = 0.696$, $\varepsilon = 0.267$] between the *p*H level and time in FFA increase from the treatment of Intralipid digested with lipase alone. This indicated that *p*H did have a significant effect on the production of FFA under such treatment. For the treatment of Intralipid digested with lipase and pepsin, there was no significant interaction

($p > 0.05$) between the *p*H level and time in FFA increase. This indicated that under this treatment, *p*H did not have a significant effect on the production of FFA.

When another between-subject factor “enzyme” (treatment with lipase or lipase and pepsin) was involved in the repeated-measures ANOVA test, there was a significant three-way interaction between enzyme, *p*H level and time [$F(5.960, 29.802) = 6.272$, $p < 0.0005$, partial $\eta^2 = 0.556$, $\varepsilon = 0.426$]. There was a significant two-way interaction between *p*H level and enzyme [$F(2, 10) = 8.509$, $p = 0.007$]. All other two-way interactions (*p*H*time or enzyme*time) showed no statistical significance ($p > 0.05$). There was a significant simple main effect of enzyme at the *p*H 4.5 [$F(1, 10) = 9.853$, $p = 0.010$], but not at *p*H 3.5 or *p*H 5.5 ($p > 0.017$, statistical significance of a simple main effect was accepted at a Bonferroni-adjusted α level of 0.017, $\alpha = 0.05/3$, as there were three *p*H levels, i.e. three main effects). This indicated that the enzyme treatment, i.e. whether pepsin was present in the digestate might have a significant impact on the increase of FFA at *p*H 4.5.

A mixed two-way ANOVA was also applied to explore whether there was a significant difference between the interaction term “time**p*H level” for treatment of lactoferrin coated Intralipid digested with lipase or lipase and pepsin, though there was no significant difference in digestion time when looking at individual treatment. For the treatment of lactoferrin coated Intralipid digested with lipase alone, there was no significant interaction ($p > 0.05$) between the *p*H level and time in FFA increase. There was also no significant interaction ($p > 0.05$) between the *p*H level and time in FFA increase when pepsin was present in the system during digestion. This indicated that *p*H might not have a significant effect on the FFA increase during digestion for the treatment of lactoferrin coated Intralipid.

When involving another between-subject factor “enzyme” (treatment with lipase or lipase and pepsin) in the repeated-measures ANOVA test, there was no significant three-way interaction between enzyme treatment, *p*H level and time ($p > 0.05$). And all two-way interactions (*p*H*time, enzyme*time or enzyme**p*H) showed no statistical significance ($p > 0.05$).

When considering the effect of lactoferrin coating on the Intralipid emulsion interface with same enzyme treatment, there was no significant three-way interaction between lactoferrin coat, *p*H level and time ($p > 0.05$) for digestion with lipase alone. There was a significant two-way interaction between time and *p*H level [$F(4.779, 21.507) = 7.605$, $p < 0.005$, partial $\eta^2 = 0.628$, $\varepsilon = 0.341$]. All other two-way interactions (lactoferrin coat*time or lactoferrin coat**p*H) showed no significant difference ($p > 0.05$). There was a significant simple main effect of the enzyme at the 120 min [$F(2, 9) = 11.357$, $p = 0.003$], but not at other time points from 15 to 120 min ($p > 0.006$, the statistical significance of a simple main effect was accepted at a Bonferroni-adjusted α level of 0.006, $\alpha = 0.05/8$, as a selection of 8-time points from 15 to 120 min was used in this test, i.e. eight main effects). There was also no significant three-way interaction between protein coat, *p*H level and time ($p > 0.05$) for digestion with lipase and pepsin ($p > 0.05$). Moreover, there was no significant difference ($p > 0.05$) for all two-way interaction under this enzyme treatment (*p*H*time, lactoferrin coat*time or lactoferrin coat**p*H). It indicated that lactoferrin coat and *p*H might not have a significant impact on the generation of FFA during digestion.

5.3.2 Morphological changes of emulsion droplets during simulated gastric digestion using SEM

Images under SEM during digestion obtained at *p*H 3.5 was shown in Figure 5.5. The emulsion droplets were embedded in agarose structure. Droplets had a round shape and smooth surface at the commencement of simulated gastric digestion (Figure 5.5 A, C and E). The surface structure of the Intralipid droplets digested in the presence of lipase alone was greatly altered throughout digestion, with noticeable dimpling evident at the interface (Figure 5.5 B).

Intralipid droplets coated with lactoferrin digested at *p*H 3.5 with lipase only did not show significant changes in size from the beginning to the end of digestion (Figure 5.5 C and D). Some surface dimpling was observed, but not to the same apparent extent to the lipase-digested uncoated emulsion. For the digestion of Intralipid coated with lactoferrin at *p*H 3.5 with lipase and pepsin, droplet size was seen to increase throughout digestion. Additionally, some of the large droplets had distinctive erosion patterns appearing at the interface. Other droplets were seen to display similar dimpling patterns similar to the uncoated emulsion after lipolysis. This surface dimpling has been reported previously, presenting comparison to studies by Pafumi *et al*. (2002) and Gallier *et al*. (2013). The image results showed that the morphology of the emulsion droplets had changed during the simulated gastric digestion.

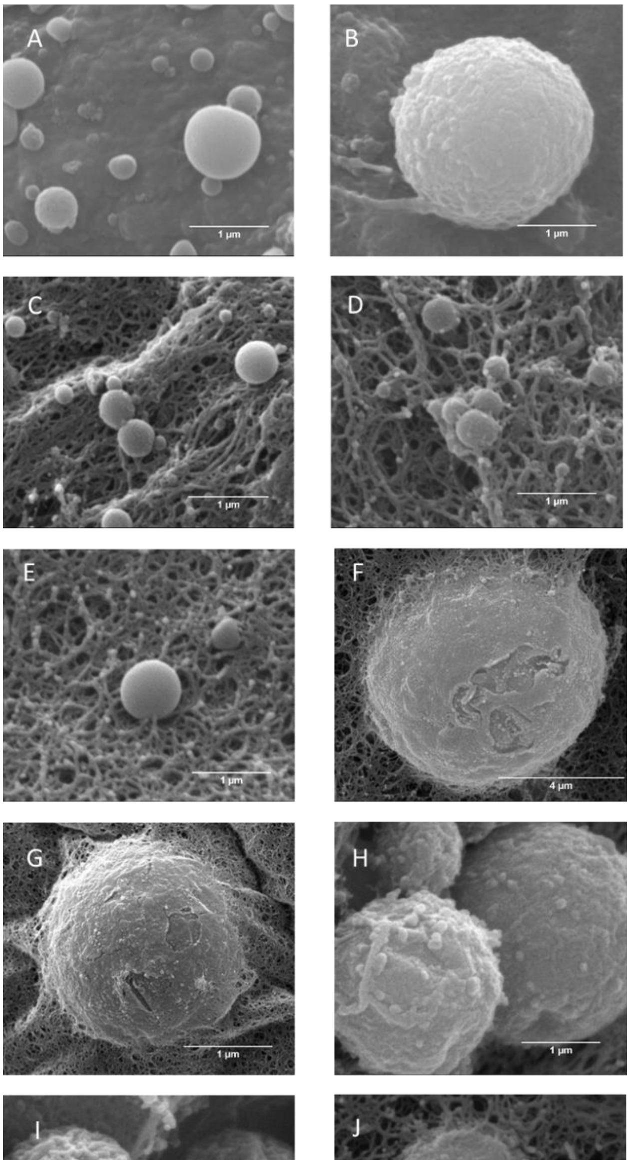


Figure 5.5 SEM images of emulsion droplets during 120 min of simulated gastric digestion at *p*H 3.5. A and B, droplets of Intralipid digested with lipase alone at 0 and 120 min; C and D, droplets of Intralipid coated with lactoferrin digested with lipase alone at 0 and 120 min; E, droplets of Intralipid coated with lactoferrin digested with lipase and pepsin at 0 min; F-J, droplets of Intralipid coated with lactoferrin digested with lipase and pepsin at 120 min.

5.3.3 Morphological changes of emulsion droplets during simulated gastric digestion using TEM

Intralipid emulsion droplets did not show any notable difference in the size at the commencement of digestion regardless of enzyme composition or *p*H (Figure 5.6 A1, B1and C1, Figure 5.7 A1, B1 and C1). The oil droplets had a diameter of 0.3-0.5 μm (in agreement with previous SEM data) and showed stability against flocculation at all *p*H levels studied. However, at the end of the simulated digestion process, the morphology of most of the oil droplets had changed with the final structure varying according to relative pH. At *p*H 3.5, they formed clusters with individual droplets displaying a high degree of anisotropy. Oil droplets appeared smaller and darker with the bright area inside the droplets, showing electron-dense and less dense regions generated during two hours of digestion (Figure 5.6 A2-A5). At *p*H 4.5, the appearance of the droplets was more uniform, noting that some electron-dense regions had accumulated at the inner surface of the droplets (Figure 5.6 B2-B4). The appearance of the large droplets (diameter larger than 5 μm) was indicative of coalescence, with less electron-dense compounds forming in round shape inside the large droplets (Figure 5.6 B4 and B5). At *p*H 5.5, some degree of anisotropy was observed, along with regions of highly disordered structures (Figure 5.6 C2-C5). Although these regions showed appreciable staining, it is difficult to determine whether these are lipids based due to the irregularity of the appearance.

For Intralipid emulsion digested with lipase and pepsin, the oil droplets at the initial state were shown in Figure 5.7 A1, B1 and C1. After two hours of digestion, oil droplets at *p*H 3.5 and 4.5 became larger. Some were merged into giant droplets with a diameter larger than 5 μm. At *p*H 3.5 in particular, the surface of the large droplets became diffuse and cracked (Figure 5.7 A2-A5, B4 and B5). Vesicles formed inside the large droplets with electron-dense regions observed inside the vesicles (Figure 5.7 A3, B2 and B3) and then released to the ambient aqueous phase (Figure 5.7 B4 and B5). Some of the striated structure were generated during simulated gastric digestion, attached on the droplet surface and then released into the ambient phase (Figure 5.7 B4 and B5). At *p*H 5.5, some others tended to break up and release the electron-dense compounds accumulated inside the droplets (Figure 5.7 C2-C5). The compounds formed inside the droplets accumulated in round beads and turned out to be very different in shapes from the droplets digested at *p*H 3.5 and 4.5 after two hours. Changes in the morphology of the droplets were more drastic when pepsin was included in the digestion process compared to when there was lipase present alone. Additionally, striated structure compounds only formed in the combined presence of pepsin and lipase.

When Intralipid emulsion droplets were coated with lactoferrin on the interface, droplets became aggregated at the commencement of digestion, with protein layer seen to located on the interface (Figure 5.8 A1, B1 and C1). Because of the contrast effect from the protein layer, droplets could be observed much more clearly under TEM. When lipase was present in the digestate alone, the size of the droplets did not change drastically after two hours of digestion at *p*H 3.5 (Figure 5.8 A2-A5). Electron-dense compounds appeared at the inner interface of the oil droplets (Figure 5.8 A2), and the droplets were increasingly less spherical (Figure 5.8 A3-A5). At *p*H 4.5, droplets had increased in size and appeared amorphous after two hours of digestion (Figure 5.8 B2-B5). Droplets at *p*H 5.5 showed diverse structural characteristics. Spherical droplets were still observed, but the staining patterns within these droplets had become distinctively disparate. When both of the enzymes (lipase and pepsin) existed in the digestate, droplets underwent many notable changes compared to conditions with lipase alone (Figure 5.9). Droplets were observed to have merged into large ones, released transparent vesicles and striated like electron-dense compounds and even appearing to have burst. Armand *et al*. (1996) also showed distorted droplets and electron-dense lipolytic products on infant formula gastric digestion in an *in vivo* study in the premature infant. There was not much different observed at different *p*H levels.

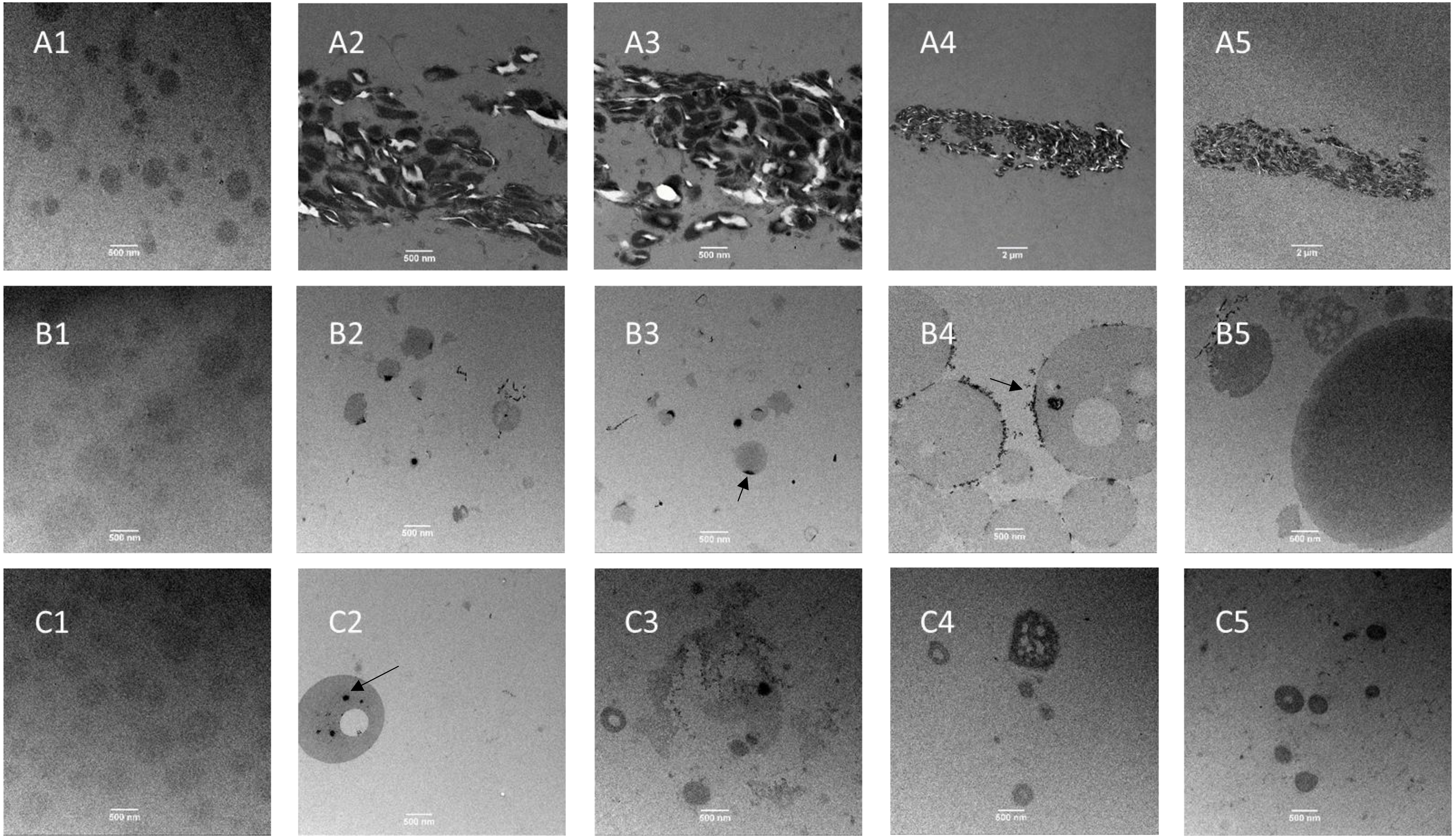


Figure 5.6 TEM images of Intralipid emulsion digested with lipase alone. A1, *p*H 3.5 at 0 min; A2-A5, *p*H 3.5 at 120 min; B1, *p*H 4.5 at 0 min; B2-B5, *p*H 4.5 at 120 min; C1, *p*H 5.5 at 0 min; C2-C5, *p*H 5.5 at 120 min. Arrows indicated electron-dense lipolytic products.

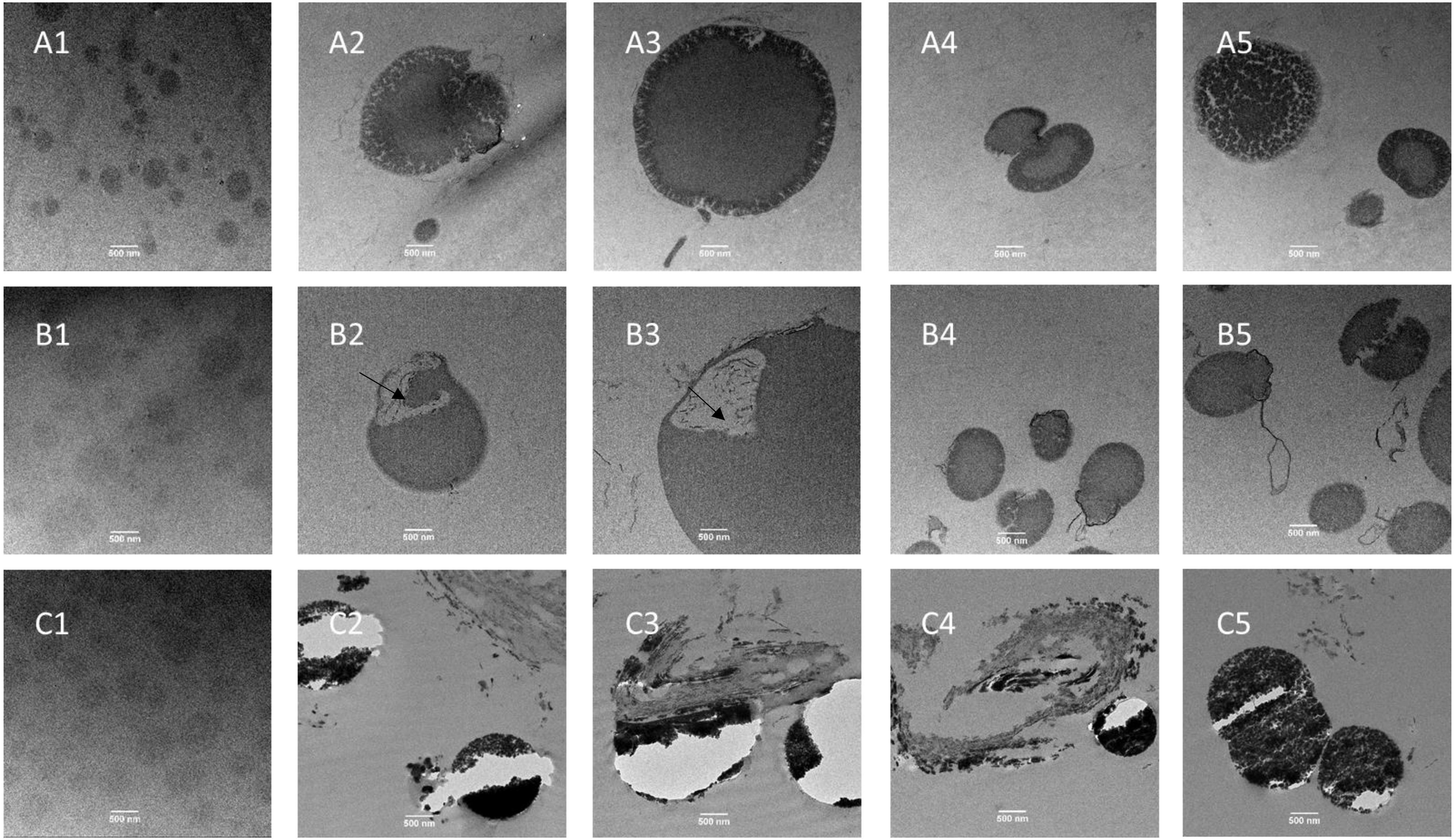


Figure 5.7 TEM images of Intralipid emulsion digested with lipase and pepsin. A1, pH 3.5 at 0 min; A2-A5, pH 3.5 at 120 min; B1, pH 4.5 at 0 min; B2-B5, pH 4.5 at 120 min; C1, pH 5.5 at 0 min; C2-C5, pH 5.5 at 120 min. Scale bar = 500 nm. Arrows indicated electron-dense lipolytic products.

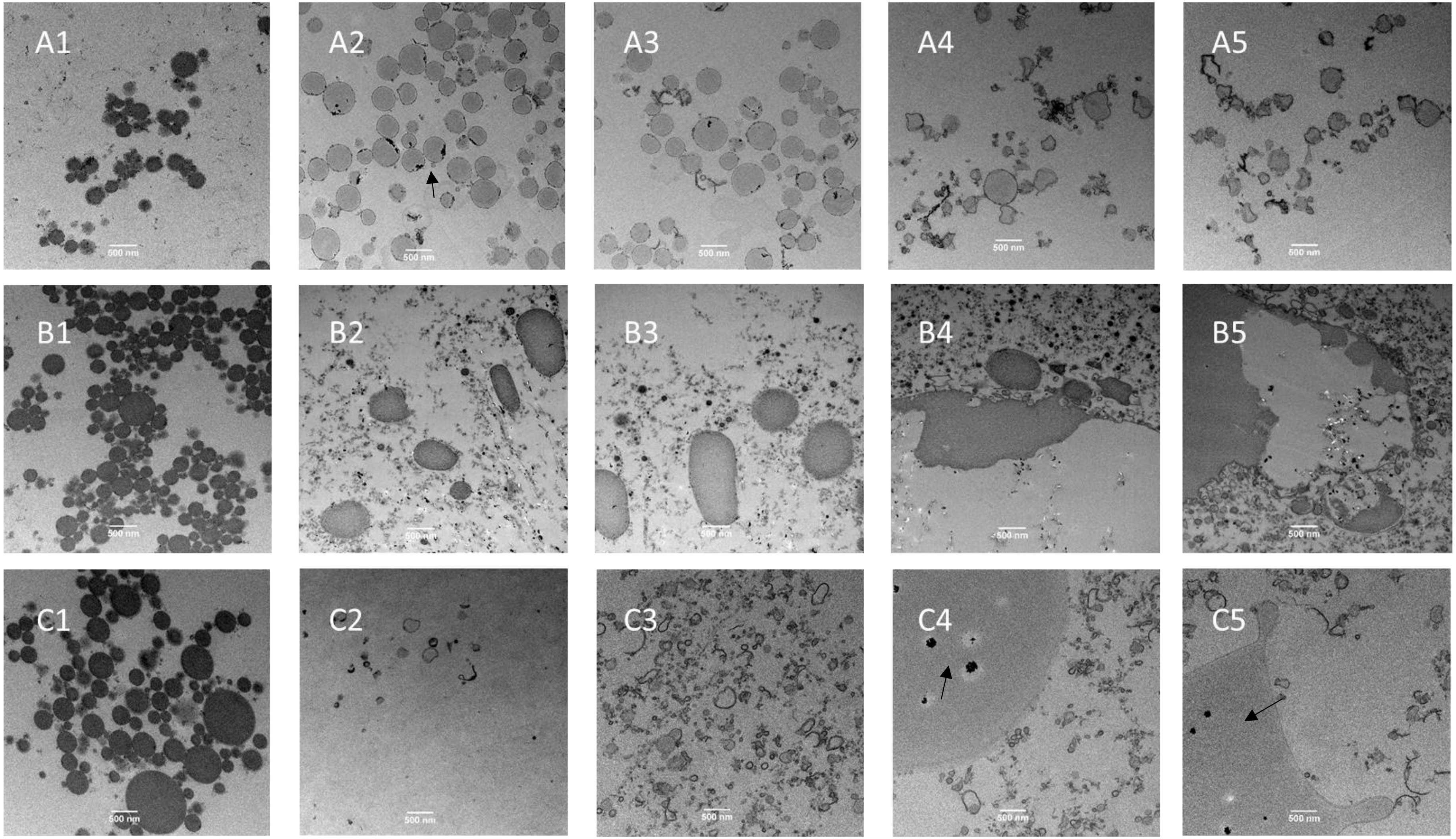


Figure 5.8 TEM images of lactoferrin Intralipid emulsion digested with lipase alone. A1, pH 3.5 at 0 min; A2-A5, pH 3.5 at 120 min; B1, pH 4.5 at 0 min; B2-B5, pH 4.5 at 120 min; C1, pH 5.5 at 0 min; C2-C5, pH 5.5 at 120 min. Scale bar = 500 nm. Arrows indicated electron-dense lipolytic products.

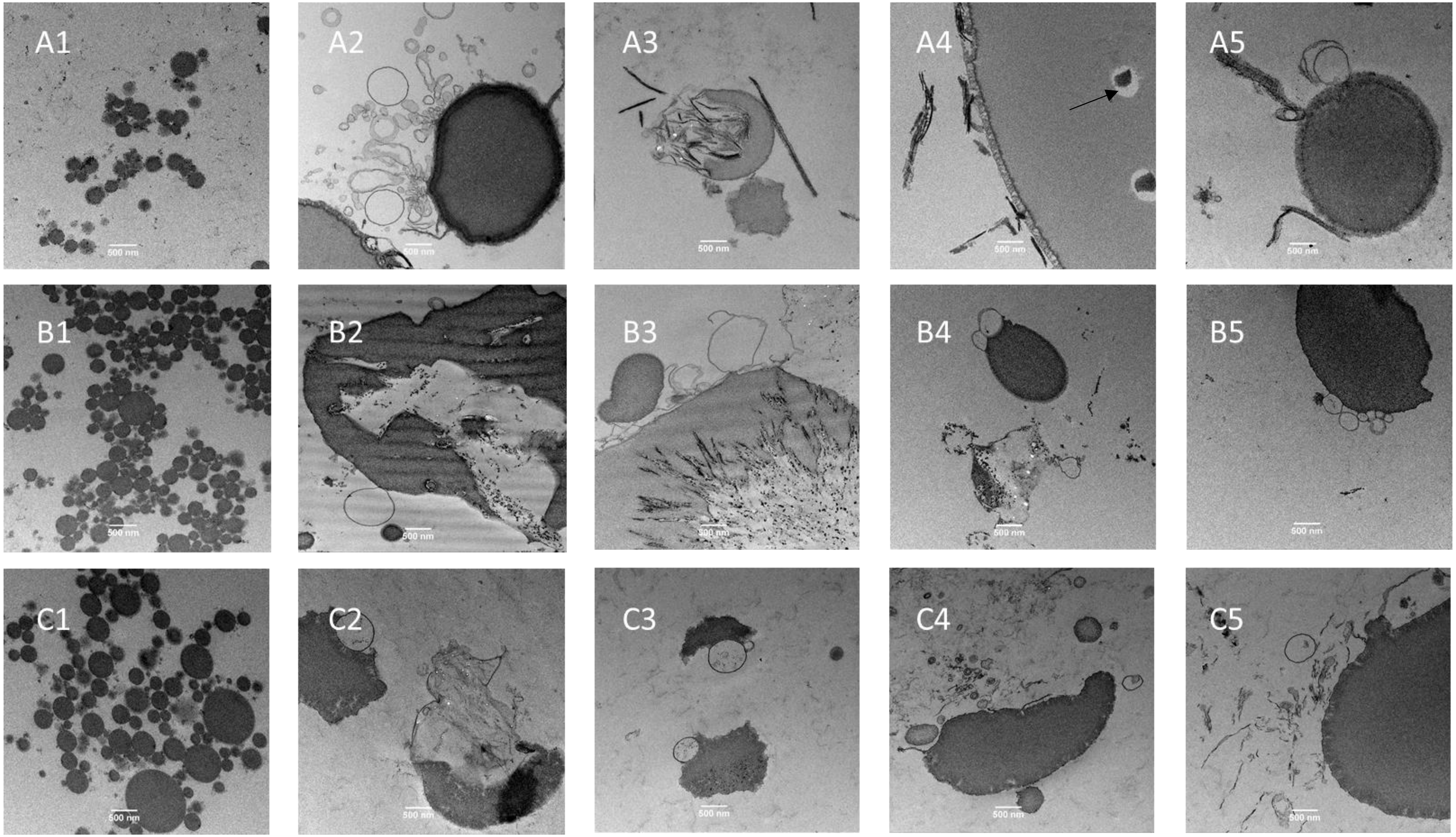


Figure 5.9 TEM images of lactoferrin coated Intralipid emulsion digested with lipase and pepsin. A1, pH 3.5 at 0 min; A2-A5, pH 3.5 at 120 min; B1, pH 4.5 at 0 min; B2-B5, pH 4.5 at 120 min; C1, pH 5.5 at 0 min; C2-C5, pH 5.5 at 120 min. Scale bar = 500 nm. Arrows indicated electron-dense lipolytic products.

5.4 Discussion

In this study, we determined that lipolysis was primarily responsible for the changes to the morphology of emulsion droplets during this simulated gastric digestion process. However, correlating the observed changes in droplet structure to the rate and extent of lipolysis was ultimately not possible, due to challenges in obtaining reliable measurements for FFA content during digestion. For the GC measurements, data showed large variations in replicates across most of the treatments (notably those samples observed to undergo phase separation during the process of digestion which hindered reliable sampling). Accordingly, it was difficult to draw reasonable conclusions regarding the extent of lipolysis from the FFA data across variable *p*H conditions, presence of lactoferrin and enzyme combination. Therefore, the GC measurement of FFA increase may not accurately reflect the actual reaction kinetics from the digestion process. The statistical analysis of linear regression for the first 30 min showed that the rate (the slope values) of lipolysis had no significant difference among all treatments. However, the data set showed that linear regression might not be a suitable way to describe the trend of lipolysis in all treatments across the overall reaction time. Since the errors in the experiment were remarkably large, it was also challenging to apply non-linear regression in order to describe the trend of FFA increase.

Findings can be considered concerning two studies by Lueamsaisuk *et al.* (2014, 2015) that had previously shown that the particle size distributions of emulsions comprising Intralipid and lactoferrin (as used in this current study) were affected by both *p*H conditions and combination of pepsin and lipase during simulated digestion over 120 min (critical findings from this study were reproduced in Figure 5.10 and 5.11). The release of FFA was not measured in these previous studies, so it was not possible to unequivocally state that structure dynamics were a consequence of lipolysis. However, it was hypothesised that emulsions not displaying any changes in emulsion structure during digestion were potentially not undergoing lipolysis. In this current study, we confirmed the liberation of FFA for all samples regardless of whether the interfacial layer comprised phospholipid or phospholipid bound to lactoferrin. This would appear to confirm that the presence of interfacial protein for these emulsions was not entirely inhibitory to lipolysis when pepsin was excluded from the digestate (i.e. negating the hypothesis that proteolysis of the interface is a requisite for lipolysis to occur for protein stabilised emulsions).

In further considering the relationship between structural changes observed during digestion and lipolysis, it was interesting to note that the electron-dense structures found near the surface of droplets after digestion (Figure 5.6-5.9) appeared independent of the inclusion of pepsin in the digestate, *p*H changes, or the curve shape of the FFA increase. Similar structures have been observed in previous studies (Armand, Hamosh, et al., 1996; Gallier et al., 2013), with the suggestion that these are polar crystal lipolytic products generated from triglyceride hydrolysis. Variance in the extent of structural changes observed in both SEM and TEM under different *p*H conditions may be due to variations in the activities of both pepsin and lipase across this *p*H range. Additionally, differences in droplet structure and morphology may also possibly be due to the impact of *p*H on the assembly of the lamellar polar lipid structures, noting that lower *p*H conditions will increasingly approach the *p*Ka of the fatty acids being liberated and thus increasingly lead to protonation of these molecules.

However, results (both microscopic and GC) would ultimately suggest that the lactoferrin layer bound to the Intralipid oil droplets did not appear to act as a barrier for lipase binding. A study on interfacial protein structure provided similar results (Maldonado-Valderrama et al., 2013). This suggested that there were gaps in the oil/water interface where the hydrophobic domain of lipase could access to the phospholipid layer, and thus bind to the interface. Subsequent liberation and adsorption of free fatty acids from lipolysis might then be expected to displace bound protein from the interface, thereby enabling further adsorption of lipase.

In our initial hypothesis, gastric lipolysis kinetics of protein stabilised emulsions are optimised by a co-dependency between pepsin and lipase. Pepsin would facilitate lipolysis by hydrolysing the adsorbed protein layer, partially removing the barrier for lipase to attach on the phospholipid-oil interface, more effectively enabling lipase binding and thus increasing the rate and extent of lipolysis. However, the addition of pepsin did not appear to change this scenario. This may occur due to several reasons. Firstly, as the isoelectric point of lactoferrin is 8.7 (Sánchez, Calvo, & Brock, 1992), lactoferrin carries positive charges from *p*H 3.5 to 5.5. The *p*H level could have a substantial impact on the charge density of proteins as it affects the degree of ionisation of proteins. Therefore, when *p*H increases from 3.5 to 5.5, the charge density of lactoferrin decreases. This may loosen the attachment of lactoferrin to the interface covered by negatively charged phospholipids where lipase could more easily overcome the protein barrier and adsorb on the oil/water interface without the help of pepsin.

Secondly, as *p*H level dropped from 5.5 to 3.5, pepsin activity would be seen to increase towards its optimum at *p*H = 2.0, increasing proteolysis and liberating more peptides. Hydrolysed hydrophobic peptides may serve to reduce the surface tension on the oil/water interface and induce emulsion instability through either flocculation and/or coalescence. For example, a study (Schröder, Berton-Carabin, Venema, & Cornacchia, 2017) showed emulsion droplets stabilised by whey protein had stronger interfaces with higher elasticity than hydrolysed proteins. Sarkar *et al*. (Sarkar, Goh, Singh, & Singh, 2009) also found emulsion droplets stabilised by *β*-lactoglobulin had extensive flocculation and coalescence in the *in vitro* gastric digestion. Such destabilisation result may usually lead to an increase of droplet size and reduction in surface area for lipase adsorption. Separating the particular roles of pepsin and lipase on lipid hydrolysis kinetics has been found to be particularly challenging both due to the profound changes in emulsion structure during digestion and as a consequence of the disparate optimum activities displayed by both enzymes. Arguably, the results would be better interpreted if the sampling problem could be resolved with an internal standard induced for GC analysis.

That said, it is worth noting that changes to emulsion droplet size relative to FFA release have been observed in several earlier studies, although the separation of lipases and pepsin was not considered in these studies (Armand, Borel, et al., 1994a; Armand et al., 1999; Bourlieu et al., 2017; de Oliveira, Deglaire, et al., 2016; Luo et al., 2019; Pafumi et al., 2002). Some of this previous research (Armand, Borel, et al., 1994a; Bourlieu et al., 2017; de Oliveira, Deglaire, et al., 2016; Luo et al., 2019; Pafumi et al., 2002) also found the release of FFA reached a plateau status after 20 to 60 min after the initiation of gastric digestion (*in vitro* and *in vivo*). This indicated that the reaction might reach equilibrium or that the lipolytic products had inhibited the lipase activity at the first hour of gastric digestion (Pafumi et al., 2002). The study by Pafumi *et al*. (2002) showed that lipases were trapped in the protrusions formed by lipolytic products on the oil/water interface and therefore inhibited lipolysis.

These findings were somewhat different from what was observed in this study. One of the reasons, as stated above, was the difficulty in sampling. The large standard errors were also seen in those studies. It should be noted that lipolysis takes place at the oil/water interface which is very different from the conventional enzymatic reactions in the aqueous environment following Michaelis-Menten kinetics. Therefore, it could be very possible that the concentration of FFA from the lipolysis in this study has not yet reached the saturation point for product inhibition, also noting that this study utilised a fungal lipase as an analogue for human gastric lipase, and therefore may not provide full equivalence regarding the type and concentrations of FFA released.

5.5 Conclusions

In conclusion, the rate of FFA during simulated gastric digestion was not able to be correlated to the differences in structural changes and droplet size as a consequence of emulsion and simulated gastric fluid composition. However, the observation of FFA synthesis for all measurements was able to indicate that lactoferrin (protein) coating on the Intralipid surface did not appear to provide a barrier to the hydrolysis by lipase, although it did result in different emulsion structural pathways during digestion. The inconclusive nature of the GC findings relative to emulsion structure dynamics during digestion was believed to be a consequence of phase separation of particular compositions during digestion, which rendered accurate sampling difficult. To address this, alternative approaches for either sampling or analysis would be recommended.

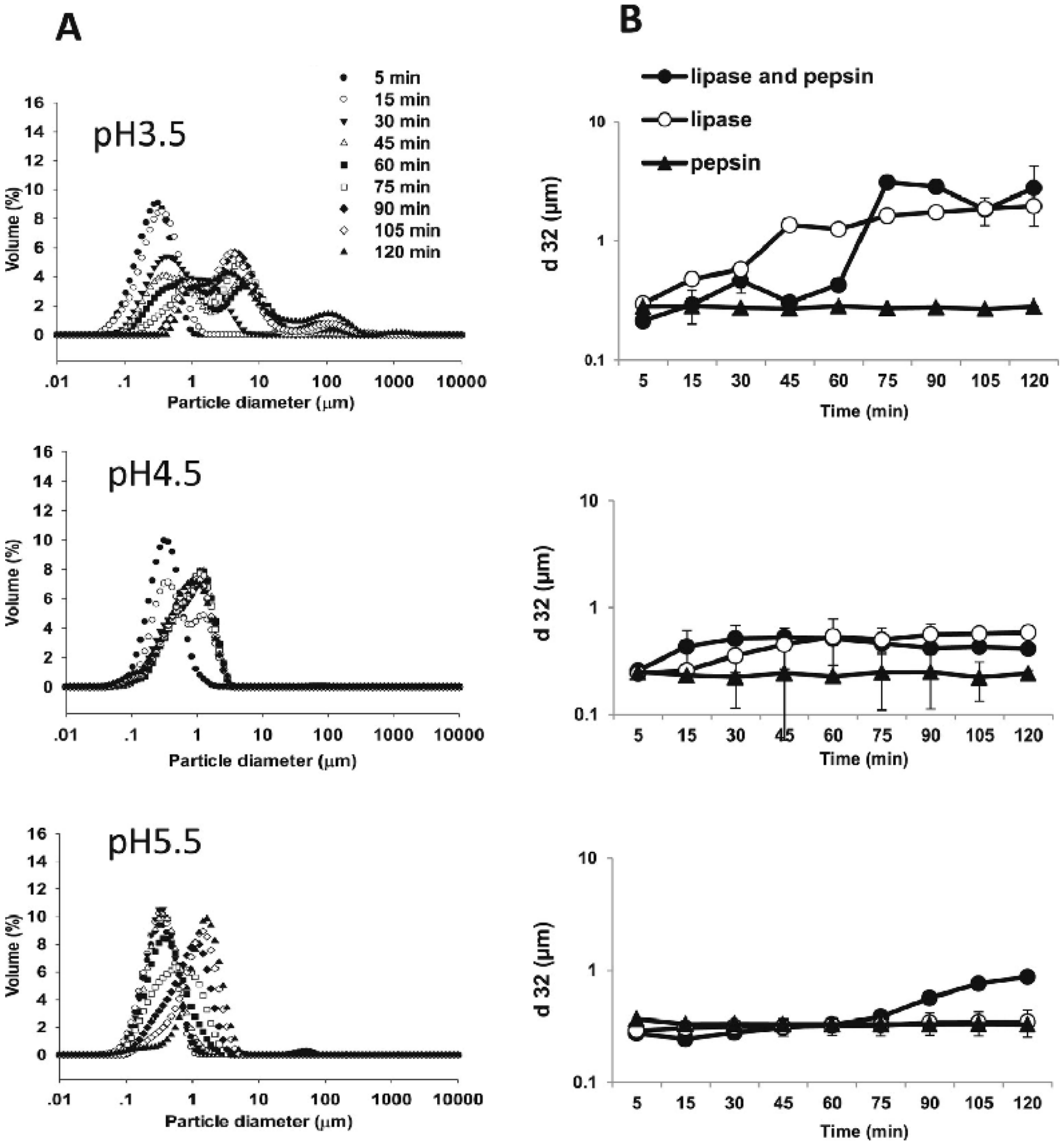


Figure 5.10 Droplet size distribution of Intralipid emulsion in a simulated infant gastric system, adapted from Luemsaisuk *et al*. (2015). A, Changes in the droplet size distribution during digestion with fungal lipase and pepsin for 120 min; B, Changes in d_{32} during digestion with fungal lipase and pepsin, pepsin alone, and lipase alone for 120 min. Emulsions were treated by a dissociating solution. Reproduce with permission from Elsevier.

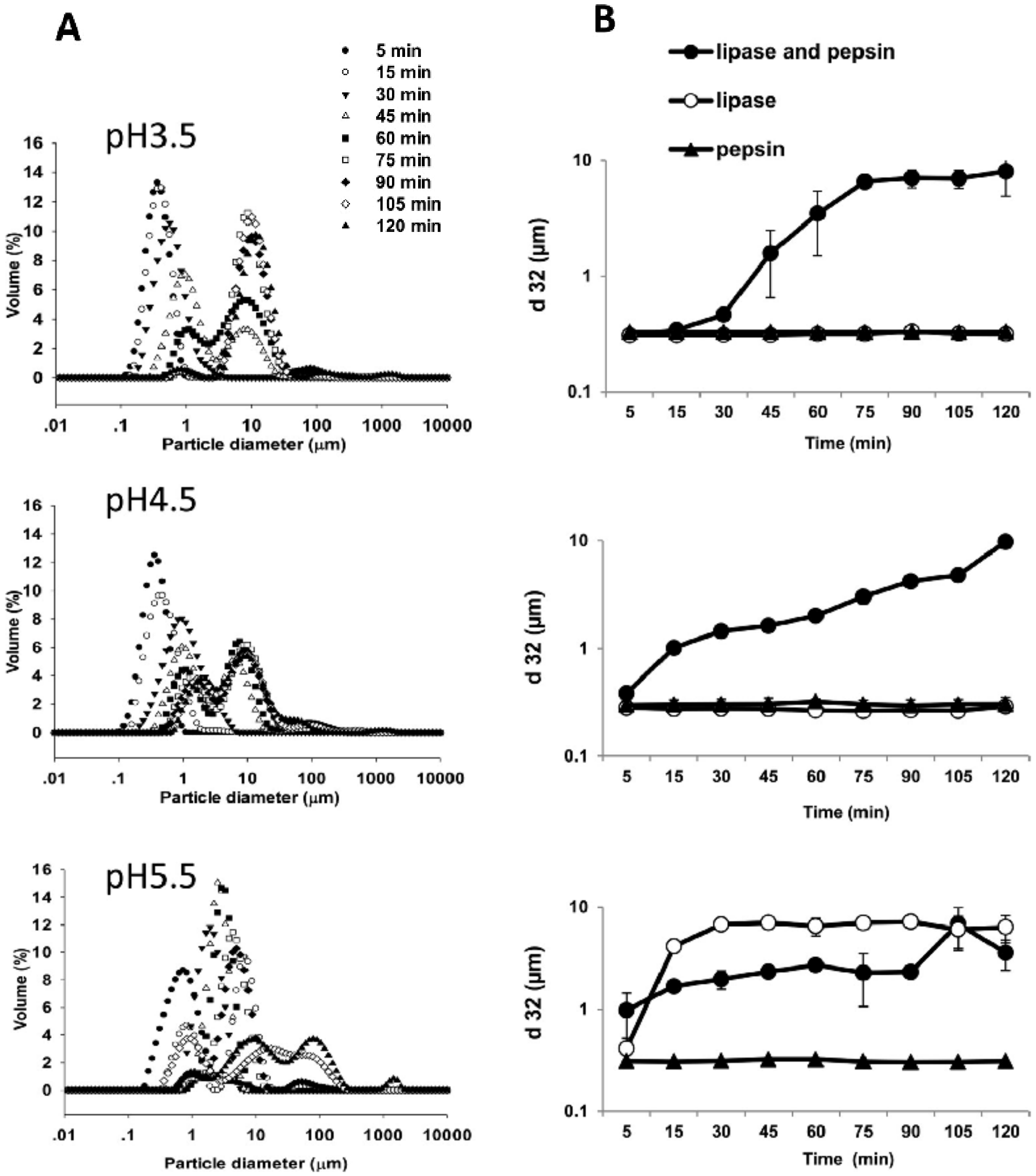


Figure 5.11 Droplet size distribution of lactoferrin coated Intralipid emulsion in a simulated infant gastric system, adapted from Luemsaisuk *et al*. (2015). A, Changes in droplet size distribution during digestion with fungal lipase and pepsin for 120 min; B, Changes in d_{32} during digestion with fungal lipase and pepsin, pepsin alone, and lipase alone for 120 min. Emulsions were treated by a dissociating solution. Reproduce with permission from Elsevier.

Chapter 6 Digestion of a powder infant formula using a simulated neonatal gastric system

6.1 Introduction

The rationale for this study was to progress findings from Chapter 4 and attempt to determine the separate and combined role of lipase and pepsin in the process of gastric lipolysis of formula emulsions. As discussed in the literature review, until recently, many prior *in vitro* studies exploring lipid digestion did not tend to include gastric lipase as part of the gastric stage of digestion. Partly this has been due to limitations around the availability of human gastric lipase or appropriate mammalian analogues, along with concerns about the functional equivalence of fungal alternatives. Additionally, the fact that previous clinical trials have determined that about 10-30% lipid hydrolysis occurs in the stomach (Armand, Borel, et al., 1994b; Armand, Borel, et al., 1996b; Armand et al., 1999; Fave et al., 2004) has tended to provide a rationale that the gastric stage of lipid digestion can be overlooked in the *in vitro* models. Fortunately, this view is now changing. However, as indicated in the literature review, gastric lipolysis is viewed as providing a more significant role in lipid digestion for neonates and provides crucial contribution ensuring effective digestion and metabolism of milk fat for infants at the very early stages of life.

There have been many additional discussions on the differences in properties (both nutritional and structural) between infant formulae and maternal milk over the decades (Carnielli et al., 1996; Hageman et al., 2019; Jensen et al., 1992; Jensen et al., 1978; Lönnerdal, 2014; López-López et al., 2001; Michalski et al., 2005; Zou, Ali, Abed, & Guo, 2017). Most of these have indicated that maternal milk, and notably milk fat, is effectively digested by infants when compared to infant formulae. While many possible mechanisms exist to account for variations in efficacy of lipid digestion, this study focusses specifically on the role of the adsorbed protein layer that provides stability to formulated emulsions post-homogenisation, and whether this interfacial layer is in some way inhibitory to the action of gastric lipase. Our previous study has determined that the protein did not form a barrier for lipase adsorption to a model protein stabilised emulsion system compared to phospholipid stabilised layer (noting that structural differences were observed). However, in standard infant formulae, protein adsorption and interfacial layer properties are also influenced by other processing and compositional variable.

In order to unravel the question of whether the interfacial layer of formulated emulsions was influential on the kinetics of lipolysis, we used a commercial infant formula as a more representative protein stabilised formulation. This commercial formulae consisted of the same amount of oil (3.5% w/v) as in Chapter 4 but comprised a different lipid and milk protein (whey and casein) composition. Aliquots sampled during simulated gastric digestion were again analysed using GC and electron microscopy to provide insights as to the separate and combined roles of lipase and pepsin on the rate of digestion and corresponding changes in droplet structure during gastric incubation.

6.2 Materials and Methods

6.2.1 Materials

A scheme of infant formula is shown below.

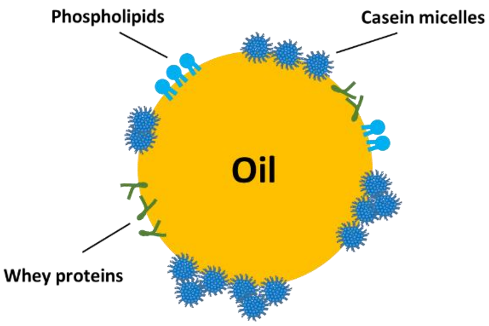


Figure 6.1 The interface of infant formula emulsion droplet which consists of a mixture of milk proteins and phospholipids.

The other chemicals used in this chapter has been described in Chapter 3.1. The ingredient list, and protein and fat information are in Appendix A4-A7.

6.2.2 *In vitro* digestion using a powder infant formula

Infant formula dilution was prepared by adding 36 mL Mill-Q water to 5 g infant formula to 40 mL final emulsion with a 3.6% (w/v) fat as the substrate. It was used as a compared emulsion system to investigate the difference of the behaviours during simulated digestion between infant formula and the Intralipid model emulsion. The ratio of this emulsion and SGF was 20:50 v/v.

The initial digestate was prepared by mixing the diluted infant formula as the substrate, and SGF at a ratio of 20:50 v/v. In this experiment, the volumes of diluted Infant formula and SGF (without the enzymes) were 40 ml and 100 ml, respectively. The mixtures were then kept in the water bath for around 10 min until they reached a temperature of 37 ^{o}C.

The hydrated fungal lipase and porcine pepsin were then added to the mixture, and this was incubated at 37 ^{o}C for 120 min under a stirring rate of 10 rpm. Starting from t = 0 min, and subsequently, at intervals of 15 min, two aliquots (0.5 or 1.0 mL) were taken for gas chromatography, scanning electron or transmitted electron microscopy measurements.

6.2.3 Analysis of lipolysis products by GC

6.2.3.1 Lipid extraction

The extraction process referred to the method in Helbig *et al*. (2012) with small modifications. An aliquot of 0.5 mL sample was taken during the *in vitro* digestion at 0, 15, 30, 45, 60, 90, 105, and 120 min. Each sample was poured into a Kimax tube containing a mix of solvents (1 mL ethanol, 1.5 mL of 1:1 (v/v) diethyl ether and heptane, and 0.1 mL of sulphuric acid (2.5 M)). Each tube was mixed by vortex for about 1 min and centrifuged for another 5 min at 1000 × g at room temperature. The supernatant from each tube was carefully removed and transferred to another Kimax tube. The residual lipidic fraction in the first Kimax tube was extracted with 1 mL of 1:1 (v/v) diethyl ether and heptane again following the previous procedure. The collected supernatant of the two extractions with an addition of 30 μL IS stock solution (20 g/L) was added was brought up to 3 mL with 1:1 (v/v) diethyl ether and heptane. Then 300 mg of anhydrous sodium sulphate was added into each tube to dry any water residue. An amount of 1 mL of the dried supernatant was carefully transferred to a GC sample vial for further analysis. The final concentration of IS was 0.2 g/L in the GC sample vial.

6.2.3.2 GC instrument validation

See Chapter 3.2.4.

6.2.3.3 GC analysis

See Chapter 3.2.4.

6.2.4 Transmission electron microscopy (TEM)

See Chapter 3.2.6.

6.2.5 Statistical Analysis

As confirmed by the use of a standard curve (Chapter 3.2.5), the AUP (mV*min) was in proportion to the concentration of free fatty acids (g/L) in the samples. Accordingly, AUP was used to represent the rate and degree of gastric lipolysis.

Pentadecanoic acid (15:0) was used as the internal standard in this study, and the AUP was corrected relative to the maximum value of the internal standard to compare parameters from different treatments:

$$Corrected\ (AUP) = \frac{AUP(max)_{internal}}{AUP(i)_{internal}} * AUP(i)$$

Where $AUP(max)_{internal}$ is the maximum area under the peak value of the internal standard, $AUP(i)_{internal}$ is the area under the peak value of the internal standard in each run, and $AUP(i)$ is the area under the peak value of the sample run. The increase of free fatty acids was indicated by corrected AUP instead.

Limited data collection over the first 30 min of digestion meant that it was not possible to effectively quantify the kinetics of lipolysis in the early stages of the reaction. To address this issue, linear regression was instead applied to data points between 30 and 120 min.

Although there were traces of free fatty acids in the digestate when no enzymes were present, the amount was negligible as these were at least one order of magnitude below the value at 30 min. In order to modulate the data from 30 min and onwards, we moved the original point of x-axis from 0 to 30 min. Therefore, the interface of the linear regression with the y-axis at 30 min was able to provide an indicator as to the initial rate of lipolysis over the first 30 min of digestion.

In order to compare the differences between each *p*H level when infant formula was digested with lipase only, the Student's t-test was used for statistical analysis. As there were only mean and standard errors, it was assumed that the variances of the population were equal (homogeneity of variances).

6.3 Results

6.3.1 Increase of free fatty acids in simulated gastric digestion

The S–26 powder infant formula showed a *p*H of 7.23, d_{43} of 1.156±0.033 μm, d_{32} of 0.251±0.002 μm, the specific surface area of 25.633±0.115 m^2/g fat and ζ-potential of –36.3±2.3 mV according to the previous study by Luemsaisuk *et al*. (2015).

The extent of lipolysis was calculated based on the internal standard of each run (Figure 6.2). The gastric lipolysis for infant formula was under 40% during 120 min of simulated digestion. The results were similar to studies in the past, which showed 10-30% of gastric lipolysis in neonates (Carriere et al., 1993). Except for *p*H 5.5 digested with lipase and pepsin, the extent of lipolysis increased along with elevated *p*H level. Moreover, the extent of infant formula digested with lipase only was higher than digested with lipase and pepsin.

Evolution of free fatty acids was treated as the marker of the degree of lipid digestion. Validation of results showed consistent responses with good reproducibility from our method and the GC unit. The results were shown in Figure 6.3. The examples of GC chromatogram could be found in the Appendix A8. The statistical analysis showed that the data from digestion a linear relationship with time, except for formula digested at *p*H 4.5 with lipase and pepsin (Table 6.1). This indicated that except for treatment at *p*H 4.5 with lipase and pepsin, all other treatments had a significant increase in free fatty acids with time.

The pair-wise comparison of the probability between treatments was shown in Table 6.2. It could be seen that the FFA increased significantly with time (Figure 6.4). From the probability table (Table 6.2), no significant difference in the slopes was observed between *p*H 3.5 and *p*H 4.5 or *p*H 5.5, or additionally between *p*H 4.5 and *p*H 5.5 in slopes. This indicated that the digestate across the three *p*H conditions showed no significant difference in the rate of lipolysis from 30 min onwards to the end of simulated digestion.

However, for the intercept, all three *p*H levels were significantly different from each other. This indicated that in the first 30 min, the rate of lipolysis would have been different among the three *p*H levels where the rate of *p*H 3.5 [a = 32.287±30.471 mV*min) < *p*H 4.5 (a = 248.715±32.824 mV*min) < (a = 534.458±40.924 mV*min)].

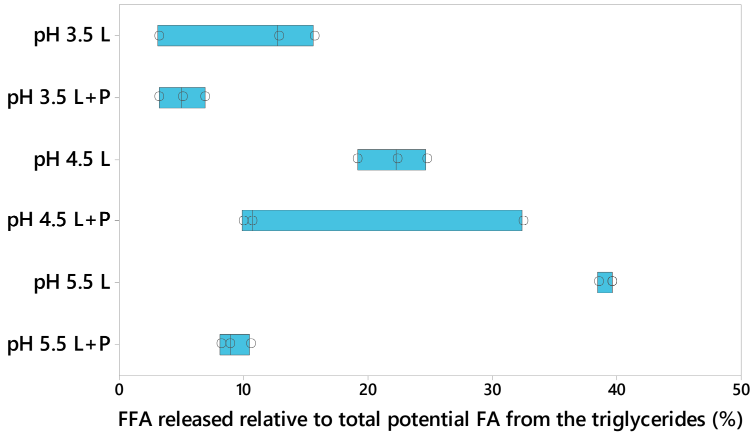


Figure 6.2 FFA released relative to the total potential FA from the triglycerides from an infant formula emulsion digested in a simulated gastric system. L, infant formula digested with lipase alone; L+P, infant formula digested with lipase and pepsin. The *p*H values were all initial *p*H levels.

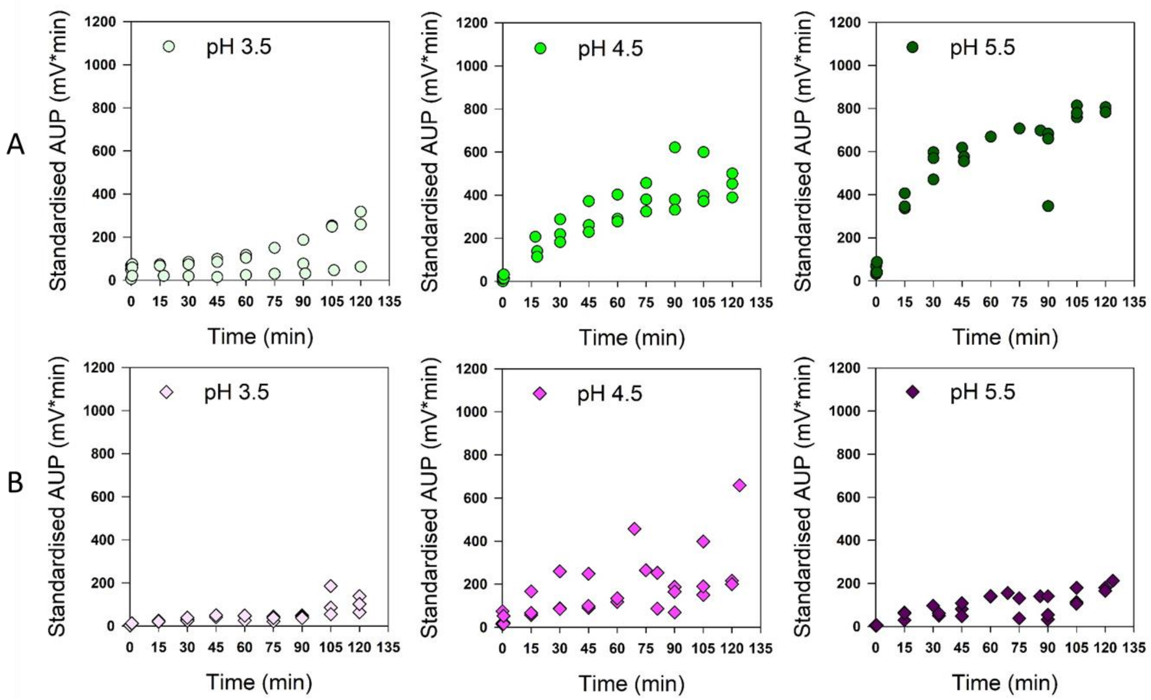


Figure 6.3 Free fatty acid increase showed by standardised AUP (mV*min) for a powder infant formula digested in a simulated gastric system. A, infant formula digested with lipase alone; B, infant formula digested with lipase and pepsin.

Table 6.1 Statistical analysis of linear regression for FFA increase under different treatments.

	T1	**T2**	**T3**	**T4**	**T5**	**T6**
a	38.287	248.715	534.458	20.690	-	67.550
SEM (a)	30.471	32.824	40.924	12.692	-	17.764
b	1.679	2.650	2.607	0.816	-	0.975
SEM (b)	0.558	0.607	0.716	0.235	-	0.322
df	1;18	1;19	1;16	1;19	1;19	1;19
F	9.058	19.068	13.247	12.080	3.583	9.163
Sig.	0.008	<0.005	0.002	0.003	0.074	0.007
R^2	0.335	0.501	0.453	0.389	-	0.325

T1, *p*H 3.5 digested with lipase; T2, *p*H 4.5 digested with lipase; T3, *p*H 5.5 digested with lipase; T4, *p*H 3.5 digested with lipase and pepsin; T5, *p*H 4.5 digested with lipase and pepsin; T6, *p*H 5.5 digested with lipase and pepsin.

a, intercept value, unit mV*min.

SEM (a), standard error of the mean of *a*.

b, slope value, unit mV.

SEM (b), standard error of the mean of *b*.

df, degrees of freedom and residual degrees of freedom.

F, F value.

Sig., *p*-value, α=0.05.

R^2, coefficient of determination.

When infant formula was digested with lipase and pepsin, the degree of lipolysis was lower relative to digestion with lipase alone. At *p*H 4.5, there was no significant increase with time, and accordingly, we were only able to compare coefficients at *p*H 3.5 and 5.5 (Figure 6.5). The slope values at *p*H 3.5 and 5.5 again showed no significant difference. The intercept value of *p*H 5.5 (a = 67.550±17.764 mV*min) was significantly higher than that of *p*H 3.5 (a = 20.690±12.692 mV*min). This indicated at the first 30 min, the rate of lipolysis for the digestate at *p*H 5.5 was slightly higher than the rate at *p*H 3.5. However, after 30 min, the rate of lipolysis was the same.

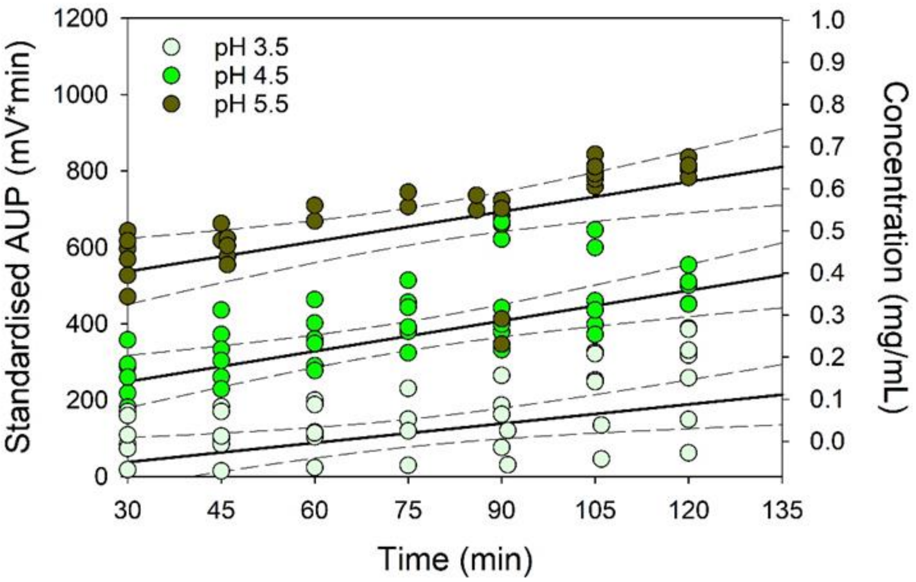


Figure 6.4 Linear regression of FFA increase of infant formula digested with lipase alone in a simulated gastric system. Solid line, regression; dash line, 95% confidence interval.

Table 6.2 Pair-wise comparison of the probability of coefficients of the linear regression of FFA increase in infant formula digested with lipase alone (α=0.05).

Intercept (a)	*p*H 3.5	*p*H 4.5	*p*H 5.5	**Slope (b)**	*p*H 3.5	*p*H 4.5	*p*H 5.5
*p*H 3.5	-	<0.001	<0.001	*p*H 3.5	-	0.247	0.309
*p*H 4.5	-	-	<0.001	*p*H 4.5	-	-	0.963
*p*H 5.5	-	-	-	*p*H 5.5	-	-	-

Furthermore, we were able to compare the intercept and slope values from treatment with lipase alone to the values from treatment with lipase and pepsin using the Student t-test (Figure 6.6). The slope values of digestion at *p*H 3.5 with lipase alone (b = -1.679±0.558 mV) or with lipase and pepsin (b = 0.816±0.235 mV) had no significant difference from digestion at *p*H 5.5 with lipase and pepsin (b = 0.975±0.322 mV), but the slope value of digestion at *p*H 5.5 with lipase (b = 2.607±0.716 mV) was significantly higher than those three treatments. The intercept values of digestion at *p*H 3.5 (with lipase alone) at t = 30 min had no significant difference from digestion at *p*H 3.5 or *p*H 5.5 (with lipase and pepsin). However, there a significant difference was observed between the intercept values of treatment at *p*H 5.5 (with lipase alone) and those of both *p*H 3.5 and *p*H 5.5 (with lipase and pepsin). This indicated that there was no significant difference between the rate of lipolysis for the first 30 min for digestion at *p*H 3.5 with lipase alone (a = 32.287±30.471 mV*min), and digestion at *p*H 3.5 (a = 20.690±12.692 mV*min) or 5.5 with lipase and pepsin (a = 67.550±17.764 mV*min). This indicated from 30 min onwards, rate of lipolysis for digestion at *p*H 3.5 (with lipase alone or with lipase and pepsin) was the same and was very similar to that of digestion at *p*H 5.5 with lipase and pepsin, whereas the rate for digestion at *p*H 5.5 with lipase was faster than the previous three treatments.

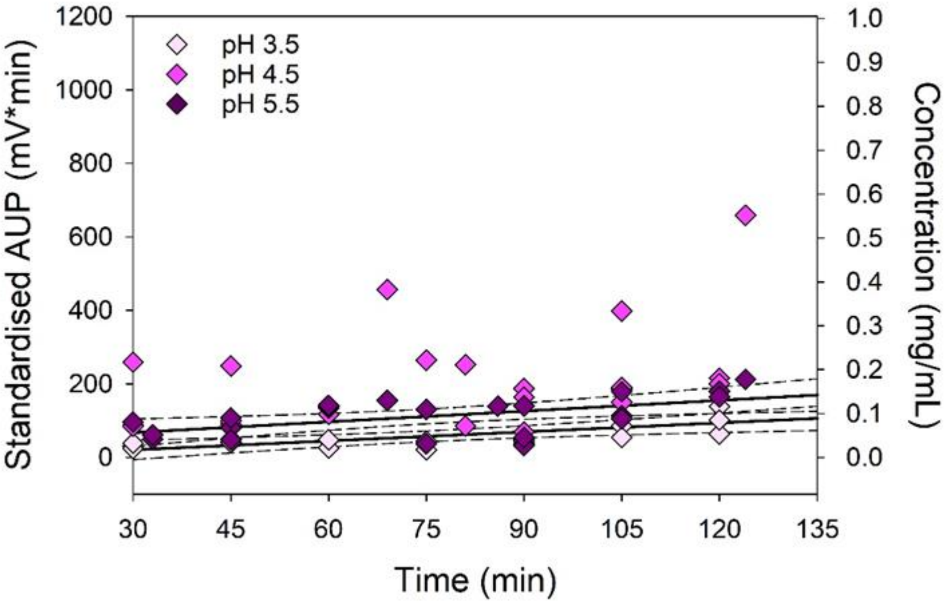


Figure 6.5 Comparison of data of FFA increase of infant formula digested with lipase and pepsin in a simulated gastric system. Solid line, regression; dash line, 95% confidence interval (regression of *p*H 4.5 did not show as the regression was not statistically significant).

Table 6.3 Pairwise comparison of the probability of coefficients of the linear regression of FFA increase in infant formula digested with lipase and pepsin (α=0.05).

Intercept (a)	*p*H 3.5	*p*H 5.5	**Slope (b)**	*p*H 3.5	*p*H 5.5
*p*H 3.5	-	0.038	*p*H 3.5	-	0.692
*p*H 5.5	-	-	*p*H 5.5	-	-

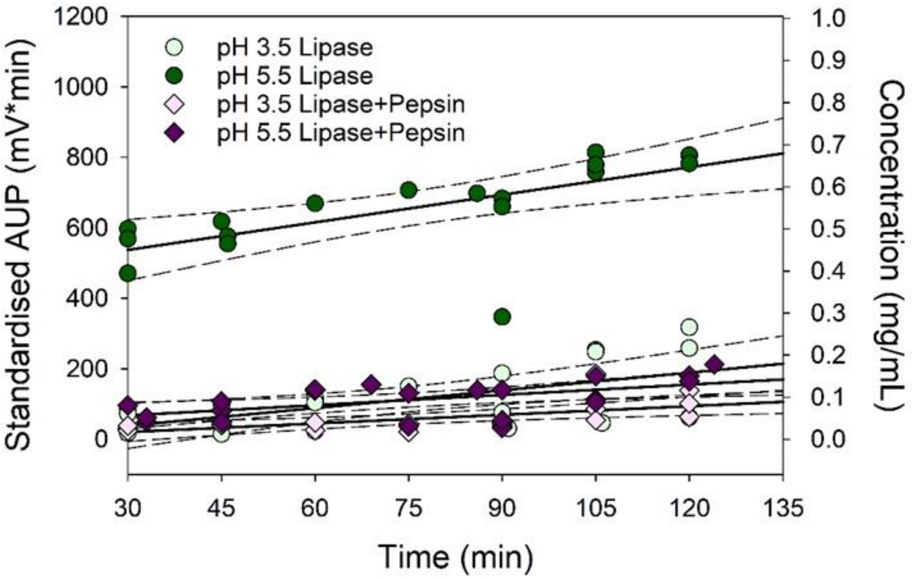


Figure 6.6 Comparison of data of FFA increase of infant formula digested with lipase alone or with lipase and pepsin in a simulated gastric system. Solid line, regression; dash line, 95% confidence interval.

Table 6.4 Pairwise comparison of the probability of coefficients of the linear regression of FFA increase in infant formula digested with lipase alone or with lipase and pepsin (α=0.05).

Intercept (a)	3.5L	5.5L	3.5LP	5.5LP	**Slope (b)**	3.5L	5.5L	3.5LP	5.5LP
3.5L	-	-	-	-	3.5L	-			
5.5L	<0.001	-	-	-	5.5L	0.309	-		
3.5LP	0.591	<0.001	-	-	3.5LP	0.155	0.016	-	
5.5LP	0.406	<0.001	0.038	-	5.5LP	0.275	0.036	0.692	-

3.5L, *p*H 3.5 digested with lipase.

5.5L, *p*H 5.5 digested with lipase.

3.5LP, *p*H 3.5 digested with lipase and pepsin.

5.5LP, *p*H 5.5 digested with lipase and pepsin.

6.3.2 Morphological changes of infant formula during simulated gastric digestion using TEM

The morphological changes of the infant formula emulsion during simulated gastric digestion was shown in Figure 6.7 and 6.8. Figure 6.7 showed the changes for lipase only and Figure 6.8 for lipase and pepsin. The initial structure (t = 0 min) of the infant formula showed an average droplet size of 0.3-0.4 μm with flocculated droplets evident at *p*H 3.5 or 4.5. At *p*H 5.5, the oil droplets displayed less propensity towards flocculation.

When infant formula was digested with lipase alone, the droplet size was observed to have reduced in size, and the droplets became amorphous after 120 min at *p*H 3.5 (Figure 6.7 A2-A5). The original morphology of the oil droplets disappeared at *p*H 4.5 after 120 min of simulated digestion (Figure 6.7 B2-B5). Also, there appeared to be evidence of some filamentous structures after 120 min of simulated digestion at this *p*H level. A *p*H 5.5, the droplets showed a propensity towards aggregations, displaying increasing anisotropy, but not being significantly reduced in particle size (Figure 6.7 C2-C5).

When the infant formula was digested with lipase and pepsin, a considerable change in structure was observed (Figure 6.8). The assembled structure appeared indicative of extensive coalescence of which, surrounded by an interconnected filamentous network, which was attributed to the self-association of hydrolysates of proteins and intact proteins on the interface of oil droplets as well as in the aqueous phase. These structures were seen to be relatively consistent across all *p*H levels studied after 120 min of simulated gastric digestion.

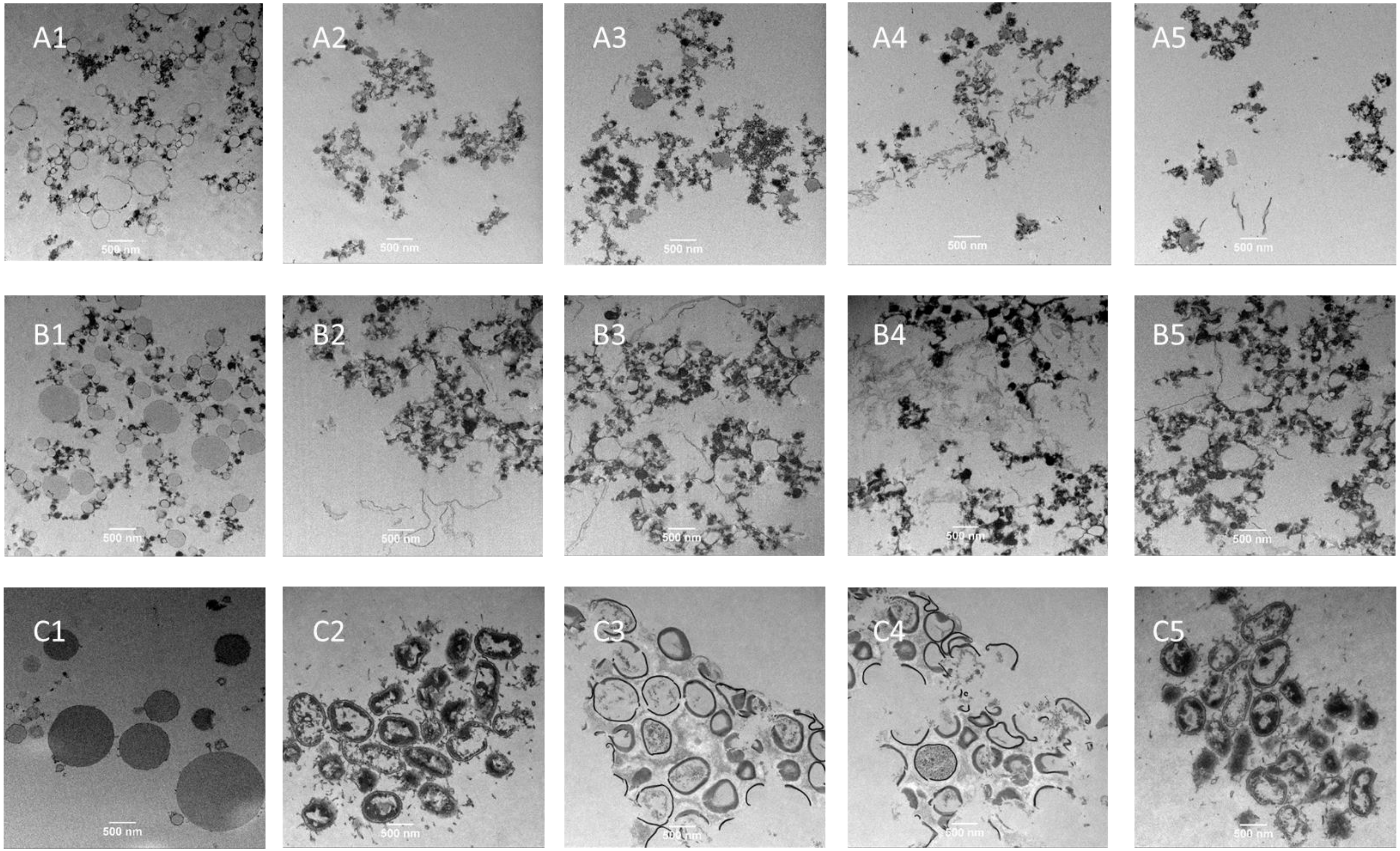


Figure 6.7 Images of TEM of Infant formula digested with lipase alone in a simulated gastric system. A1, *p*H 3.5 at 0 min; A2-A5, *p*H 3.5 at 120 min; B1, *p*H 4.5 at 0 min; B2-B5, *p*H 4.5 at 120 min; C1, *p*H 5.5 at 0 min; C2-C5, *p*H 5.5 at 120 min. Scale bar = 500 nm.

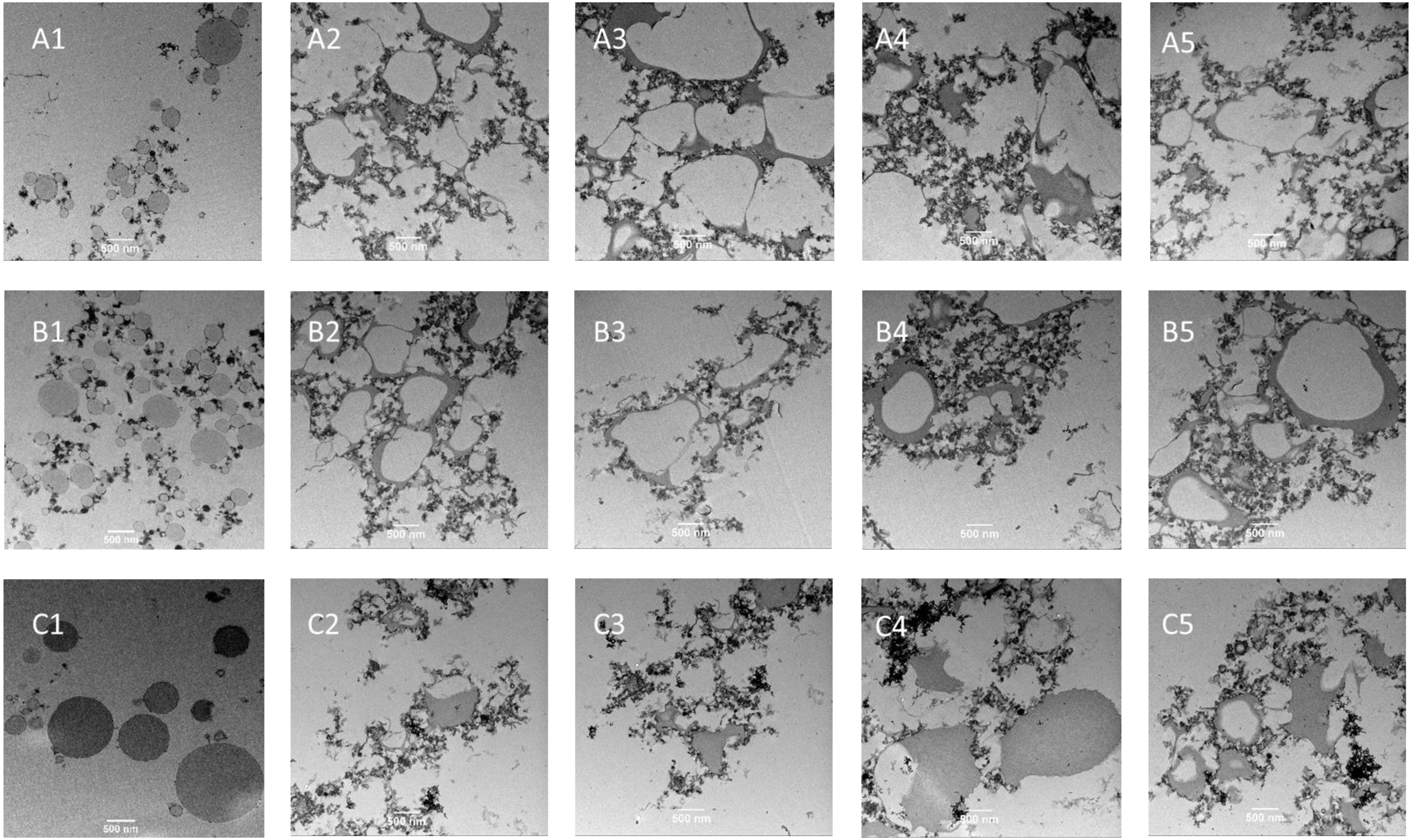


Figure 6.8 Images of TEM of Infant formula digested with lipase and pepsin in a simulated gastric system. A1, *p*H 3.5 at 0 min; A2-A5, *p*H 3.5 at 120 min; B1, *p*H 4.5 at 0 min; B2-B5, *p*H 4.5 at 120 min; C1, *p*H 5.5 at 0 min; C2-C5, *p*H 5.5 at 120 min. Scale bar = 500 nm.

6.4 Discussion

The gastric digestion of formula emulsions was studied under *in vitro* conditions, with the rationale that separate applications of lipase and combined lipase-pepsin would provide an indication of their respective roles in the lipolysis of emulsion droplets. Digestion behaviours, both in terms of generation of fatty acid and emulsion structure were seen to vary according to both *p*H and enzyme combination.

6.4.1 Lipolysis from 0 to 30 min – lipase alone

For emulsion digestion in the absence of pepsin, findings from GC indicated that the rate of lipolysis showed significant difference among three different *p*H conditions for the samples containing lipase in the absence of pepsin, as determined by the intercept values at t = 30 min (Figure 6.4). Noting that the extent of lipolysis increased as *p*H was increased from 3.5 to 5.5, this would suggest that this effect was primarily governed by the relative *p*H activity of the enzyme. As HGL lipase is an acid-stable lipase and possesses an optimal *p*H of 3.0 to 6.0 (Carriere et al., 1991; Gargouri, Sarda, et al., 1986; Roussel et al., 1999; Ville et al., 2002), this allows it to digest triglycerides across a broad spectrum of digestive *p*H, though it does become increasingly less active towards fasting *p*H conditions (Gargouri, Sarda, et al., 1986). In contrast, the fungal gastric lipase used in this study has its optimally active at *p*H 7.0 (pK_a of histidine is 6.5 in its Ser-His-Asp catalytic triad) (Aloulou & Carrière, 2008; Salah et al., 2006). Thus, it is not necessarily surprising that the relative rate of digestion would decline under increasingly acidic conditions, as indicated in Figure 6.5.

The extent of lipolysis observed at *p*H 5.5 also indicates that the proteinaceous interface of the droplets does not appear to be inhibitory to lipase adsorption. Decreasing *p*H towards the isoelectric point might, however, be expected to influence both the interfacial structure of the protein and continuous phase structure which could in turn influence the lipolysis behaviours observed. Figure 5.6 has shown that, at the commencement of digestion, at *p*H 5.5 the emulsion droplets were stable and non-interacting. In contrast, at *p*H 4.5 and 3.5, flocculation of the emulsion was observed. As *p*H approaches the isoelectric point of the proteins, interactions between the adsorbed protein layer of the emulsion droplets and the surrounding serum protein phase could lead to the formation of coagulated protein-droplet structures. It can be speculated that the access of the lipase enzyme is optimised when droplets are non-aggregated, and for which the entirety of the surface is available for lipase adsorption. In contrast, aggregated structures may reduce the availability of binding sites for lipase adsorption. While it is argued that the relative *p*H activity of the enzyme, the predominant effect of extent of lipase during the first 30 min of digestion, and the variations in emulsion structure, as observed in Figure 6.7, may separately impact on the degree of lipolysis during the early stages of digestion.

6.4.2 Lipolysis from 30 to 120 minutes – lipase alone

While significant differences in rates of lipolysis were observed over the first 30 min of digestion, it is interesting to note that the relative rate of change of lipolysis after 30 min through to the end of digestion was shown to be equivalent across all three *p*H conditions.

One further curious observation was that no plateau levels of FFA were observed for any of the samples regardless of relative *p*H over 120 min of digestion. Approximately 10-30% of lipolysis occurs during gastric digestion, with the remainder occurring in the small intestine. The lower the levels of lipolysis in the stomach are usually attributed to the accumulation of FFA at the interface of fat droplets (Pafumi et al., 2002). In the absence of bile salts, which assist in the solubilisation and removal of FFA from the interfacial layer, the accumulation of surface fatty acids ultimately becomes inhibitory to the adsorption of gastric lipase and limits its access to the triglyceride substrate. That no plateau level was observed may be due to fungal, rather than human gastric lipase, being used in the study. Additionally, it may be argued that the reaction has not proceeded to the point of droplet surface saturation by free fatty acids had occurred.

While GC data shows that the rate of increase of FFA to be comparable across all *p*H conditions, noticeable structural differences were observed to the emulsion droplets during this period. At *p*H 3.5 and 4.5, after 120 min of digestion, flocculated structures could still be observed. As has already been discussed, the formation of coagulated protein-fat networks under conditions approached protein *p*I was observed at commencement of digestion. In the absence of pepsin, it seems reasonable that these structures would persist for the duration of incubation, and lipolysis appears to have little influence on the emulsion structure under these conditions.

After 120 minutes, droplets at *p*H 5.5 was also observed to be agglomerated (noting that aggregation was not observed at the onset of digestion). Flocculation under theses *p*H conditions is considered a direct consequence of lipolysis. Based on the observations in Figure 6.7, it is suggested that fatty acid displacement of the adsorbed protein layer of emulsion droplets during lipolysis reduces both charge stabilisation and droplet repulsion. These adsorbed polar lipids can form a variety of crystalline and soft condensed layers at the surface of oil droplets depending on fatty acid types (Pafumi et al., 2002). This surface crystallisation may be a mechanism for the droplet anisotropy observed in Figure 5.6 and is similar to the observations made by Gallier *et al*. (2013) in their study where a lipid-structured phase was observed at the interface of milk fat globules when digested milk in a gastric digestion study conducted in rats. Moreover, such interfaces can be rendered sticky, leading to droplet aggregation as observed by Pafumi *et al*. and others (Gallier et al., 2013; Pafumi et al., 2002).

6.4.3 Lipolysis from 0 to 120 min – lipase and pepsin

The inclusion of pepsin to the gastric fluid was seen to alter the digestion behaviours of the emulsion depending on *p*H conditions. As indicated in the findings, the impact on lipolysis was most noticeable at *p*H 5.5, displaying a considerable decrease in rate and extent of lipolysis during the first 30 min of digestion when compared to the sample digested by lipase alone. At *p*H 3.5, no significant changes were observed relative to the sample with lipase alone, noting that the overall extent of lipolysis was lowest under theses *p*H conditions. At *p*H 4.5, the raw data suggests that inclusion of pepsin results in a reduction in the extent of lipolysis relative to the sample containing lipase alone, however, the high degree of variability across samples at this *p*H makes it difficult to verify this finding.

The observation that the inclusion of pepsin to the simulated gastric fluid appears to reduce the rate and extent of lipolysis (at least at *p*H 5.5) appears contradictory to the initial hypothesis of the research that interfacial pepsinolysis would facilitate lipase adsorption for protein-stabilised emulsions by rendering the adsorbed layer more hydrophobic. There are several possible mechanisms to explain these observed behaviours. The first of these relates to the design of the study, and the use of the fungal lipase as an alternative to human gastric lipase. From a biological viewpoint, human gastric lipase is a highly glycosylated enzyme and is resistant to pepsinolysis, which is essential for it to retain activity during digestion. The use of fungal lipase analogue presents the possibility (as it may not as glycosylated as human gastric lipase) that the lipase is hydrolysed during digestion by the inclusion of pepsin, leading to a loss of activity. While this would certainly account for the generic low extent of lipolysis observed under all *p*H conditions, it would be noted that fungal lipases have been used widely in the *in vitro* studies in combination and speculated to not be compromised in terms of activity due to hydrolysis of the enzyme by pepsin. Additionally, while a significant reduction in the degree of lipolysis was observed at *p*H 5.5 when pepsin is included, the activity of the pepsin should be relative, low at these *p*H conditions, at least likely to affect the functionality of the lipase.

Another possible explanation for the differences in measured results is related to sampling issues during analysis, which in turn can be related to significant changes to emulsion structure during digestion when pepsin was included in the simulated gastric fluid. The sampling method of emulsions for GC analysis was made challenging due to increasing separation of the emulsion during gastric digestion under conditions where both enzymes were present.

This separation is believed to be primarily a consequence of proteolysis of the interfacial layer, which is responsible for the change in structures observed in TEM in Figure 6.8. After 120 min digestion completed, absence of the initial spherical droplet distribution was observed across all three *p*H conditions. Instead, a network of aggregated filaments was observed surrounding highly anisotropic structures. These filaments were attributed as assemblies of protein formed through a combination of *p*H and protein hydrolysis. The darker anisotropic regions within the protein network, most notably at *p*H 5.5, are assumed to be coalescent fat droplets. However, at *p*H 3.5 and 4.5, the voids within the protein network appeared unstained, suggesting an absence of fat. These are interpreted as being ghost structures caused by the increasing destabilisation of the emulsion leading to extensive coalescence and phase separation of oil from with the structures. The observations are supported by previous work using particle size analysis which showed increasing particle size of formula milk during digestion with lipase and pepsin (Lueamsaisuk et al., 2014).

Proteolysis of the interfacial layer could cause detachment of polar polypeptides and retention of hydrophobic domains at the oil/water interface promoting flocculation of droplets, even for *p*H conditions away from the *p*I of the proteins. Secondly, loss of mechanical stabilisation of the interface arising from proteolysis promotes coalescence of droplets which is exacerbated by increased droplet-droplet contact as a result of flocculation.

As stated earlier, the loss of emulsion structure during digestion was problematic in trying to achieve homogeneous sampling throughout incubation. However, it is also worth noting that the extensive coalescence observed in the TEM images (Figure 6.8) would have the effect of decreasing the relative surface area of the emulsion during digestion, thereby reducing the availability of binding sites for lipase adsorption. This would itself contributed to a decrease in relative extent of lipolysis, providing a third possible explanation as to the apparent reduction in overall lipolysis relative to emulsion digested with lipase alone.

6.5 Conclusions

In summary, this study provided further insight into the effect of protein interface in the process of gastric lipolysis by studying a commercial powder infant formula in a simulated gastric system. The results showed that proteins on the interface of the oil droplets do not appear to block lipases from accessing their substrate at the oil core. The rate of lipolysis was dominated by the ambient *p*H level when there was only the presence of lipase in the simulated digestive system. The higher the *p*H, the closer it was to the optimum activity, and the faster the rate could be achieved. When there were pepsin and lipase presented in the digestive system together, pepsin did not act as a facilitator to the protein barrier on the oil/water interface. On the contrary, pepsin could inhibit lipase on the interface as those fungi originated lipase might not be as highly glycosylated as human gastric lipase. Furthermore, proteolysis of the interfacial layer could cause the loss of mechanical strength of the protein interface, which leads to destabilisation of the interface and coalescence of the droplets. Moreover, the coalesced oil droplets could also reduce the active interface for lipases to adsorb and act on their substrates. For our *in vitro* study, since fungal lipase was still used as the analogue of gastric lipase, a replacement of mammal lipases was closer to the stereoselectivity, conformation, and optimal *p*H to human gastric lipase would better confirm those findings.

Chapter 7 Summary and recommendations

7.1 Summary

As has been discussed in the literature review, human studies of infant fat digestion have observed that maternal milk emulsion is more efficiently digested in comparison with infant formulae. There are many potential structural and physiological levers that may contribute to this observation, and it would be unrealistic to attempt to address all of these in a single study. Instead, this study has focussed on the specific events occurring during the gastric stage of digestion. This study aimed to unravel the effect of structural dynamics – in particular, how interfacial layer composition impacts on both proteolysis (in the case of protein stabilised emulsions) and lipase adsorption. Furthermore, as to whether ongoing changes to emulsion structure during digestion arising from either proteolysis and/or lipolysis might further influence the kinetics of lipolysis during gastric incubation. Following these objectives, this study has shown several relevant findings.

In this study, in order to investigate how differences in interfacial composition between maternal milk and infant formulae could lead to different gastric lipolysis outcomes, three different types of interfaces (phospholipid, phospholipid-protein and protein) with same fat content (3.6% w/v, different fat compositions) were investigated. The main difference of the interface was whether there were dairy proteins being partitioned into the interfaces of the oil droplets. As discussed in Chapter 2.5, the initial characteristics of the different interfaces (with or without the partitioning of protein) may change under the impacts of the neonatal gastric fluids, especially the dynamic gastric *p*H. For the monolayer phospholipid interface, the microscopic results in Chapter 5 showed that *p*H did not have an impact on the size or morphological characteristics of the oil droplets. Similar findings were observed in the study of Lueamsaisuk *et al*. (2015) of maternal milk fat droplets that were majorly consisted of phospholipid. This may imply that the acidic environment could have little influence on the relative stability of oil droplets from the maternal milk at the commencement of gastric digestion. When milk proteins were adsorbed at the interface after homogenisation, aggregation of these droplets was induced when the *p*H level approached the *p*I point of the proteins. Protein coated and phospholipid coated simulated milk emulsions already started to show differences at the commencement of gastric digestion. Flocculation of protein-stabilised droplets during digestion was further exacerbated to proteolysis of the interfacial layer. The detachment of interfacial polar domains would be expected to render the interface both more hydrophobic and structurally weaker, leading to greater attraction and increasing incidence of coalescence. Arguably the significant differences in structure development between phospholipid and proteinaceous emulsions might be expected to manifest itself in changes to the relative rate and extent of lipolysis, which was the additional focus of this study.

The study attempted to correlate the kinetics of lipolysis during *in vitro* gastric digestion to structural dynamics. However, confirming this hypothesis was difficult due to challenges in getting statistically significant lipolysis data. While some samples showed good reproducibility (i.e. for fatty acid generation over the incubation period) within sample sets, other compositions showed significant variation within a given sample set. Visual observations showed this to be mainly the case of emulsions that underwent significant destabilisation and phase separation during the digestive process, which made, homogeneous sampling very difficult. However, given that all samples displayed evidence of lipolysis (regardless of the extent of variation within a sample set), it could be argued that the protein coating, either for lactoferrin-coated Intralipid droplets or combined whey/casein in the case of the infant formulae, was not ultimately inhibitory to the adsorption of lipase (even in the absence of pepsin). This is somewhat counter to the original hypothesis of the research, which postulated that the lipolysis of protein-stabilised emulsions may be less effective under conditions for which pepsin is less able to hydrolyse the interfacial layer (such as those in early infancy, and where a combination of lower pepsin activity and higher gastric *p*H conditions may render gastric proteolysis less effective). Curiously enough, the inclusion of pepsin as part of the simulated gastric fluid appeared to induce far greater structural changes (particularly flocculation and coalescence) to the emulsion systems compared to samples where it was excluded. Unfortunately, the large variation in GC data made it difficult to determine the impact of these structural changes on the digestibility of the emulsions.

A final interesting observation of the GC analysis showed that lipolysis did not reach equilibrium during the incubation period. This may be due to insufficient reaction time to saturate the interface with fatty acids that would consequently inhibit further lipase adsorption and lipid hydrolysis as suggested from the study by Pafumi *et al*. (2002).

In conclusion, the research findings presented in this thesis has built the bridge of understanding the relations between the structural dynamics and gastric lipolysis for infant milk with different types of interfaces in a simulated neonatal gastric system. Especially using low shear rate in this study could allow the shear rate in the model more closely simulating the physiological low shear environment in the digestion of human gastric phase compare to high shear rate that were commonly used in other *in vitro* models. The findings also gave new insights into the separate and combined action of digestive enzymes that are crucial for infant fat digestion in the gastric phase. However, several limitations presented in this thesis such as the use of fungal lipase, static pH over time in the model and show that future work is necessary to unveil the influences of interfaces and fat composition of infant milk droplets to the digestibility of fat in the infant gastric phase, the impacts on the lipid digestion in the small intestine and mechanisms in human physiology.

7.2 Recommendations for future work

- Sampling for GC measurement was an ongoing issue across the studies in this thesis. The phase separation due to enzymatic reactions has caused difficulties in sampling and reduced the accuracy of lipolysis data. The large variation in the data points has made the statistical analysis very challenging. Though the sampling issue has not been reported in the previous *in vitro* studies (possibly due to less extreme structural transformations), it is still a key point to resolve in experimental designs, either in terms of the sampling model for GC or in the context of any other analysis that might be useful for determining fatty acid composition.
- Further optimisation of the *in vitro* model may be useful. For example, our simulated gastric digestion has used a very low stirring rate (10 rpm) instead of the commonly-used high stirring rate (100 rpm) in many *in vitro* studies. This low shear rate was much closer to the physiological circumstance in the infant's stomach, which created a much lower shearing force compared to those studies. The concern of the high stirring rate (high shearing forces) is that it could lead to more collisions for the droplets and thin the protein interface (Miller & Möbius, 1998) which may increase the probability for the occurrence of droplet instability. A faster encounter of digestive enzymes to the oil/water interface may also lead to an elevated reaction rate. These two factors may together mislead the interpretation of the results. Ensuring that the mechanical action of samples during simulated digestion is close to physiological equivalents is key to obtain relevant data.

- The use of three types of interfaces in this thesis in order to mimic the difference between infant formulae and maternal milk has provided clues for their impacts on infant gastric fat digestion. However, since the triple-layered structure of human milk fat globule is much more complicated than the artificial-built interfaces for the oil droplets in model emulsions and infant formulae. Studies that involve fresh human breast milk would be still demanding to verify the findings in this thesis.
- Fungal lipase was used as an analogue of human gastric lipase in this thesis due to the difficulty and expenses of sourcing commercial suppliers for recombinant human gastric lipase. However, as discussed in Chapter 2.4, it was not a fully equivalent alternative for human gastric lipase. Examples for better alternatives are lipases from mammal origins such as dog and rabbit gastric lipase, which have been already included in many *in vitro* digestive system designs (Brodkorb et al., 2019; Sams, Paume, Giallo, & Carriere, 2016). The future *in vitro* study designs for gastric lipid digestion should preferably use these lipases instead of lipases from fungal origins.
- Human trials and *in vivo* studies, including direct incubation and collection of gastric content would be needed to support the evidence presented in this thesis. The complications of physiological circumstances in the gastric phase of infants would change the scenario compared to studies conducted *in vitro*.
- Measurement for lipolytic products such as FFA, MG, DG and TG for improved accuracy would be needed in future studies. The GC and ^{1}H NMR methods presented in this thesis have provided possibilities of investigating novel and accurate methodology for the measurement of these compounds. The development of the methodology for monitoring human gastric lipolysis in real-time (including *p*H and lipolytic products) could provide valuable new insights for the impacts of the dynamic environment in the gastric phase on fat digestion. Additional challenges in this context will be ensuring baseline levels of free fatty acids are calculated before digestion and ensuring effective data collection starts with the addition of digestive enzymes (i.e. at t = 0 min), as well as being able to entirely inactive digestive enzymes on the completion of the incubation period.
- The surface mechanisms and surface structural changes during gastric lipolysis are also promising and exciting new areas to be explored in the future. Some studies have shown that gastric lipase exhibited different adsorption behaviours to the interface of milk emulsions (Bourlieu-Lacanal et al., 2015; Bourlieu et al., 2020). Advanced

microscopic methodology such as atomic force microscopy (AFM) could be further developed to investigate the interfacial structure and mechanical properties of lipid digestion in the human gastric phase.

Appendices

A1 Parameters of the linear regression of the normalised FFA increase for the first 30 min for Intralipid and lactoferrin coated Intralipid digestion

	***p*H3.5 CL**	***p*H4.5 CL**	***p*H5.5 CL**	***p*H3.5 CLP**	***p*H4.5 CLP**	***p*H5.5 CLP**	***p*H3.5 LfL**	***p*H4.5 LfL**	***p*H5.5 LfL**	***p*H3.5 LfLP**	***p*H4.5 LfLP**	***p*H5.5 LfLP**
a	-1.124	-1.070	-0.962	-1.151	-1.046	-1.118	-	-	-1.094	-1.137	-0.783	-1.177
SEM (a)	0.102	0.126	0.117	0.060	0.064	0.011	-	-	0.056	0.220	0.194	0.031
b	0.043	0.019	0.016	0.026	0.031	0.029	-	-	0.016	0.047	0.046	0.027
SEM (b)	0.005	0.007	0.007	0.003	0.004	0.001	-	-	0.003	0.011	0.012	0.002
df	1;7	1;9	1;10	1;10	1;10	1;9	1;6	1;9	1;10	1;7	1;8	1;9
F	65.932	6.666	5.546	54.831	67.165	1481.960	0.187	0.173	23.809	17.335	14.075	246.035
Sig.	<0.001	0.030	0.040	<0.001	<0.001	<0.001	0.681	0.688	0.001	0.004	0.006	<0.001
R^2	0.904	0.425	0.357	0.846	0.870	0.994	0.030	0.019	0.704	0.712	0.638	0.965

CL, Intralipid digested with lipase alone.

CLP, Intralipid digested with lipase and pepsin.

LfL, lactoferrin coated Intralipid digested with lipase alone.

LfLP, lactoferrin coated Intralipid digested with lipase and pepsin.

a, intercept value.

SEM (a), standard error of the mean of *a*.

b, slope value.

SEM (b), standard error of the mean of *b*.

df, degrees of freedom and residual degrees of freedom.

F, F value.

Sig., *p*-value, α=0.05.

R^2, coefficient of determination

A2 Pair-wise comparison of the probability of coefficients of the linear regression of FFA increase in Intralipid and lactoferrin coated Intralipid digestion

	***p*H 3.5 CL**	***p*H 4.5 CL**	***p*H 5.5 CL**	***p*H 3.5 CLP**	***p*H 4.5 CLP**	***p*H 5.5 CLP**	***p*H 3.5 LfL**	***p*H 4.5 LfL**	***p*H 5.5 LfL**	***p*H 3.5 LfLP**	***p*H 4.5 LfLP**	***p*H 5.5 LfLP**
***p*H 3.5 CL**	-	0.707	0.673	0.732	0.823	0.740	-	-	0.588	0.957	0.969	0.730
***p*H 4.5 CL**	-	-	0.963	0.897	0.834	0.841	-	-	0.956	0.712	0.726	0.879
***p*H 5.5 CL**	-	-	-	0.854	0.793	0.797	-	-	1.000	0.679	0.692	0.836
***p*H 3.5 CLP**	-	-	-	-	0.916	0.932	-	-	0.812	0.739	0.760	0.980
***p*H 4.5 CLP**	-	-	-	-	-	0.962	-	-	0.742	0.809	0.826	0.926
***p*H 5.5 CLP**	-	-	-	-	-	-	-	-	0.714	0.759	0.785	0.948
***p*H 3.5 LfL**	-	-	-	-	-	-	-	-	-	-	-	-
***p*H 4.5 LfL**	-	-	-	-	-	-	-	-	-	-	-	-
***p*H 5.5 LfL**	-	-	-	-	-	-	-	-	-	0.624	0.648	0.779
***p*H 3.5 LfLP**	-	-	-	-	-	-	-	-	-	-	0.991	0.746
***p*H 4.5 LfLP**	-	-	-	-	-	-	-	-	-	-	-	0.770
***p*H 5.5 LfLP**	-	-	-	-	-	-	-	-	-	-	-	-

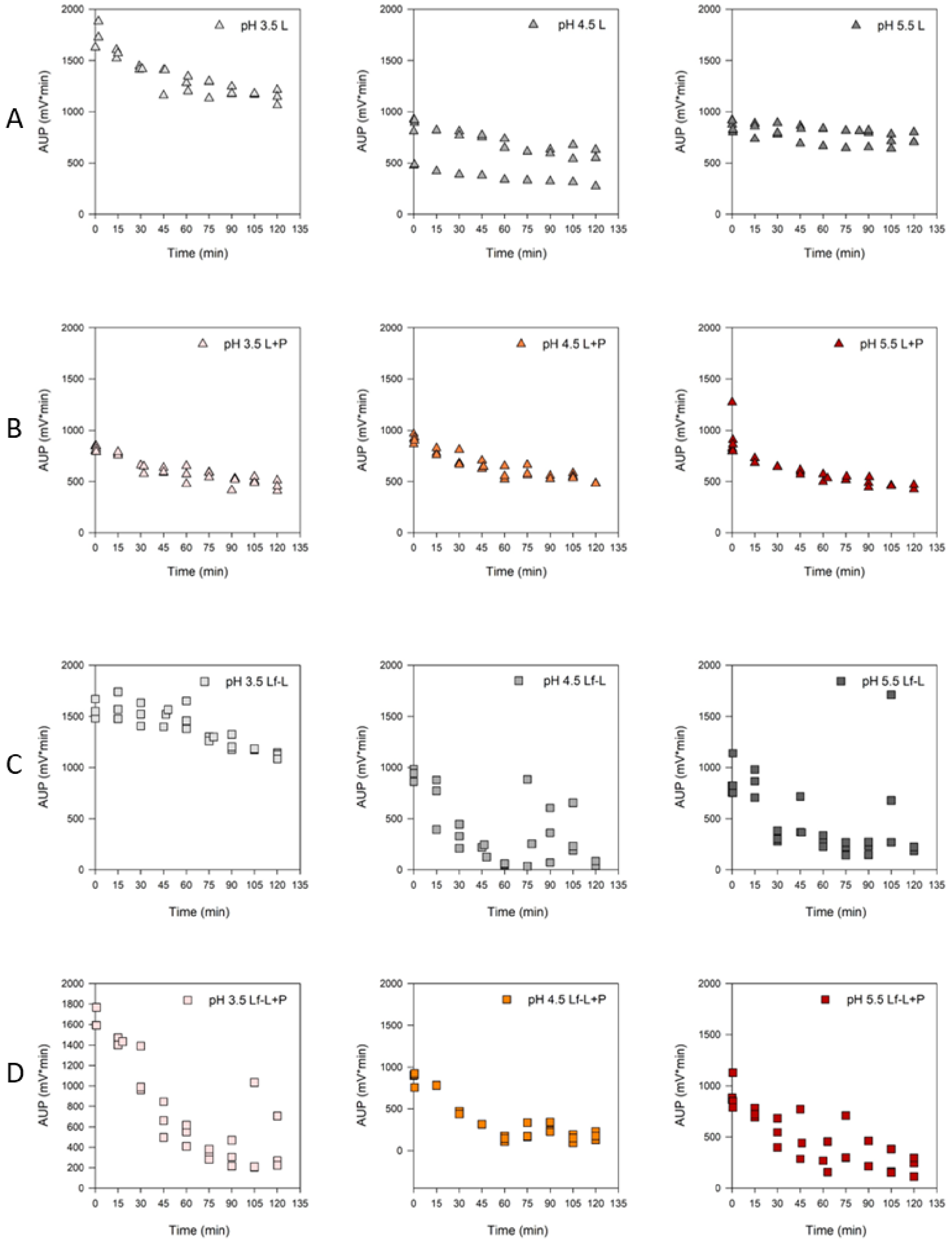
CL, Intralipid digested with lipase alone.

CLP, Intralipid digested with lipase and pepsin.

LfL, lactoferrin coated Intralipid digested with lipase alone.

LfLP, lactoferrin coated Intralipid digested with lipase and pepsin.

A3 Changes in the triglycerides for digestion of a protein emulsion using a simulated neonatal gastric system



A, Intralipid digested with lipase alone (L); B, Intralipid digested with lipase and pepsin (L+P); C, lactoferrin coated Intralipid digested with lipase alone (Lf-L); D, lactoferrin coated Intralipid digested with lipase and pepsin (Lf-L+P)

A4 Ingredient information for S-26® Gold Newborn infant formula

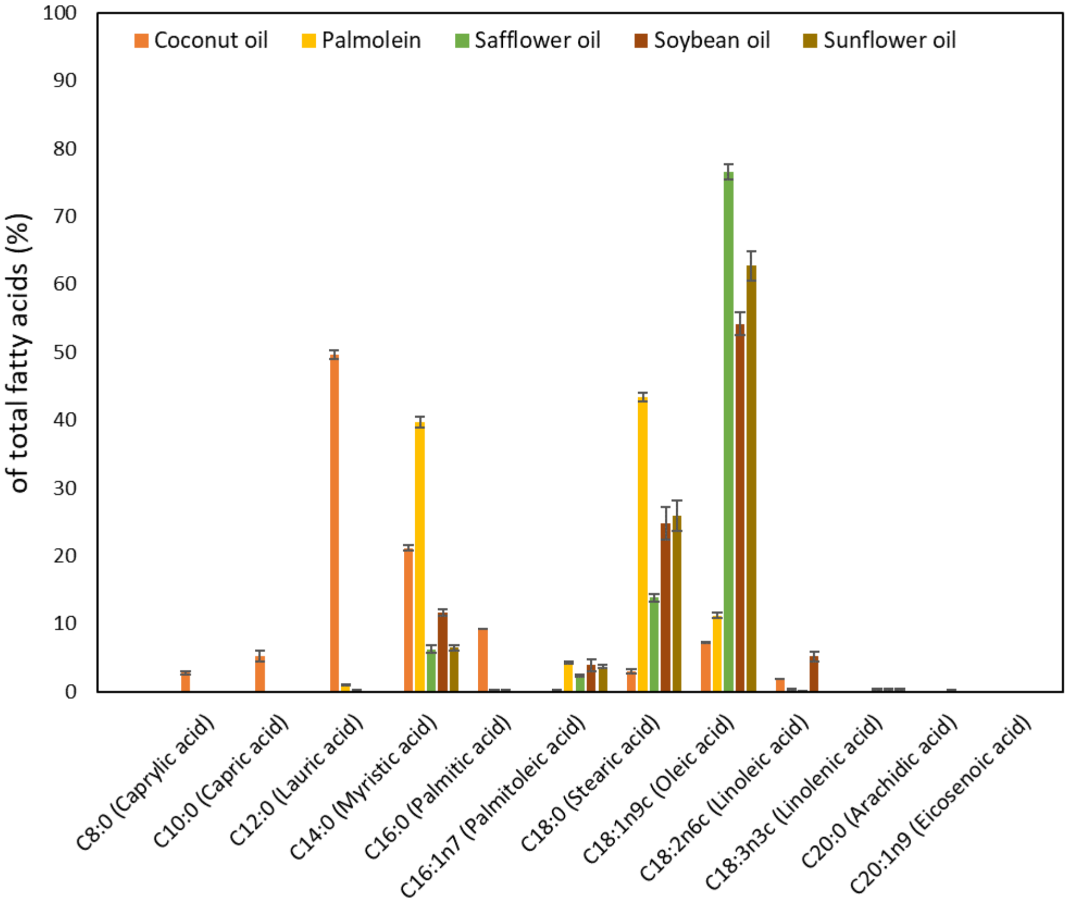
Average as per 100 mL prepared (12.7 g powder + 90 mL water)	
Energy	281 kJ
Protein	1.5 g
Whey protein (60%)	0.9 g
Casein (40%)	0.6 g
Fat	3.6 g
Linoleic acid	580 mg
α-linolenic acid	42 mg
Carbohydrate	7.2

Ingredients
Milk solids, vegetable oils (including soybean), emulsifier (soy lecithin), taurine, nucleotides (cytidine-5′-monophosphate, disodium uridine-5′-monophosphate, adenosine-5′-monophosphate, disodium inosine-5′-monophosphate, disodium guanosine-5′-monophosphate), L-carnitine.

[*] The information in the table was retrieved from https://www.meandmychild.co.nz/products/newborn-to-6-months/s-26-original-newborn/ (04/07/2020).

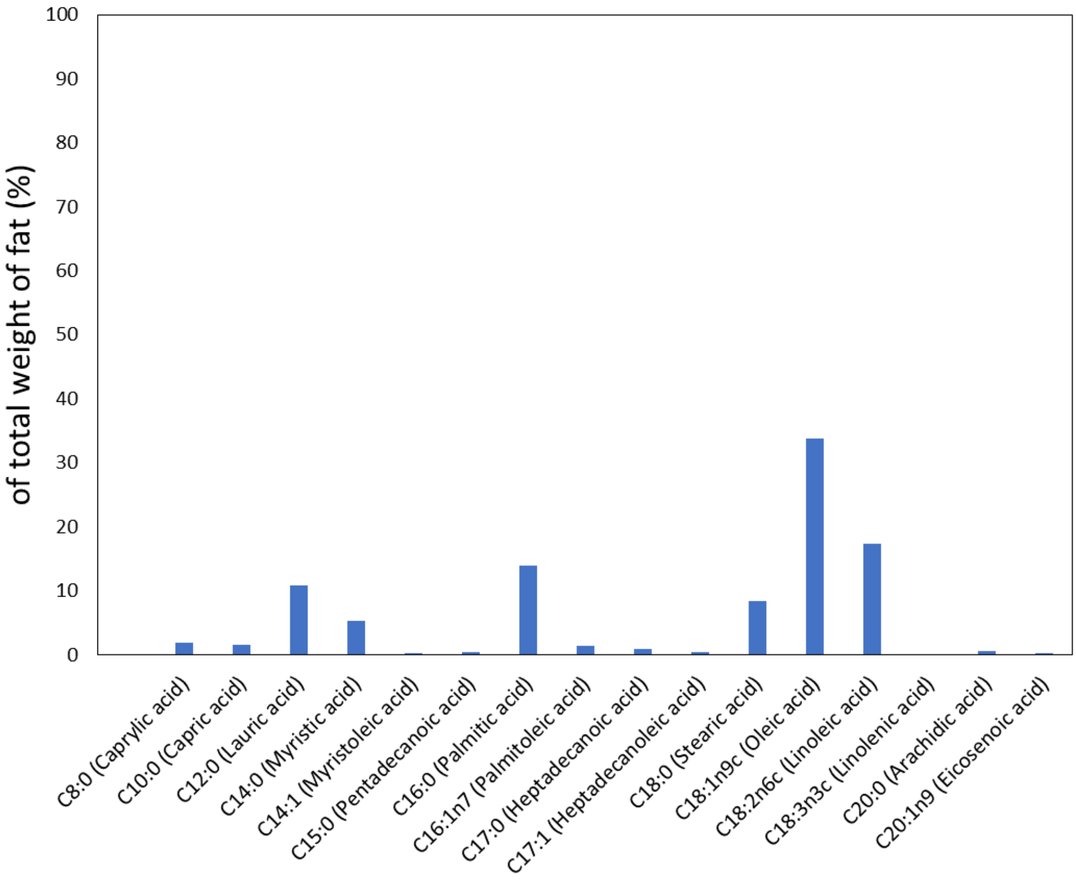
A5 Fatty acid profile of the oil blend of S-26® Gold Newborn infant formula

According to the oil blend on the ingredient label of S-26® Gold Newborn Infant formula, the oil blend was consist of coconut oil, palm oil, soybean oil, sunflower and safflower oil. There was no indication of the percentage of each oil in the oil blend. The figure below showed the fatty acid profile of each oil in the oil blend of this infant formula. Data in this figure was adjusted from Dorni *et al*. (2018).

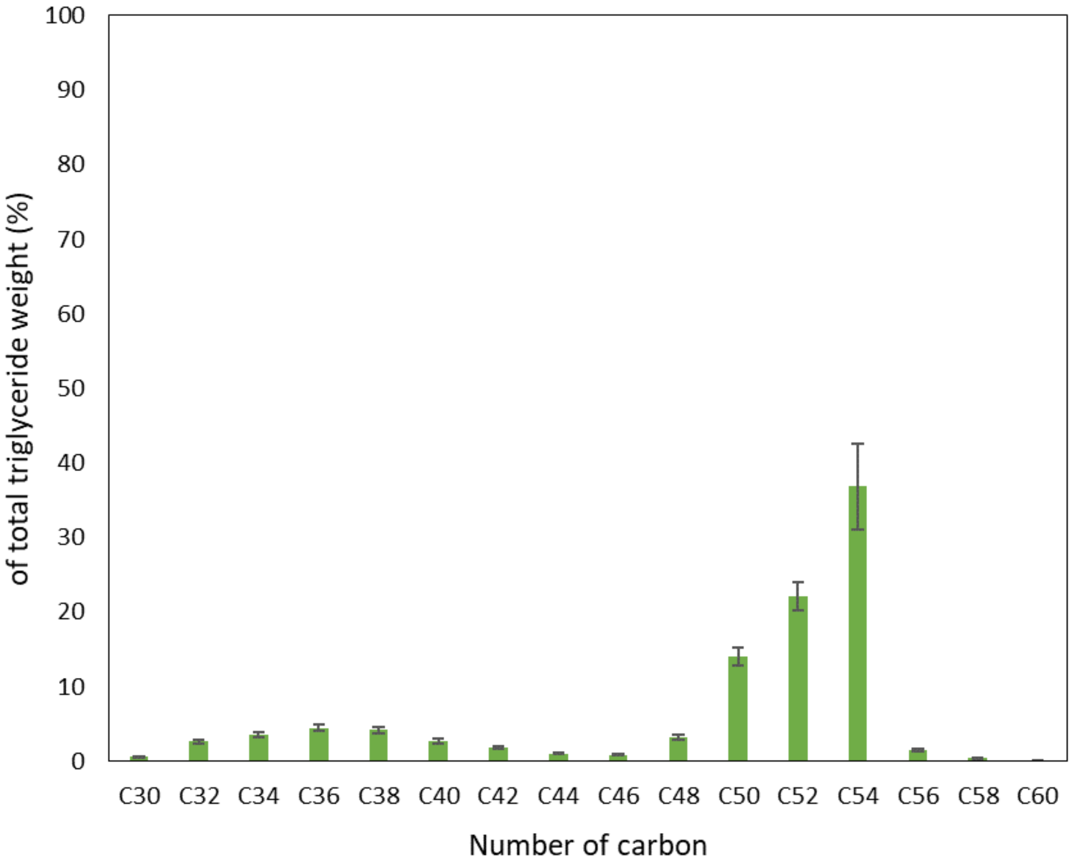


A6 Fatty acid profile of S-26® Gold Newborn infant formula

The figure below shows the fatty acid profile of S-26® Gold Newborn infant formula from Gibson and Kneebone (1981).

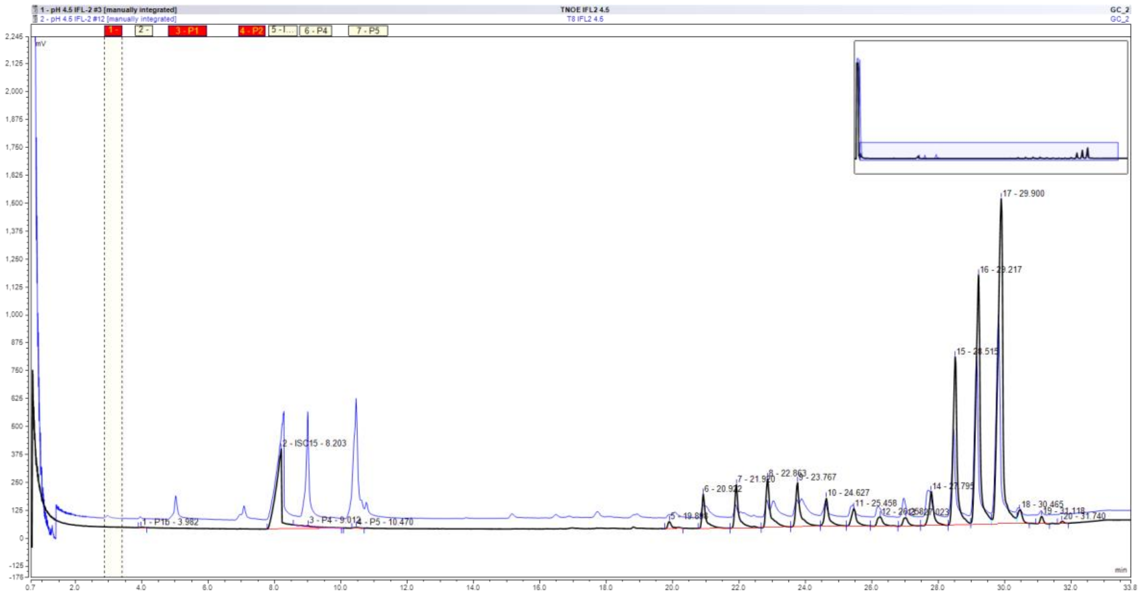


A7 Triglyceride profile of S-26® Gold Newborn infant formula from GC analysis

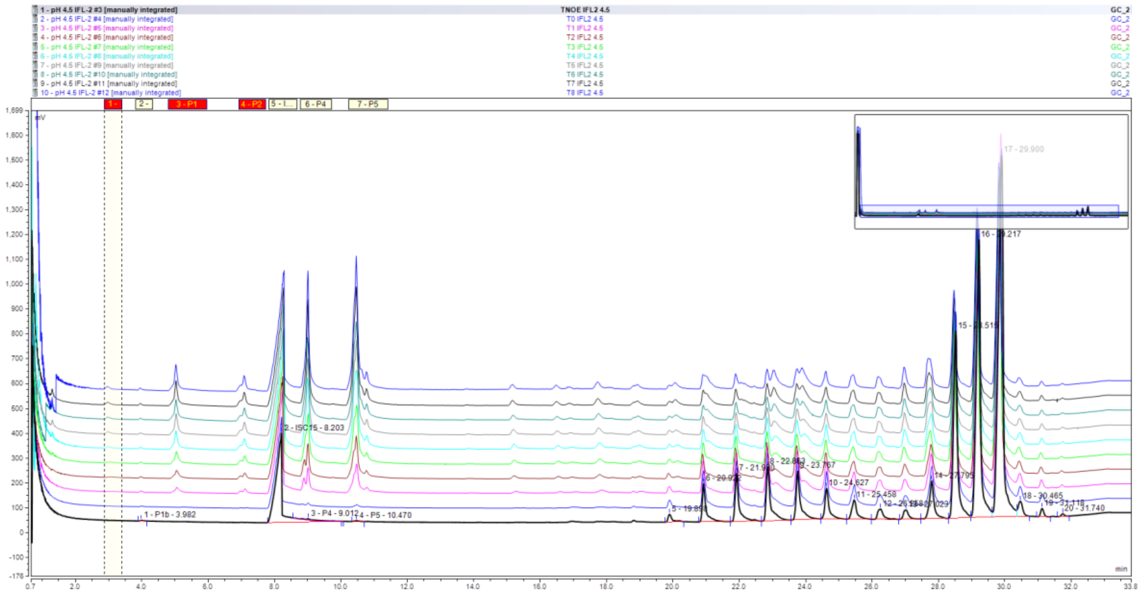


A8 GC chromatogram examples of triglycerides in the digestion of a powder infant formula using a simulated neonatal gastric system

Overlapped chromatograms of infant formula digested with lipase alone at *p*H 4.5 (blue and black lines showed the beginning and the end of simulated digestion).



Overlapped chromatograms of infant formula digested with lipase alone at *p*H 4.5 (blue and black lines showed the beginning and the end of digestion, other colours showed the changes throughout the 120 min simulated digestion).



Bibliography

Abrahamse, E., Minekus, M., van Aken, G. A., van de Heijning, B., Knol, J., Bartke, N., . . . Ludwig, T. (2012). Development of the digestive system—experimental challenges and approaches of infant lipid digestion. *Food Digestion, 3*(1-3), 63-77.

Agunod, M., Yamaguchi, N., Lopez, R., Luhby, A. L., & Glass, G. B. J. (1969). Correlative study of hydrochloric acid, pepsin, and intrinsic factor secretion in newborns and infants. *The American journal of digestive diseases, 14*(6), 400-414.

Aloulou, A., & Carrière, F. (2008). Gastric lipase: An extremophilic interfacial enzyme with medical applications. *Cellular and Molecular Life Sciences, 65*(6), 851-854. https://doi.org/10.1007/s00018-008-7546-z

Armand, M., Borel, P., Dubois, C., Senft, M., Peyrot, J., Salducci, J., . . . Lairon, D. (1994a). *Characterization of emulsions and lipolysis of dietary lipids in the human stomach* (Vol. 266).

Armand, M., Borel, P., Dubois, C., Senft, M., Peyrot, J., Salducci, J., . . . Lairon, D. (1994b). Characterization of emulsions and lipolysis of dietary lipids in the human stomach. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 266*(3), G372-G381.

Armand, M., Borel, P., Pasquier, B., Dubois, C., Senft, M., Andre, M., . . . Lairon, D. (1996a). *Physicochemical characteristics of emulsions during fat digestion in human stomach and duodenum* (Vol. 271).

Armand, M., Borel, P., Pasquier, B., Dubois, C., Senft, M., Andre, M., . . . Lairon, D. (1996b). Physicochemical characteristics of emulsions during fat digestion in human stomach and duodenum. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 271*(1), G172-G183.

Armand, M., Hamosh, M., DiPalma, J. S., Gallagher, J., Benjamin, S. B., Philpott, J. R., . . . Hamosh, P. (1995). Dietary fat modulates gastric lipase activity in healthy humans. *The American journal of clinical nutrition, 62*(1), 74-80.

Armand, M., Hamosh, M., Mehta, N. R., & Angelus, P. A. (1994). Fat digestion: Gastric lipolysis is higher in mother's own milk than in formula-fed very low birth weight infants. *Pediatr Res, 35*(2), 124A.

Armand, M., Hamosh, M., Mehta, N. R., Angelus, P. A., Philpott, J. R., Henderson, T. R., . . . Hamosh, P. (1996). Effect of human milk or formula on gastric function and fat digestion in the premature infant. *Pediatr Res, 40*(3), 429-437.

Armand, M., Pasquier, B., Andre, M., Borel, P., Senft, M., Peyrot, J., . . . Lairon, D. (1999). Digestion and absorption of 2 fat emulsions with different droplet sizes in the human digestive tract. *American Journal of Clinical Nutrition, 70*(6), 1096-1106.

Astwood, J. D., Leach, J. N., & Fuchs, R. L. (1996). Stability of food allergens to digestion in vitro. *Nature Biotechnology, 14*(10), 1269-1273. https://doi.org/10.1038/nbt1096-1269

Ballard, O., & Morrow, A. L. (2013). Human milk composition: Nutrients and bioactive factors. *Pediatric Clinics, 60*(1), 49-74. https://doi.org/10.1016/j.pcl.2012.10.002

Barrett, A. J., Woessner, J. F., & Rawlings, N. D. (2012). *Handbook of proteolytic enzymes* (Vol. 1): Elsevier.

Barrett, K. E., Barman, S. M., & Boitano, S. (2010). *Ganong's review of medical physiology*: New Delhi: McGraw Hill, 2010.

Bauer, E., Jakob, S., & Mosenthin, R. (2005). Principles of physiology of lipid digestion. *Asian-Australasian Journal of Animal Sciences, 18*(2), 282-295.

Berendsen, P. B., & Blanchette-Mackie, E. J. (1979). Milk lipid absorption and chylomicron production in the suckling rat. *The Anatomical Record, 195*(3), 397-412. https://doi.org/10.1002/ar.1091950301

Bergenståhl, B., & Claesson, P. M. (1997). Surface forces in emulsions. In K. Larsson & S. Friberg (Eds.), *Food Emulsions, Third edition* (pp. 57-109). New York: Marcel Dekker Inc.

Bernback, S., & Blackberg, L. (1989). Human gastic lipase - the N-terminal tetrapeptide is enssential for lipid-binding and lipase activity. *European Journal of Biochemistry, 182*(3), 495-499. https://doi.org/10.1111/j.1432-1033.1989.tb14855.x

Bernback, S., Blackberg, L., & Hernell, O. (1990). The complete digestion of human-milk triacylglycerol invitro requires gastric lipase, pancreatic colipase-dependent lipase, and bile-salt stimulated plipase. *Journal of Clinical Investigation, 85*(4), 1221-1226. https://doi.org/10.1172/jci114556

Berton, A., Rouvellac, S., Robert, B., Rousseau, F., Lopez, C., & Crenon, I. (2012). Effect of the size and interface composition of milk fat globules on their in vitro digestion by the human pancreatic lipase: Native versus homogenized milk fat globules. *Food Hydrocolloids, 29*(1), 123-134. https://doi.org/10.1016/j.foodhyd.2012.02.016

Bodmer, M. W., Angal, S., Yarranton, G. T., Harris, T. J. R., Lyons, A., King, D. J., . . . Lowe, P. A. (1987). Molecular cloning of a human gastric lipase and expression of the enzyme in yeast. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression, 909*(3), 237-244. http://dx.doi.org/10.1016/0167-4781(87)90083-2

Borel, P., Armand, M., Ythier, P., Dutot, G., Melin, C., Senft, M., . . . Lairon, D. (1994). Hydrolysis of emulsions with different triglycerides and droplet sizes by gastric lipase in vitro. Effect on pancreatic lipase activity. *The Journal of Nutritional Biochemistry, 5*(3), 124-133. http://dx.doi.org/10.1016/0955-2863(94)90083-3

Bourlieu-Lacanal, C., Madhoueni, W., Paboeuf, G., Desport, C., De Oliveira, S., Pezennec, S., . . . Bouhallab, S. (2015). *Human and bovine milk membrane extracts interfacial behavior and their influence on gastric lipase adsorption.* Paper presented at the 13. Euro Fed Lipid Congress" Fats, Oils and Lipids: New Challenges in Technology, Quality Control and Health".

Bourlieu, C., Dupont, D., Kroell, F., Ménard, O., de Oliveira, S. C., Le Gouar, Y., . . . Pladys, P. (2017). Impact of human milk pasteurization on gastric digestion in preterm infants: A randomized controlled trial. *The American journal of clinical nutrition, 105*(2), 379-390. https://doi.org/10.3945/ajcn.116.142539

Bourlieu, C., Mahdoueni, W., Paboeuf, G., Gicquel, E., Ménard, O., Pezennec, S., . . . Vié, V. (2020). Physico-chemical behaviors of human and bovine milk membrane extracts and their influence on gastric lipase adsorption. *Biochimie, 169*, 95-105. https://doi.org/10.1016/j.biochi.2019.12.003

Bourlieu, C., Menard, O., Bouzerzour, K., Mandalari, G., Macierzanka, A., Mackie, A. R., & Dupont, D. (2014). Specificity of infant digestive conditions: Some clues for developing relevant in vitro models. *Critical Reviews in Food Science and Nutrition, 54*(11), 1427-1457. https://doi.org/10.1080/10408398.2011.640757

Bourlieu, C., Ménard, O., De La Chevasnerie, A., Sams, L., Rousseau, F., Madec, M.-N., . . . Dupont, D. (2015). The structure of infant formulas impacts their lipolysis, proteolysis and disintegration during in vitro gastric digestion. *Food Chemistry, 182*(0), 224-235. http://dx.doi.org/10.1016/j.foodchem.2015.03.001

Bourlieu, C., Rousseau, F., Briard-Bion, V., Madec, M. N., & Bouhallab, S. (2012). Hydrolysis of native milk fat globules by microbial lipases: Mechanisms and modulation of interfacial quality. *Food Research International, 49*(1), 533-544. https://doi.org/10.1016/j.foodres.2012.07.036

Bracken, F. J. (1953). Infant feeding in the American colonies. *Journal of the American Dietetic Association, 29*(4), 349-358.

Brenna, J. T. (2013). Fatty acid analysis by high resolution gas chromatography and mass spectrometry for clinical and experimental applications. *Current Opinion in Clinical Nutrition and Metabolic Care, 16*(5), 548-554. https://doi.org/10.1097/MCO.0b013e328363bc0a

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., . . . Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols* https://doi.org/10.1038/s41596-018-0119-1

Capolino, P., Guérin, C., Paume, J., Giallo, J., Ballester, J.-M., Cavalier, J.-F., & Carrière, F. (2011). In vitro gastrointestinal lipolysis: Replacement of human digestive lipases by a combination of rabbit gastric and porcine pancreatic extracts. *Food Digestion, 2*(1-3), 43-51. https://doi.org/10.1007/s13228-011-0014-5

Carnielli, V. P., Luijendijk, I. H., Van Beek, R. H., Boerma, G. J., Degenhart, H. J., & Sauer, P. J. (1995). Effect of dietary triacylglycerol fatty acid positional distribution on plasma lipid classes and their fatty acid composition in preterm infants. *The American journal of clinical nutrition, 62*(4), 776-781.

Carnielli, V. P., Luijendijk, I. H. T., Van Goudoever, J. B., Sulkers, E. J., Boerlage, A. A., Degenhart, H. J., & Sauer, P. J. J. (1996). Structural position and amount of palmitic acid in infant formulas: Effects on fat, fatty acid, and mineral balance. *Journal of Pediatric Gastroenterology and Nutrition, 23*(5), 553-560.

Carriere, F., Barrowman, J. A., Verger, R., & Laugier, R. (1993). Secretion and contribution to lypolysis of gastric and pancreatic lipases during a test meal in humans. *Gastroenterology, 105*(3), 876-888. https://doi.org/10.1016/0016-5085(93)90908-U

Carriere, F., Moreau, H., Raphel, V., Laugier, R., Benicourt, C., Junien, J.-L., & Verger, R. (1991). Purification and biochemical characterization of dog gastric lipase. *European Journal of Biochemistry, 202*(1), 75-83. https://doi.org/10.1111/j.1432-1033.1991.tb16346.x

Carrière, F., Rogalska, E., Cudrey, C., Ferrato, F., Laugier, R., & Verger, R. (1997). In vivo and in vitro studies on the stereoselective hydrolysis of tri- and diglycerides by gastric and pancreatic lipases. *Bioorganic & Medicinal Chemistry, 5*(2), 429-435. http://dx.doi.org/10.1016/S0968-0896(96)00251-9

Cavell, B. (1983). Postprandial gastric acid secretion in infants. *Acta Pædiatrica, 72*(6), 857-860. https://doi.org/10.1111/j.1651-2227.1983.tb09830.x

Chahinian, H., Snabe, T., Attias, C., Fojan, P., Petersen, S. B., & Carrière, F. (2006). How gastric lipase, an interfacial enzyme with a Ser-His-Asp catalytic triad, acts optimally at acidic pH. *Biochemistry, 45*(3), 993-1001.

Chatterton, D. E. W., Rasmussen, J. T., Heegaard, C. W., Sørensen, E. S., & Petersen, T. E. (2004). In vitro digestion of novel milk protein ingredients for use in infant formulas: Research on biological functions. *Trends in Food Science & Technology, 15*(7), 373-383. https://doi.org/10.1016/j.tifs.2003.12.004

Chen, Q., Bläckberg, L., Nilsson, Å., Sternby, B., & Hernell, O. (1994). Digestion of triacylglycerols containing long-chain polyenoic fatty acids in vitro by colipase-dependent pancreatic lipase and human milk bile salt-stimulated lipase. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 1210*(2), 239-243.

Christie, W. W., & Han, X. (2012). Gas chromatographic analysis of fatty acid derivatives. In W. W. C. Han (Ed.), *Lipid Analysis (Fourth edition)* (pp. 159-180): Woodhead Publishing. http://dx.doi.org/10.1533/9780857097866.159

de Maria, P. D., Fernandez-Alvaro, E., ten Kate, A., & Bargeman, G. (2009). Role of apparent pK a of carboxylic acids in lipase-catalyzed esterifications in biphasic systems. *Journal of Molecular Catalysis B: Enzymatic, 59*(1), 220-224.

de Oliveira, S. C., Bourlieu, C., Ménard, O., Bellanger, A., Henry, G., Rousseau, F., . . . Deglaire, A. (2016). Impact of pasteurization of human milk on preterm newborn in vitro digestion: Gastrointestinal disintegration, lipolysis and proteolysis. *Food Chemistry, 211*, 171-179. https://doi.org/10.1016/j.foodchem.2016.05.028

de Oliveira, S. C., Deglaire, A., Ménard, O., Bellanger, A., Rousseau, F., Henry, G., . . . Bourlieu, C. (2016). Holder pasteurization impacts the proteolysis, lipolysis and disintegration of human milk under in vitro dynamic term newborn digestion. *Food Research International, 88*, 263-275. https://doi.org/10.1016/j.foodres.2015.11.022

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*(5), 436-457.

Dickinson, E. (2001). Milk protein interfacial layers and the relationship to emulsion stability and rheology. *Colloids and Surfaces B: Biointerfaces, 20*(3), 197-210.

Dickinson, E. (2010). Flocculation of protein-stabilized oil-in-water emulsions. *Colloids and Surfaces B: Biointerfaces, 81*(1), 130-140. https://doi.org/10.1016/j.colsurfb.2010.06.033

Dickinson, E., Valerie, J. P., David, S. H., & Frans, A. M. L. (1997). Self-consistent-field modelling of adsorbed casein Interaction between two protein-coated surfaces. *Journal of the Chemical Society, Faraday Transactions, 93*(9), 1785-1790. https://doi.org/10.1039/A608417F

Dorni, C., Sharma, P., Saikia, G., & Longvah, T. (2018). Fatty acid profile of edible oils and fats consumed in India. *Food Chemistry, 238*, 9-15.

Dressman, J. B., Berardi, R. R., Dermentzoglou, L. C., Russell, T. L., Schmaltz, S. P., Barnett, J. L., & Jarvenpaa, K. M. (1990). Upper gastrointestinal (GI) pH in young, healthy men and women. *Pharmaceutical Research, 7*(7), 756-761.

Dupont, D., Mandalari, G., Molle, D., Jardin, J., Léonil, J., Faulks, R. M., . . . Mackie, A. R. (2010). Comparative resistance of food proteins to adult and infant in vitro digestion models. *Molecular Nutrition and Food Research, 54*(6), 767-780. https://doi.org/10.1002/mnfr.200900142

EFSA Panel on Dietetic Products, N., & Allergies. (2013). Scientific opinion on nutrient requirements and dietary intakes of infants and young children in the European Union. *EFSA Journal, 11*(10), 3408. 10.2903/j.efsa.2013.3408

Ergan, F., & André, G. (1989). Simple high performance liquid chromatography methods for monitoring lipase reactions. *Lipids, 24*(1), 76-78.

Fave, G., Coste, T. C., & Armand, M. (2004). Physicochemical properties of lipids: New strategies to manage fatty acid bioavailability. *Cellular and Molecular Biology, 50*(7), 815-831.

Fomon, S. J. (1967). Infant nutrition. *Infant nutrition.*

Fomon, S. J. (2001). Infant feeding in the 20th century: Formula and beikost. *The Journal of Nutrition, 131*(2), 409S-420S. https://doi.org/10.1093/jn/131.2.409S

Fordtran, J. S., & Sleisenger, M. H. (1989). *Gastrointestinal disease: Pathophysiology, diagnosis, management*: WB Saunders.

Friberg, S. (1997). Emulsion stability. In K. Larsson & S. Friberg (Eds.), *Food Emulsions, Third edition* (pp. 1-55). New York: Marcel Dekker Inc.

Gallier, S. (2010). *Understanding the structure of the bovine milk fat globule and its membrane by means of microscopic techniques and model systems.* (Thesis, Doctor of Philosophy), University of Otago, Retrieved from http://hdl.handle.net/10523/450

Gallier, S., Cui, J., Olson, T. D., Rutherfurd, S. M., Ye, A. Q., Moughan, P. J., & Singh, H. (2013). In vivo digestion of bovine milk fat globules: Effect of processing and interfacial structural changes. I. Gastric digestion. *Food Chemistry, 141*(3), 3273-3281. https://doi.org/10.1016/j.foodchem.2013.06.020

Gallier, S., & Singh, H. (2012). Behavior of almond oil bodies during in vitro gastric and intestinal digestion. *Food & Function, 3*(5), 547-555. https://doi.org/10.1039/c2fo10259e

Gallier, S., Ye, A., & Singh, H. (2012). Structural changes of bovine milk fat globules during in vitro digestion. *Journal of Dairy Science, 95*(7), 3579-3592. https://doi.org/10.3168/jds.2011-5223

Garcia, C., Antona, C., Robert, B., Lopez, C., & Armand, M. (2014). The size and interfacial composition of milk fat globules are key factors controlling triglycerides bioavailability in simulated human gastro-duodenal digestion. *Food Hydrocolloids, 35*, 494-504. https://doi.org/10.1016/j.foodhyd.2013.07.005

Gargouri, Y., Julien, R., Bois, A. G., Verger, R., & Sarda, L. (1983). Studies on the detergent inhibition of pancreatic lipase activity. *Journal of Lipid Research, 24*(10), 1336-1342.

Gargouri, Y., Pieroni, G., Rivière, C., Lowe, P. A., Saunière, J., Sarda, L., & Verger, R. (1986). Importance of human gastric lipase for intestinal lipolysis: An in vitro study. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism, 879*(3), 419-423. http://dx.doi.org/10.1016/0005-2760(86)90234-1

Gargouri, Y., Sarda, L., Piéroni, G., Rivière, C., Lowe, P., Ferrato, F., & Verger, R. (1986). Kinetic assay of human gastric lipase on short and long chain triacylglycerol emulsions. In L. Freysz, H. Dreyfus, R. Massarelli, & S. Gatt (Eds.), *Enzymes of Lipid Metabolism II* (Vol. 116, pp. 19-22): Springer US. https://doi.org/10.1007/978-1-4684-5212-9_2

German, J. B., Dillard, C. J., & Ward, R. E. (2002). Bioactive components in milk. *Current Opinion in Clinical Nutrition & Metabolic Care, 5*(6), 653-658.

Gerstenberger, H., Ruh, H., Brickman, M., Leslie, H., & Ochsner, R. (1919). Studies in the adaptation of an artificial food to human milk: II. A report of three year's clinical experience with the feeding of SMA (Syntetic Milk Adapted). *American Journal of Diseases of Children, 17*(1), 1-37.

Gibson, R. A., & Kneebone, G. M. (1981). Fatty acid composition of infant formulae. *Journal of Paediatrics and Child Health, 17*(1), 46-53.

Golding, M. (2013). The colloidal state and its relationship to lipid digestion. *Formulation Engineering of Foods*, 196-232.

Golding, M. (2014). Exploring the relationship between fat surface area and lipid digestibility. In M. Boland, M. Golding, & H. Singh (Eds.), *Food Structures, Digestion and Health* (pp. 145-167). San Diego: Academic Press. http://dx.doi.org/10.1016/B978-0-12-404610-8.00005-0

Golding, M., & Wooster, T. J. (2010). The influence of emulsion structure and stability on lipid digestion. *Current Opinion in Colloid & Interface Science, 15*(1-2), 90-101. https://doi.org/10.1016/j.cocis.2009.11.006

Golding, M., Wooster, T. J., Day, L., Xu, M., Lundin, L., Keogh, J., & Clifton, P. (2011). Impact of gastric structuring on the lipolysis of emulsified lipids. *Soft Matter, 7*(7), 3513-3523. https://doi.org/10.1039/c0sm01227k

Goncharova, K., Pierzynowski, S. G., Grujic, D., Kirko, S., Szwiec, K., Wang, J., . . . Prykhodko, O. (2014). A piglet with surgically induced exocrine pancreatic insufficiency as an animal model of newborns to study fat digestion. *British Journal of Nutrition, 112*(12), 2060-2067. https://doi.org/10.1017/s0007114514003286

Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., & Alric, M. (2012). Relevance and challenges in modeling human gastric and small intestinal digestion. *Trends in Biotechnology, 30*(11), 591-600. http://dx.doi.org/10.1016/j.tibtech.2012.08.001

Gunning, A. P., & Morris, V. J. (2018). Getting the feel of food structure with atomic force microscopy. *Food Hydrocolloids, 78*, 62-76. https://doi.org/10.1016/j.foodhyd.2017.05.017

Guo, M. (2013). *Human milk and infant formula*: Elsevier.

Gurnida, D. A., Rowan, A. M., Idjradinata, P., Muchtadi, D., & Sekarwana, N. (2012). Association of complex lipids containing gangliosides with cognitive development of 6-month-old infants. *Early Human Development, 88*(8), 595-601.

Gusntone, F. D. (1991). ^{13}C-NMR studies of mono-, di-and tri-acylglycerols leading to qualitative and semiquantitative information about mixtures of these glycerol esters. *Chemistry and physics of lipids, 58*(3), 219-224. https://doi.org/10.1016/0009-3084(91)90095-S

Hageman, J. H., Keijer, J., Dalsgaard, T. K., Zeper, L., Carrière, F., Feitsma, A. L., & Nieuwenhuizen, A. G. (2019). Free fatty acid release from vegetable and bovine milk fat-based infant formulas and human milk during two-phase in vitro digestion. *Food & Function*

Hamosh, M. (1979a). Fat digestion in the newborn: Role of lingual lipase and preduodenal digestion. *Pediatric Research, 13*(5), 615-622. https://doi.org/10.1203/00006450-197905000-00008

Hamosh, M. (1979b). A review - fat digestion in the newborn - role of lingual lipase and pre-duodenal digestion. *Pediatric Research, 13*(5), 615-622. https://doi.org/10.1203/00006450-197905000-00008

Hamosh, M. (1990). *Lingual and gastric lipases: Their role in fat digestion*.

Hamosh, M. (1996). Digestion in the newborn. *Clinics in Perinatology, 23*(2), 191-209.

Hamosh, M. (2001). Bioactive factors in human milk. *Pediatric clinics of North America*, *48*(1), 69-+. https://doi.org/10.1016/s0031-3955(05)70286-8

Hamosh, M., Bitman, J., Wood, D. L., Hamosh, P., & Mehta, N. R. (1985). Lipids in milk and the first steps in their digestion. *Pediatrics, 75*(1), 146-150.

Hamosh, M., Iverson, S. J., Kirk, C. L., & Hamosh, P. (1994). Milk lipids and neonatal fat digestion: Relationship between fatty acid composition, endogenous and exogenous digestive enzymes and digestion of milk fat.

Hamosh, M., Peterson, J. A., Henderson, T. R., Scallan, C. D., Kiwan, R., Ceriani, R. L., . . . Hamosh, P. (1999). *Protective function of human milk: The milk fat globule.* Paper presented at the Seminars in Perinatology.

Hamosh, M., Scanlon, J. W., Ganot, D., Likel, M., Scanlon, K. B., & Hamosh, P. (1981). Fat digestion in the newborn: Characterization of lipase in gastric aspirates of premature and term infants. *Journal of Clinical Investigation, 67*(3), 838.

Hansson, L., Bläckberg, L., Edlund, M., Lundberg, L., Strömqvist, M., & Hernell, O. (1993). Recombinant human milk bile salt-stimulated lipase. Catalytic activity is retained in the absence of glycosylation and the unique proline-rich repeats. *Journal of Biological Chemistry, 268*(35), 26692-26698.

Heine, W. E., Klein, P. D., & Reeds, P. J. (1991). The importance of α-lactalbumin in infant nutrition. *The Journal of Nutrition, 121*(3), 277-283.

Helbig, A., Silletti, E., Timmerman, E., Hamer, R. J., & Gruppen, H. (2012). In vitro study of intestinal lipolysis using pH-stat and gas chromatography. *Food Hydrocolloids, 28*(1), 10-19. http://dx.doi.org/10.1016/j.foodhyd.2011.11.007

Henderson, T. R., Hamosh, M., Armand, M., Mehta, N. R., & Hamosh, P. (1998). Gastric proteolysis in the preterm infant: Protein digestion is limited and is not affected by diet, human milk or formula† 580. *Pediatric Research, 43*, 101-101.

Hendricks, G., & Guo, M. (2014). Bioactive components in human milk. *Human milk biochemistry and infant formula manufacturing*, 33-54.

Hernell, O., & Bläckberg, L. (1994). Human milk bile salt-stimulated lipase: Functional and molecular aspects. *The Journal of Pediatrics, 125*(5), S56-S61.

Hernell, O., & Olivecrona, T. (1974a). Human milk lipases II. Bile salt-stimulated lipase. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 369*(2), 234-244.

Hernell, O., & Olivecrona, T. (1974b). Human milk lipases. I. Serum-stimulated lipase. *Journal of Lipid Research, 15*(4), 367-374.

Hileti-Telfer, D. (2003). Infant Foods | Milk formulas. In B. Caballero (Ed.), *Encyclopedia of Food Sciences and Nutrition (Second Edition)* (pp. 3270-3276). Oxford: Academic Press. https://doi.org/10.1016/B0-12-227055-X/00629-5

Hiol, A., Jonzo, M. D., Rugani, N., Druet, D., Sarda, L., & Comeau, L. C. (2000). Purification and characterization of an extracellular lipase from a thermophilic Rhizopus oryzae strain isolated from palm fruit. *Enzyme and Microbial Technology, 26*(5), 421-430.

Hui, D. Y., & Howles, P. N. (2002). Carboxyl ester lipase: Structure-function relationship and physiological role in lipoprotein metabolism and atherosclerosis. *Journal of Lipid Research, 43*(12), 2017-2030. https://doi.org/10.1194/jlr.R200013-JLR200

Huppertz, T., & Kelly, A. (2006). Physical chemistry of milk fat globules. In *Advanced Dairy Chemistry Volume 2 Lipids* (pp. 173-212): Springer.

Hur, S. J., Decker, E. A., & McClements, D. J. (2009). Influence of initial emulsifier type on microstructural changes occurring in emulsified lipids during in vitro digestion. *Food Chemistry, 114*(1), 253-262. https://doi.org/10.1016/j.foodchem.2008.09.069

Hur, S. J., Lim, B. O., Decker, E. A., & McClements, D. J. (2011). In vitro human digestion models for food applications. *Food Chemistry, 125*(1), 1-12. http://dx.doi.org/10.1016/j.foodchem.2010.08.036

Inglingstad, R. A., Devold, T. G., Eriksen, E. K., Holm, H., Jacobsen, M., Liland, K. H., . . . Vegarud, G. E. (2010). Comparison of the digestion of caseins and whey proteins in equine, bovine, caprine and human milks by human gastrointestinal enzymes. *Dairy Science & Technology, 90*(5), 549-563. https://doi.org/10.1051/dst/2010018

Innis, S. M. (1991). Essential fatty acids in growth and development. *Progress in Lipid Research, 30*(1), 39-103.

Innis, S. M. (2004). Polyunsaturated fatty acids in human milk - an essential role in infant development. In L. K. Pickering, A. L. Morrow, G. M. RuizPalacios, & R. J. Schanler (Eds.), *Protecting Infants through Human Milk: Advancing the Scientific Evidence* (Vol. 554, pp. 27-43). New York: Kluwer Academic/Plenum Publ.

Innis, S. M. (2007). Fatty acids and early human development. *Early Human Development, 83*(12), 761-766.

Innis, S. M., & Kuhnlein, H. V. (1988). Long-chain n-3 fatty acids in breast milk of Inuit women consuming traditional foods. *Early Human Development, 18*(2), 185-189.

Ipsen, J. H., Karlström, G., Mourtisen, O. G., Wennerström, H., & Zuckermann, M. J. (1987). Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 905*(1), 162-172.

Ipsen, J. H., Mouritsen, O. G., & Bloom, M. (1990). Relationships between lipid membrane area, hydrophobic thickness, and acyl-chain orientational order. The effects of cholesterol. *Biophysical journal, 57*(3), 405.

Iverson, S. J., Kirk, C. L., Hamosh, M., & Newsome, J. (1991). Milk lipid digestion in the neonatal dog: The combined actions of gastric and bile salt stimulated lipases. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism, 1083*(1), 109-119. http://dx.doi.org/10.1016/0005-2760(91)90131-Z

Jensen, R. G. (1996). The lipids in human milk. *Progress in Lipid Research, 35*(1), 53-92. http://dx.doi.org/10.1016/0163-7827(95)00010-0

Jensen, R. G., Bitman, J., Carlson, S. E., Couch, S. C., Hamosh, M., & Newburg, D. S. (1995a). *Handbook of milk composition*: Academic press.

Jensen, R. G., Bitman, J., Carlson, S. E., Couch, S. C., Hamosh, M., & Newburg, D. S. (1995b). *Human milk lipids* (R. G. Jensen Ed. Vol. 1995). San diego, CA: Academic Press.

Jensen, R. G., Ferris, A. M., & Lammi-Keefe, C. J. (1992). Lipids in human milk and infant formulas. *Annual review of nutrition, 12*(1), 417-441. https://doi.org/10.1146/annurev.nu.12.070192.002221

Jensen, R. G., Hagerty, M. M., & McMahon, K. E. (1978). Lipids of human milk and infant formulas: A review. *The American journal of clinical nutrition, 31*(6), 990-1016.

Johnson, L. R. (2014). *Gastrointestinal physiology* (8 ed.). Philadelphia, PA: Elsevier Mosby.

Johnston, N., Dettmar, P. W., Bishwokarma, B., Lively, M. O., & Koufman, J. A. (2007). Activity/Stability of human pepsin: Implications for reflux attributed laryngeal disease. *The Laryngoscope, 117*(6), 1036-1039. https://doi.org/10.1097/MLG.0b013e31804154c3

Keenan, T. W., & Patton, S. (1995). The milk lipid globule membrane. *Handbook of milk composition*, 5-50.

Kenmogne-Domguia, H. B., Meynier, A., Viau, M., Llamas, G., & Genot, C. (2012). Gastric conditions control both the evolution of the organization of protein-stabilized emulsions and the kinetic of lipolysis during in vitro digestion. *Food & Function, 3*(12), 1302-1309. https://doi.org/10.1039/c2fo30031a

Keogh, J. B., Wooster, T. J., Golding, M., Day, L., Otto, B., & Clifton, P. M. (2011). Slowly and rapidly digested fat emulsions are equally satiating but their triglycerides are differentially absorbed and metabolized in humans. *Journal of Nutrition, 141*(5), 809-815. https://doi.org/10.3945/jn.110.131110

Kim, D. A., Cornec, M., & Narsimhan, G. (2005). Effect of thermal treatment on interfacial properties of β-lactoglobulin. *Journal of colloid and interface science, 285*(1), 100-109. https://doi.org/10.1016/j.jcis.2004.10.044

Kleinman, R. E., Barness, L. A., & Finberg, L. (2003). History of pediatric nutrition and fluid therapy. *Pediatric Research, 54*, 762. https://doi.org/10.1203/01.PDR.0000088018.17598.C5

Koletzko, B., Rodriguez-Palmero, M., Demmelmair, H., Fidler, N., Jensen, R., & Sauerwald, T. (2001). Physiological aspects of human milk lipids. *Early Human Development, 65*, S3-S18. https://doi.org/10.1016/s0378-3782(01)00204-3

Kong, F., & Singh, R. P. (2010). A human gastric simulator (HGS) to study food digestion in human stomach. *Journal of Food Science, 75*(9), E627-E635.

Kunz, C., & Lönnerdal, B. (1992). Re‐evaluation of the whey protein/casein ratio of human milk. *Acta Paediatrica, 81*(2), 107-112.

Landberg, E., Huang, Y., Strömqvist, M., Mechref, Y., Hansson, L., Lundblad, A., . . . Påhlsson, P. (2000). Changes in glycosylation of human bile-salt-stimulated lipase during lactation. *Archives of biochemistry and biophysics, 377*(2), 246-254.

Law, A. J., & Leaver, J. (2000). Effect of pH on the thermal denaturation of whey proteins in milk. *Journal of Agricultural and Food Chemistry, 48*(3), 672-679.

Le, T. T., Van de Wiele, T., Do, T. N. H., Debyser, G., Struijs, K., Devreese, B., . . . Van Camp, J. (2012). Stability of milk fat globule membrane proteins toward human enzymatic gastrointestinal digestion. *Journal of Dairy Science, 95*(5), 2307-2318. https://doi.org/10.3168/jds.2011-4947

Lebenthal, E., & Lee, P. (1980). Development of functional response in human exocrine pancreas. *Pediatrics, 66*(4), 556-560.

Lentle, R. G., & Janssen, P. W. (2011). *The physical processes of digestion*: Springer Science & Business Media.

Lentle, R. G., Janssen, P. W., Goh, K., Chambers, P., & Hulls, C. (2010). Quantification of the effects of the volume and viscosity of gastric contents on antral and fundic activity in the rat stomach maintained ex vivo. *Digestive Diseases and Sciences, 55*(12), 3349-3360.

Li, Y., Hu, M., & McClements, D. J. (2011). Factors affecting lipase digestibility of emulsified lipids using an in vitro digestion model: Proposal for a standardised pH-stat method. *Food Chemistry, 126*(2), 498-505. https://doi.org/10.1016/j.foodchem.2010.11.027

Lien, E. L., Richard, C., & Hoffman, D. R. (2018). DHA and ARA addition to infant formula: Current status and future research directions. *Prostaglandins, Leukotrienes and Essential Fatty Acids, 128*, 26-40. https://doi.org/10.1016/j.plefa.2017.09.005

Lin, Y., Fusek, M., Lin, X., Hartsuck, J. A., Kezdy, F. J., & Tang, J. (1992). pH dependence of kinetic parameters of pepsin, rhizopuspepsin, and their active-site hydrogen bond mutants. *Journal of Biological Chemistry, 267*(26), 18413-18418.

Lindahl, A., Ungell, A.-L., Knutson, L., & Lennernäs, H. (1997). Characterization of fluids from the stomach and proximal jejunum in men and women. *Pharmaceutical Research, 14*(4), 497-502. https://doi.org/10.1023/A:1012107801889

Lolli, V., Dall'Asta, M., Del Rio, D., & Caligiani, A. (2018). In vitro digestibility of cyclopropane fatty acids in Grana Padano cheese: A study combining ^{1}H NMR and GC-MS techniques. *Journal of Food Engineering, 237*, 226-230.

Lönnerdal, B. (1985). Biochemistry and physiological function of human milk proteins. *The American journal of clinical nutrition, 42*(6), 1299-1317.

Lönnerdal, B. (2003). Nutritional and physiologic significance of human milk proteins. *The American journal of clinical nutrition, 77*(6), 1537S-1543S.

Lönnerdal, B. (2014). Infant formula and infant nutrition: Bioactive proteins of human milk and implications for composition of infant formulas. *The American journal of clinical nutrition, 99*(3), 712S-717S. https://doi.org/10.3945/ajcn.113.071993

Lönnerdal, B. (2016a). Bioactive proteins in human milk: Health, nutrition, and implications for infant formulas. *The Journal of Pediatrics, 173*, S4-S9.

Lönnerdal, B. (2016b). Human milk: Bioactive proteins/peptides and functional properties. In *Protein in Neonatal and Infant Nutrition: Recent Updates* (Vol. 86, pp. 97-107): Karger Publishers.

López-López, A., Castellote-Bargalló, A. I., Campoy-Folgoso, C., Rivero-Urgel, M., Tormo-Carnicé, R., Infante-Pina, D., & López-Sabater, M. C. (2001). The influence of dietary palmitic acid triacylglyceride position on the fatty acid, calcium and magnesium contents of at term newborn faeces. *Early Human Development, 65*, S83-S94. https://doi.org/10.1016/S0378-3782(01)00210-9

Lopez, C. (2011). Milk fat globules enveloped by their biological membrane: Unique colloidal assemblies with a specific composition and structure. *Current Opinion in Colloid & Interface Science, 16*(5), 391-404. https://doi.org/10.1016/j.cocis.2011.05.007

Lopez, C., & Menard, O. (2011). Human milk fat globules: Polar lipid composition and in situ structural investigations revealing the heterogeneous distribution of proteins and the lateral segregation of sphingomyelin in the biological membrane. *Colloids and Surfaces B-Biointerfaces, 83*(1), 29-41. https://doi.org/10.1016/j.colsurfb.2010.10.039

Lueamsaisuk, C. (2015). *The dynamics of milk emulsion structure during in vitro neonatal gastric digestion: a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Food Technology at Massey University, Palmerston North, New Zealand.* Massey University,

Lueamsaisuk, C., Lentle, R. G., MacGibbon, A. K. H., Matia-Merino, L., & Golding, M. (2014). Factors influencing the dynamics of emulsion structure during neonatal gastric digestion in an in vitro model. *Food Hydrocolloids, 36*(0), 162-172. http://dx.doi.org/10.1016/j.foodhyd.2013.09.013

Lueamsaisuk, C., Lentle, R. G., MacGibbon, A. K. H., Matia-Merino, L., & Golding, M. (2015). The effect of lactoferrin on physical changes in phospholipid stabilised emulsions during neonatal in vitro gastric digestion: Does synergism of pepsin and lipase promote lipolysis in protein-stabilised emulsions? *Food Hydrocolloids, 43*(0), 785-793. http://dx.doi.org/10.1016/j.foodhyd.2014.08.010

Luo, J., Wang, Z., Li, Y., Chen, C., Ren, F., & Guo, H. (2019). The simulated in vitro infant gastrointestinal digestion of droplets covered with milk fat globule membrane polar lipids concentrate. *Journal of Dairy Science, 102*(4), 2879-2889. https://doi.org/10.3168/jds.2018-15044

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

Madden, M. C., Van Winkle, W. B., Kirk, K., Pike, M. M., Pohost, G. M., & Wolkowicz, P. E. (1993). ^{1}H-NMR spectroscopy can accurately quantitate the lipolysis and oxidation of cardiac triacylglycerols. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 1169*(2), 176-182.

Malaki Nik, A., Wright, A. J., & Corredig, M. (2010). Surface adsorption alters the susceptibility of whey proteins to pepsin-digestion. *Journal of colloid and interface science, 344*(2), 372-381. https://doi.org/10.1016/j.jcis.2010.01.006

Maldonado-Valderrama, J., Gunning, A. P., Ridout, M. J., Wilde, P. J., & Morris, V. J. (2009). The effect of physiological conditions on the surface structure of proteins: Setting the scene for human digestion of emulsions. *The European Physical Journal E, 30*(2), 165-174.

Maldonado-Valderrama, J., Holgado Terriza, J. A., Torcello-Gomez, A., & Cabrerizo-Vilchez, M. A. (2013). In vitro digestion of interfacial protein structures. *Soft Matter, 9*(4), 1043-1053. https://doi.org/10.1039/c2sm26843d

Maldonado-Valderrama, J., Wilde, P. J., Mulholland, F., & Morris, V. J. (2012). Protein unfolding at fluid interfaces and its effect on proteolysis in the stomach. *Soft Matter, 8*(16), 4402-4414.

Manson, W. G., & Weaver, L. T. (1997). Fat digestion in the neonate. *Archives of Disease in Childhood, 76*(3), F206-F211.

Manuel, R. D. (1999). *Calf pregastric lipase-a kinetic study.* (PhD Thesis), The University of Auckland, Retrieved from http://hdl.handle.net/2292/519

Marciani, L., Faulks, R., Wickham, M. S. J., Bush, D., Pick, B., Wright, J., . . . Spiller, R. C. (2009). Effect of intragastric acid stability of fat emulsions on gastric emptying, plasma lipid profile and postprandial satiety. *British Journal of Nutrition, 101*(6), 919-928. https://doi.org/10.1017/s0007114508039986

Marciani, L., Wickham, M. S. J., Singh, G., Bush, D., Pick, B., Cox, E. F., . . . Gowland, P. A. (2007). Enhancement of intragastric acid stability of a fat emulsion meal delays gastric emptying and increases cholecystokinin release and gallbladder contraction. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 292*(6), G1607-G1613.

Martin, C. R., Ling, P.-R., & Blackburn, G. L. (2016). Review of infant feeding: Key features of breast milk and infant formula. *Nutrients, 8*(5), 279. https://doi.org/10.3390/nu8050279

Martini, M., Salari, F., & Altomonte, I. (2016). The macrostructure of milk lipids: The fat globules. *Critical Reviews in Food Science and Nutrition, 56*(7), 1209-1221. https://doi.org/10.1080/10408398.2012.758626

Marze, S., Gaillard, C., & Roblin, P. (2015). In vitro digestion of emulsions: High spatiotemporal resolution using synchrotron SAXS. *Soft Matter, 11*(26), 5365-5373. https://doi.org/10.1039/c5sm01205h

Mason, S. (1962). Some aspects of gastric function in the newborn. *Archives of Disease in Childhood, 37*(194), 387.

McClements, D. J. (2015). *Food emulsions: Principles, practices, and techniques*. Boca Raton: CRC press.

McClements, D. J., Decker, E. A., & Park, Y. (2009). Controlling lipid bioavailability through physicochemical and structural approaches. *Critical Reviews in Food Science and Nutrition, 49*(1), 48-67. https://doi.org/10.1080/10408390701764245

McClements, D. J., & Li, Y. (2010). Review of in vitro digestion models for rapid screening of emulsion-based systems. *Food & Function, 1*(1), 32-59. https://doi.org/10.1039/c0fo00111b

McMurry, J. E., Hoeger, C. A., Ballantine, D. S., & Peterson, V. E. (2012). *Fundamentals of general, organic, and biological chemistry*: Pearson Higher Ed.

McSweeney, S. L., Healy, R., & Mulvihill, D. M. (2008). Effect of lecithin and monoglycerides on the heat stability of a model infant formula emulsion. *Food Hydrocolloids, 22*(5), 888-898.

Mehta, N. R., Jones, J. B., & Hamosh, M. (1982). Lipases in preterm human milk: Ontogeny and physiologic significance. *Journal of Pediatric Gastroenterology and Nutrition, 1*(3), 317-326.

Mellema, M., & Isenbart, J. G. (2004). Effect of Acidification and Heating on the Rheological Properties of Oil-Water Interfaces with Adsorbed Milk Proteins. *Journal of Dairy Science, 87*(9), 2769-2778. https://doi.org/10.3168/jds.S0022-0302(04)73404-9

Ménard, O., Bourlieu, C., De Oliveira, S. C. s., Dellarosa, N., Laghi, L., Carrière, F., . . . Deglaire, A. (2018). A first step towards a consensus static in vitro model for simulating full-term infant digestion. *Food Chemistry, 240*, 338-345.

Ménard, O., Cattenoz, T., Guillemin, H., Souchon, I., Deglaire, A., Dupont, D., & Picque, D. (2014). Validation of a new in vitro dynamic system to simulate infant digestion. *Food Chemistry, 145*(0), 1039-1045. http://dx.doi.org/10.1016/j.foodchem.2013.09.036

Ménard, O., Picque, D., & Dupont, D. (2015). The DIDGI® System. In *The Impact of Food Bioactives on Health* (pp. 73-81): Springer.

Merck KGaA (Producer). (2020). Shigemi 5 mm Symmetrical NMR microtube assembly matched with D2O, bottom length 8 mm. Retrieved from https://www.sigmaaldrich.com/catalog/product/aldrich/z543349?lang=en&region=NZ

Michalski, M. C., Briard, V., Michel, F., Tasson, F., & Poulain, P. (2005). Size distribution of fat globules in human colostrum, breast milk, and infant formula. *Journal of Dairy Science, 88*(6), 1927-1940.

Miled, N., Bussetta, C., De caro, A., Riviere, M., Berti, L., & Canaan, S. (2003). Importance of the lid and cap domains for the catalytic activity of gastric lipases. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 136*(1), 131-138. https://doi.org/10.1016/s1096-4959(03)00183-0

Miled, N., Canaan, S., Dupuis, L., Roussel, A., Rivière, M., Carrière, F., . . . Verger, R. (2000). Digestive lipases: From three-dimensional structure to physiology. *Biochimie, 82*(11), 973-986. http://dx.doi.org/10.1016/S0300-9084(00)01179-2

Miller, R., & Möbius, D. (1998). *Proteins at liquid interfaces*: Elsevier Amsterdam.

Minekus, M. (2015). The TNO Gastro-Intestinal Model (TIM). In K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, & H. Wichers (Eds.), *The Impact of Food Bioactives on Health* (pp. 37-46): Springer International Publishing. https://doi.org/10.1007/978-3-319-16104-4_5

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., . . . Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food - an international consensus. *Food & Function, 5*(6), 1113-1124. https://doi.org/10.1039/c3fo60702j

Mitchell, D., McClure, B., & Tubman, T. (2001). Simultaneous monitoring of gastric and oesophageal pH reveals limitations of conventional oesophageal pH monitoring in milk fed infants. *Archives of Disease in Childhood, 84*(3), 273-276.

Moreau, H., Abergel, C., Carriére, F., Ferrato, F., Fontecilla-Camps, J. C., Cambillau, C., & Verger, R. (1992). Isoform purification of gastric lipases: Towards crystallization. *Journal of Molecular Biology, 225*(1), 147-153. http://dx.doi.org/10.1016/0022-2836(92)91032-K

Motouri, M., Matsuyama, H., Yamamura, J.-i., Tanaka, M., Aoe, S., Iwanaga, T., & Kawakami, H. (2003). Milk sphingomyelin accelerates enzymatic and morphological maturation of the intestine in artificially reared rats. *Journal of Pediatric Gastroenterology and Nutrition, 36*(2), 241-247.

Mouritsen, O. G. (2010). The liquid-ordered state comes of age. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 1798*(7), 1286-1288.

Mu, H. L., & Hoy, C. E. (2004). The digestion of dietary triacylglycerols. *Progress in Lipid Research, 43*(2), 105-133. https://doi.org/10.1016/s0163-7827(03)00050-x

Muller, D., McCollum, J., Trompeter, R., & Harries, J. (1975). Proceedings: studies on the mechanism of fat absorption in congenital isolated lipase deficiency. *Gut, 16*(10), 838-838.

Mun, S., Decker, E. A., Park, Y., Weiss, J., & McClements, D. J. (2006). Influence of interfacial composition on in vitro digestibility of emulsified lipids: Potential mechanism for chitosan's ability to inhibit fat digestion. *Food Biophysics, 1*(1), 21-29.

Ng, S. (2000). Quantitative analysis of partial acylglycerols and free fatty acids in palm oil by ^{13}C nuclear magnetic resonance spectroscopy. *Journal of the American Oil Chemists' Society, 77*(7), 749-755. https://doi.org/10.1007/s11746-000-0120-7

Nguyen, T. T. P., Bhandari, B., Cichero, J., & Prakash, S. (2015). A comprehensive review on in vitro digestion of infant formula. *Food Research International, 76*, 373-386. https://doi.org/10.1016/j.foodres.2015.07.016

Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2014). A method based on ^{1}H NMR spectral data useful to evaluate the hydrolysis level in complex lipid mixtures. *Food Research International, 66*, 379-387.

Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2015). Usefulness of ^{1}H NMR in assessing the extent of lipid digestion. *Food Chemistry, 179*, 182-190. https://doi.org/10.1016/j.foodchem.2015.01.104

Oka, T., & Morihara, K. (1970). Specificity of pepsin: Size and property of the active site. *Febs Letters, 10*(4), 222-224.

Omari, T. I., & Davidson, G. P. (2003). Multipoint measurement of intragastric pH in healthy preterm infants. *Archives of Disease in Childhood - Fetal and Neonatal Edition, 88*(6), F517-F520. https://doi.org/10.1136/fn.88.6.F517

Oosting, A., van Vlies, N., Kegler, D., Schipper, L., Abrahamse-Berkeveld, M., Ringler, S., . . . van der Beek, E. M. (2014). Effect of dietary lipid structure in early postnatal life on mouse adipose tissue development and function in adulthood. *British Journal of Nutrition, 111*(02), 215-226.

Packard, V. S. (2012). *Human milk and infant formula*. New York: Academic Press.

Pafumi, Y., Lairon, D., de la Porte, P. L., Juhel, C., Storch, J., Hamosh, M., & Armand, M. (2002). Mechanisms of inhibition of triacylglycerol hydrolysis by human gastric lipase. *Journal of Biological Chemistry, 277*(31), 28070-28079. https://doi.org/10.1074/jbc.M202839200

Pal, A., Indireshkumar, K., Schwizer, W., Abrahamsson, B., Fried, M., & Brasseur, J. G. (2004). Gastric flow and mixing studied using computer simulation. *Proceedings of the Royal Society of London B: Biological Sciences, 271*(1557), 2587-2594.

Palacios, L. E., & Wang, T. (2005). Egg-yolk lipid fractionation and lecithin characterization. *Journal of the American Oil Chemists' Society, 82*(8), 571-578. 10.1007/s11746-005-1111-4

Porter, C. J., Trevaskis, N. L., & Charman, W. N. (2007). Lipids and lipid-based formulations: Optimizing the oral delivery of lipophilic drugs. *Nature Reviews Drug Discovery, 6*(3), 231-248.

Poulsen, K. R., Snabe, T., Petersen, E. I., Fojan, P., Neves-Petersen, M. T., Wimmer, R., & Petersen, S. B. (2005). Quantization of pH: Evidence for acidic activity of triglyceride lipases. *Biochemistry, 44*(34), 11574-11580.

Prosser, C., Svetashev, V., Vyssotski, M., & Lowry, D. (2010). Composition and distribution of fatty acids in triglycerides from goat infant formulas with milk fat. *Journal of Dairy Science, 93*(7), 2857-2862.

Radbill, S. X. (1981). Infant feeding through the ages. *Clinical Pediatrics, 20*(10), 613-621. https://doi.org/10.1177/000992288102001001

Raikos, V. (2010). Effect of heat treatment on milk protein functionality at emulsion interfaces. A review. *Food Hydrocolloids, 24*(4), 259-265.

Rawlings, N. D., & Salvesen, G. (2013). *Handbook of Proteolytic Enzymes* (3 ed.): Academic Press.

Reddy, I. M., Kella, N. K. D., & Kinsella, J. E. (1988). Structural and conformational basis of the resistance of beta-lactoglobulin to peptic and chymotryptic digestion. *Journal of Agricultural and Food Chemistry, 36*(4), 737-741. https://doi.org/10.1021/jf00082a015

Rogalska, E., Cudrey, C., Ferrato, F., & Verger, R. (1993). Stereoselective hydrolysis of triglycerides by animal and microbial lipases. *Chirality, 5*(1), 24-30. https://doi.org/10.1002/chir.530050106

Rogalska, E., Ransac, S., & Verger, R. (1990). Stereoselectivity of lipases. II. Stereoselective hydrolysis of triglycerides by gastric and pancreatic lipases. *Journal of Biological Chemistry, 265*(33), 20271-20276.

Roman, C., Carriere, F., Villeneuve, P., Pina, M., Millet, V., Simeoni, U., & Sarles, J. (2007). Quantitative and qualitative study of gastric lipolysis in premature infants: Do MCT-enriched infant formulas improve fat digestion? *Pediatr Res, 61*(1), 83-88.

Roudani, S., Miralles, F., Margotat, A., Escribano, M.-J., & Lombardo, D. (1995). Bile salt-dependent lipase transcripts in human fetal tissues. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression, 1264*(1), 141-150.

Roussel, A., Canaan, S., Egloff, M.-P., Rivière, M., Dupuis, L., Verger, R., & Cambillau, C. (1999). Crystal structure of human gastric bipase and model of lysosomal acid lipase, two lipolytic enzymes of medical interest. *Journal of Biological Chemistry, 274*(24), 16995-17002. https://doi.org/10.1074/jbc.274.24.16995

Ruby, M. V., Davis, A., Link, T. E., Schoof, R., Chaney, R. L., Freeman, G. B., & Bergstrom, P. (1993). Development of an in vitro screening test to evaluate the in vivo bioaccessibility of ingested mine-waste lead. *Environmental science & technology, 27*(13), 2870-2877.

Rueda, A., Seiquer, I., Olalla, M., Gimnez, R., Lara, L., & Cabrera-Vique, C. (2014). Characterization of fatty acid profile of argan oil and other edible vegetable oils by gas chromatography and discriminant analysis. *Journal of Chemistry, 2014*, 8. https://doi.org/10.1155/2014/843908

Rüegg, M., & Blanc, B. (1981). The fat globule size distribution in human milk. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 666*(1), 7-14.

Russell, T. L., Berardi, R. R., Barnett, J. L., Dermentzoglou, L. C., Jarvenpaa, K. M., Schmaltz, S. P., & Dressman, J. B. (1993). Upper gastrointestinal pH in seventy-nine healthy, elderly, North American men and women. *Pharmaceutical Research, 10*(2), 187-196.

Sakai, K., Ogawa, M., Takashima, H., Mizunuma, T., & Manabe, S. (1997). Peptic digestibility of proteins in different heat-treated commercial cow's milks. *Journal of Japanese Society of Nutrition and Food Science (Japan)*

Sakai, K., Yoshino, K., Satter, M. A., Ota, F., Nii, Y., Fukuta, K., . . . Yamamoto, S. (2000). Effects of pH variation and NaCl on in vitro digestibility of cow's milk proteins in commercially available infant formulas. *Journal of Nutritional Science and Vitaminology*, *46*(6), 325-328. https://doi.org/10.3177/jnsv.46.325

Salah, R. B., Mosbah, H., Fendri, A., Gargouri, A., Gargouri, Y., & Mejdoub, H. (2006). Biochemical and molecular characterization of a lipase produced by Rhizopus oryzae. *FEMS microbiology letters*, *260*(2), 241-248.

Salentinig, S., Phan, S., Hawley, A., & Boyd, B. J. (2015). Self-assembly structure formation during the digestion of human breast milk. *Angewandte Chemie International Edition*, *54*(5), 1600-1603. https://doi.org/10.1002/anie.201408320

Sams, L., Paume, J., Giallo, J., & Carriere, F. (2016). Relevant pH and lipase for in vitro models of gastric digestion. *Food & Function*, *7*(1), 30-45. https://doi.org/10.1039/c5fo00930h

Sánchez, L., Calvo, M., & Brock, J. H. (1992). Biological role of lactoferrin. *Archives of Disease in Childhood*, *67*(5), 657-661. https://doi.org/10.1136/adc.67.5.657

Sarkar, A., Goh, K. K., & Singh, H. (2009). Colloidal stability and interactions of milk-protein-stabilized emulsions in an artificial saliva. *Food Hydrocolloids*, *23*(5), 1270-1278.

Sarkar, A., Goh, K. K., Singh, R. P., & Singh, H. (2009). Behaviour of an oil-in-water emulsion stabilized by β-lactoglobulin in an in vitro gastric model. *Food Hydrocolloids*, *23*(6), 1563-1569.

Sarkar, A., Goh, K. K. T., & Singh, H. (2010). Properties of oil-in-water emulsions stabilized by β-lactoglobulin in simulated gastric fluid as influenced by ionic strength and presence of mucin. *Food Hydrocolloids*, *24*(5), 534-541. http://dx.doi.org/10.1016/j.foodhyd.2009.12.005

Scheuble, N., Geue, T., Windhab, E. J., & Fischer, P. (2014). Tailored interfacial rheology for gastric stable adsorption layers. *Biomacromolecules*, *15*(8), 3139-3145. https://doi.org/10.1021/bm500767c

Schröder, A., Berton-Carabin, C., Venema, P., & Cornacchia, L. (2017). Interfacial properties of whey protein and whey protein hydrolysates and their influence on O/W emulsion stability. *Food Hydrocolloids, 73*, 129-140. https://doi.org/10.1016/j.foodhyd.2017.06.001

Schulze, K. (2006). Imaging and modelling of digestion in the stomach and the duodenum. *Neurogastroenterology & Motility, 18*(3), 172-183. https://doi.org/10.1111/j.1365-2982.2006.00759.x

Schuman, A. J. (2003). A concise history of infant formula (twists and turns included). *Contemporary Pediatrics, 20*(2), 91-98.

Schwizer, W., Steingoetter, A., & Fox, M. (2006). Magnetic resonance imaging for the assessment of gastrointestinal function. *Scandinavian Journal of Gastroenterology, 41*(11), 1245-1260.

Simonin, C., Rüegg, M., & Sidiropoulos, D. (1984). Comparison of the fat content and fat globule size distribution of breast milk from mothers delivering term and preterm. *The American journal of clinical nutrition, 40*(4), 820-826.

Singh, H. (2006). The milk fat globule membrane—a biophysical system for food applications. *Current Opinion in Colloid & Interface Science, 11*(2-3), 154-163.

Singh, H., & Gallier, S. (2017). Nature's complex emulsion: The fat globules of milk. *Food Hydrocolloids, 68*, 81-89.

Singh, H., & Sarkar, A. (2011). Behaviour of protein-stabilised emulsions under various physiological conditions. *Advances in Colloid and Interface Science, 165*(1), 47-57. https://doi.org/10.1016/j.cis.2011.02.001

Sitohy, M., Chobert, J.-M., Dalgalarrondo, M., & Haertlé, T. (2001). Factors influencing pepsinolysis of methyl-, ethyl-, and propyl-ester derivatives of β-lactoglobulin. *Journal of Food Biochemistry, 25*(3), 181-198. https://doi.org/10.1111/j.1745-4514.2001.tb00733.x

Spyros, A., & Dais, P. (2000). Application of ^{31}P NMR spectroscopy in food analysis. 1. Quantitative determination of the mono- and diglyceride composition of olive oils. *Journal of Agricultural and Food Chemistry, 48*(3), 802-805. https://doi.org/10.1021/jf9910990

Steingoetter, A., Radovic, T., Buetikofer, S., Curcic, J., Menne, D., Fried, M., . . . Wooster, T. J. (2015). Imaging gastric structuring of lipid emulsions and its effect on gastrointestinal function: A randomized trial in healthy subjects. *The American journal of clinical nutrition* https://doi.org/10.3945/ajcn.114.100263

Stevens, E. E., Patrick, T. E., & Pickler, R. (2009). A history of infant feeding. *The Journal of perinatal education, 18*(2), 32-39. https://doi.org/10.1624/105812409X426314

Stipanuk, M. H., & Caudill, M. A. (2013). *Biochemical, physiological, and molecular aspects of human nutrition*: Elsevier Health Sciences.

Talbot, G. (2015). *Specialty oils and fats in food and nutrition: Properties, processing and applications*. Amsterdam: Woodhead Publishing.

Thao, T. P. N., Bhesh, B., Cichero, J., & Sangeeta, P. (2015). Gastrointestinal digestion of dairy and soy proteins in infant formulas: An in vitro study. *Food Research International, 76*(Part 3), 348-358. https://doi.org/10.1016/j.foodres.2015.07.030

Thomson, C. A., Delaquis, P. J., & Mazza, G. (1999). Detection and measurement of microbial lipase activity: A review. *Critical Reviews in Food Science and Nutrition, 39*(2), 165-187.

Thureen, P. J. (2006). *Neonatal nutrition and metabolism*: Cambridge University Press.

Timby, N., Domellöf, E., Hernell, O., Lönnerdal, B., & Domellöf, M. (2014). Neurodevelopment, nutrition, and growth until 12 mo of age in infants fed a low-energy, low-protein formula supplemented with bovine milk fat globule membranes: a randomized controlled trial. *The American journal of clinical nutrition, 99*(4), 860-868.

Tomita, M., Wakabayashi, H., Yamauchi, K., Teraguchi, S., & Hayasawa, H. (2002). Bovine lactoferrin and lactoferricin derived from milk: production and applications. *Biochemistry and Cell Biology, 80*(1), 109-112. 10.1139/o01-230

Trout, G. E., & Fruton, J. S. (1969). Side-chain specificity of pepsin. *Biochemistry, 8*(10), 4183-4190.

Uauy, R., & Castillo, C. (2003). Lipid requirements of infants: Implications for nutrient composition of fortified complementary foods. *Journal of Nutrition, 133*(9), 2962S-2972S.

Uauy, R., Hoffman, D. R., Mena, P., Llanos, A., & Birch, E. E. (2003). Term infant studies of DHA and ARA supplementation on neurodevelopment: Results of randomized controlled trials. *The Journal of Pediatrics, 143*(4), 17-25.

Untersmayr, E., & Jensen-Jarolim, E. (2008). The role of protein digestibility and antacids on food allergy outcomes. *Journal of Allergy and Clinical Immunology, 121*(6), 1301-1308.

Veeraragavan, K. (1990). A simple and sensitive method for the estimation of microbial lipase activity. *Analytical Biochemistry, 186*(2), 301-305.

Venable, J. H., & Coggeshall, R. (1965). A simpllified lead citrate stain for use in electron microscopy. *The Journal of Cell Biology, 25*(2), 407. https://doi.org/10.1083/jcb.25.2.407

Versantvoort, C., & Rompelberg, C. (2004). Development and applicability of an in vitro digestion model in assessing the bioaccessibility of contaminants from food.

Versantvoort, C. H., Oomen, A. G., Van de Kamp, E., Rompelberg, C. J., & Sips, A. J. (2005). Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. *Food and Chemical Toxicology, 43*(1), 31-40.

Vié, V., Paboeuf, G., Cavalier, J.-F., Fanny, G. H., Deglaire, A., Bouhallab, S., . . . Bourlieu-Lacanal, C. (2015). *Mechanism of adsorption of gastric lipase in model membranes of milk fat globules.* Paper presented at the 13. Euro Fed Lipid Congress" Fats, Oils and Lipids: New Challenges in Technology, Quality Control and Health".

Ville, E., Carriere, F., Renou, C., & Laugier, R. (2002). Physiological study of pH stability and sensitivity to pepsin of human gastric lipase. *Digestion, 65*(2), 73-81. https://doi.org/10.1159/000057708

Vlahov, G. (1996). Improved quantitative^{13}C nuclear magnetic resonance criteria for determination of grades of virgin olive oils. The normal ranges for diglycerides in olive oil. *Journal of the American Oil Chemists' Society, 73*(9), 1201-1203. https://doi.org/10.1007/bf02523385

Walstra, P. (1999). *Dairy technology: Principles of milk properties and processes*: CRC Press.

Wargo, W. F. (2016). The history of infant formula: Quality, safety, and standard methods. *Journal of Aoac International, 99*(1), 7-11.

Wegman, M. E. (2001). Infant mortality in the 20th century, dramatic but uneven progress. *The Journal of Nutrition, 131*(2), 401S-408S.

Wells, J. (1996). *Nutritional considerations in infant formula design.* Paper presented at the Seminars in Neonatology.

WHO. (2001). *Report of the expert consultation of the optimal duration of exclusive breastfeeding, Geneva, Switzerland, 28-30 March 2001*. World Health Organization.

Wickes, I. G. (1953). A history of infant feeding. *Archives of Disease in Childhood, 28*(139), 232. https://doi.org/10.1136/adc.28.139.232

Wilde, P., Mackie, A., Husband, F., Gunning, P., & Morris, V. (2004). Proteins and emulsifiers at liquid interfaces. *Advances in Colloid and Interface Science, 108*, 63-71.

Wildman, R. E., & Medeiros, D. M. (1999). *Advanced human nutrition*: CRC press.

Xu, X., & Akoh, C. (2002). *Enzymatic production of Betapol and other specialty fats*. New York: Marcel Dekker, Inc.

Ye, A., Cui, J., & Singh, H. (2011). Proteolysis of milk fat globule membrane proteins during in vitro gastric digestion of milk. *Journal of Dairy Science, 94*(6), 2762-2770.

Zhu, X., Ye, A., Verrier, T., & Singh, H. (2013). Free fatty acid profiles of emulsified lipids during in vitro digestion with pancreatic lipase. *Food Chemistry*, *139*(1–4), 398-404. http://dx.doi.org/10.1016/j.foodchem.2012.12.060

Zou, X., Ali, A. H., Abed, S. M., & Guo, Z. (2017). Current knowledge of lipids in human milk and recent innovations in infant formulas. *Current Opinion in Food Science*, *16*, 28-39. https://doi.org/10.1016/j.cofs.2017.06.010