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

Chalida Lueamsaisuk

2015

Summary

Efficient fat digestion is an essential part of neonatal development. In this respect, it is noteworthy that the process by which infants digest fat differs from that in adults; key differences include the immaturity of the pancreatic function and elevated gastric pH. The digestion of emulsified lipids may accordingly be rendered less efficient in ambient conditions in the infant gastric lumen. For example, it may be postulated that covariation in optimal conditions of proteolytic and lipolytic digestion may differently affect the digestion and disruption of the droplet membrane, the interfacial accessibility of lipase and the subsequent fatty acid production.

Differences between formulated emulsion structures may therefore influence the rate of digestion; previous human studies have indicated that infants digest formula feeds more slowly than they do breast milk (Splinter and Schreiner, 1999). To further explore this observation, the lipid digestion of native biological milk (human breast milk), commercial infant formulae (liquid and powder), and model emulsions (Intralipid® containing lactoferrin) were investigated in an *in vitro* gastric system. The aim was to gain a better understanding on the changes in emulsion structure and fat digestibility with various interfacial layers and pH environments under simulated gastric conditions.

The introduction and a rationale for the focus of this thesis are shown in **Chapter 1**. **Chapter 2** gives a critical overview and review of the literature pertaining to this thesis, and presents possible explanations of how the properties of milk fat globules and their membranes are related to the digestion outcome in the digestive system of

infants. The review also examines the effects of physicochemical factors on emulsion stability. Then, **Chapter 3** presents the general materials and methods used in the experimental work.

The first experimental design is described in **Chapter 4.** This chapter compares the characteristics and physicochemical properties of different types of milks. Infant formulae are prepared from cow's milk and designed to mimic human milk as much as possible. However, even with the advances of technology, there are still differences observed between the breast milk and commercial infant formulae. Therefore the microstructure, droplet size and droplet charge of these different types of milk (human milk, raw cow's milk, commercial liquid formulae and commercial powder formulae) were examined before studying the emulsion digestibility under simulated infant physiological conditions.

Chapter 5 gives a description on how digestion affects emulsion structure of a typical formula emulsion at different pH levels (2–5.5) in an *in vitro* system that replicates the shear rates that would normally be encountered in the infant stomach. The system is designed to simulate infant gastric conditions using different combinations of porcine pepsin and fungal lipase (*Rhizopus oryzae*). Thus, digestion in the presence and absence of proteolytic and lipolytic enzymes was evaluated by observing changes in microstructure, particle size and surface charge.

In liquid infant formulae, droplet size increased progressively by coalescence during *in vitro* digestion at pHs between 3.5 and 4.5 when both lipase and protease were present, but not when either enzyme was omitted or when pH levels were outside this range. Coalescence was augmented by shear, notably at rates above the normal

physiological range. The fidelity of *in vitro* systems did not appear to be compromised by the use of fungal lipases but compromised by the use of inappropriately high stirring rates. The stability and structural properties of formula emulsions appeared to be influenced by disruption of the proteinaceous oil/water interface during digestion, being most susceptible to the concerted activity of pepsin and gastric lipase over a limited range of pH. Given that the onset of secretion of pepsin, lipase and hydrochloric acid does not occur synchronously in the developing infant stomach, inappropriately formulated milks may lower digestive efficiency.

Chapter 6 progresses the findings from chapter 5, employing a model phospholipid–stabilised emulsion which was digested alone, and in combination with the milk protein lactoferrin. It was postulated that the lactoferrin would form an electrostatic layer-on-layer complex with the phospholipid allowing comparison to be made between digestion of the phospholipid–stabilised emulsion and the emulsion stabilised by lactoferrin–phospholipid complex.

Lipolysis of untreated Intralipid®, as evidenced by the increase in droplet size i.e. d_{43} and by confocal microscopy, took place at pH levels between 3.5 and 5.5. Coalescence was evident with lipase alone and with mixtures of pepsin and lipase at pH 3.5, but did not occur in the presence of pepsin alone. Conversely, no coalescence was evident on digestion of Intralipid® treated with lactoferrin, with lipase alone at pH levels below 5.5. However, coalescence of droplets in treated Intralipid® did take place at pH levels above 2 when both pepsin and lipase were present. Changes in surface potential indicated that interfacial proteolysis was required for lipase-mediated coalescence to occur. Findings indicated that the

interaction of lactoferrin with the oil/water interface of soybean oil droplets may have inhibited the action of lipase pending digestion by pepsin.

The findings of Chapter 5 and 6 demonstrate the co-dependent role of proteolytic and lipolytic enzymes on the stability of emulsions during digestion, and the contribution of pH on enzymatic function. This knowledge should be a key factor for the design of emulsion structures in infant formula emulsions.

Chapter 7 describes how digestion affects the structure of human breast milk. Fat droplets showed no significant propensity towards flocculation and aggregation during incubation both with and without either enzyme at all pH. Additionally, the breast milk emulsion was seen to be resistant to coalescence across all pH's and enzymatic conditions studied. The difference in structural behaviour is attributed to variance in lipid composition of the MFG relative to the emulsion systems studied in chapters 5 and 6. Accordingly, it is suggested that the by-products of lipolysis of the breast milk emulsion may serve to stabilise droplets rather than cause instability. Thus the MFGM of maternal milk is not considered inhibitory to the action of either of these two enzymes (porcine pepsin and fungal lipase) under *in vitro* simulation of infant gastric conditions.

Chapter 8 describes the overall conclusions and addresses the major findings and recommendations for future work.

Acknowledgements

I would like to express the deepest appreciation to my supervisor Professor Matt Golding: he continually and persuasively conveyed a spirit of adventure in regard to research and useful critiques of this research work. I can't say thank him enough for his tremendous support and help. I would like to thank my co-supervisors, Professor Roger G. Lentle, Dr. Lara Matia-Merino and Dr. Alastair MacGibbon, whose work demonstrated to me that concern for global affairs supported by an "engagement" in comparative literature and modern technology, should always transcend academia and provide a quest for our times. Without their supervision and constant help this dissertation would not have been possible.

I would like to thank the Fonterra Research and Development Centre for providing me the funding during my PhD research and providing the travel budget for attending overseas conferences. I also want to thank to Rajabhat Suratthani University, Thailand for the scholarships and financial support for the whole life of my study.

I wish to thank various people. I am really grateful to Ms. Michelle Tamehana (Institute of Food, Nutrition and Human Health), Ms. Janiene Gilliland and Mr. Chris Hall (Riddet Institute), Ms. Yvonne van der Does, Mr. Bert Fong and Mr. Mark Reynolds (Fonterra Research and Development Centre) for providing trainings, technical help and scientific suggestions. I am thankful to all the staff in the Institute of Food, Nutrition and Human Health, the Riddet Institute and the Fonterra Research and Development Centre.

I would like to express my deep gratitude to Ms. Lois Wilkinson (Centre of Teaching and Learning) for proof reading my thesis. I am indeed thankful to Mr. Yichao Liang (Kendison), Ms. Leong Shu-Fen (Claudia) and Ms. Teo Kai Zhi for their help during did the lab experiment and invaluable assistance.

I would like to thank all of my Thai friends in Palmerston North; Ms. Piyamas, Ms. Parussaya, Ms. Sureewan, Ms. Lalida, Ms. Supornpan, Mr. Chumpol, Ms. Janyawat, Ms. Komkiew, Ms. Sasiphattra, Ms. Patcha, Ms. Chanapa, Mr. Atthapon, Ms. Araya, Mr. Parinya, Mr. Tawan, Ms. Paweena, Ms. Shutiwan, Ms. Widchaporn, Ms. Aurathai, Ms. Thewaporn, Ms. Rattanawan and Ms. Jantana for their love, warm, kindness and support during the past four years. Also thanks to Mr. Thanawat and Ms. Nittha for their extremely entertaining which made my life so much happy.

Special thanks to my lovely officemates; Ms. Sandra Kim, Ms. Elham Khanipour, Dr. Sina Hosseiniparvar, Mr. Ian (Yuen Feung) Lim, Dr. Jeremy Smith, Ms. Heyley Stewart, Ms. HaoranWang, Ms. Noor Sofalina Sofian Seng, Ms. Teresa Wegrzyn for their love and support.

I am also sincerely thankful to Prof. Ian Warrington and his family for their help, support and keeping the Thai students' connection alive in Palmerston North.

Furthermore I would also like to thank my parents, Mr. Songsak and Assist. Professor Chollada Lueamsaisuk for their endless love and support. I would like to thank my cousin, Assoc. Professor Sunee Longprasert as well for her assistance and guidance I feel motivated and encouraged every time I talk to her. Thanks to my close friends and relatives for being their always. Last but not least, I would like to

thank my brother, my sister-in-law and my lovely nephew, Mr. Sakkapong, Ms. Kamonchanok and Mstr. Bhubadi (Nong Aron) for their love and motivation.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

α-lac	Alpha-lactalbumin
β–lg	Beta-lactoglobulin
BS	Bile salt
BSDL	Bile salt-dependent lipase
BSSL	Bile salt-stimulated lipase
C	Carbon
CSLM	Confocal scanning laser microscopy
DAGs	Diacylglycerols
EDTA	Disodium ethylene diamine tetra-acetate
FA	Fatty acid
FFA	Free fatty acid
HCl	Hydrochloric acid
LC–PUFA	Long chain polyunsaturated fatty acids
MAGs	Monoacylglycerols
MCFA	Medium chain fatty acid
MCT	Medium chain triglyceride
MFG	Milk fat globule
MFGM	Milk fat globule membrane
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
p*I*	Isoelectric point

PUFA	Polyunsaturated fatty acid
RO	Reverse osmosis
SGF	Simulated gastric fluid
TAGs	Triacylglycerols
Tween 20	Polyoxyethylene sorbitan monolaurate
UHT	Ultra high temperature

Chapter 1 Introduction

"Breastfeeding from a woman who is in good health and nutritional status provides a complete food which is unique to the species. There is no better nutrition for healthy infants both at term and during the early months of life" (Thompson, 1998). Whilst breast milk is the best nutritional choice for infants, breastfeeding may not be possible for all women. It depends on their comfort level, lifestyle, and any specific medical considerations they might have. Thus, good infant formula provides a necessary alternative. Currently, many studies are investigating the modifications necessary to make formula milk as similar as possible to human milk, not only from a compositional and nutritional perspective, but also in relation to how the physical form of their components behave to limit or promote digestion.

Milk is a dispersion of fat globules and casein micelles in an aqueous phase, consisting of dissolved and suspended components such as whey proteins, lactose, minerals and vitamins (Brans et al., 2004, Raikos et al., 2009). Fats are essential components of the diet, and have a critical role in the growth and development of the neonate. Accordingly, knowledge of how the neonate digests fats is of growing importance (Manson and Weaver, 1997). Fat is a major component of milk, but there are differences in structure, composition and nutritional value among human breast milk, cows' milk and infant formula. These differences can potentially affect the efficiency of digestion of each type of milk, with previous human studies indicating that infants digest and absorb formula more slowly than breast milk (Andersson et al., 2007, Splinter and Schreiner, 1999). One of the important factors that affects the digestion of milk is the milk fat globule membrane (MFGM) (Keenan and Patton, 1995, Ye et al., 2010). The core of the milk fat globule (MFG) contains more than 98

percent of triacylglycerols, which are broken down by enzymes in the upper gastrointestinal tract before absorption; this core is surrounded by a thin membrane. The MFGM, has a similar general composition to that of biological membranes, containing phospholipids, cholesterol, glycoproteins and enzymes. This MFGM protects the interior from coalescence and degradation (Dewettinck et al., 2008, Manson and Weaver, 1997).

In order to understand how milk emulsions are digested, it is necessary to know how physicochemical properties and the interfacial composition of MFG change during the process of digestion. The purpose of this study is to investigate the relationship between emulsion structure and digestion by using an appropriate *in vitro* model that simulates the infant physiological conditions in the stomach. The proposed research is necessary for maximising the digestibility of formula emulsions that more closely resemble that of maternal milks.

Previous studies have shown that the rate of lipid digestion may be influenced by the ability of lipase enzymes to bind to emulsion interfaces, which in turn can be limited by emulsion size and interfacial composition (Mansson, 2008). In this respect, the interfacial composition of commercial formulae differs considerably from that of biological milks, with additional variation existing between manufacturers as a consequence of both composition and processing conditions. To be able to study the physical changes within various feeding compositions during digestion, it is first necessary to define the physicochemical properties of milk and formula emulsions (i.e. structure, size distribution, surface composition, and zeta-potential). Differences in these parameters have the potential to affect the efficiency of digestion.

The objectives of this research are:

1. To compare the physicochemical and structural properties of breast milk, commercial infant formulae, and model emulsions.
2. To develop an appropriate *in vitro* digestion model that simulates infant gastric digestion.
3. To investigate the changes in interfacial emulsion composition and droplet size during the digestion of a model emulsion using the *in vitro* model developed.
4. To compare and interpret the digestive behaviours of infant formulae and model emulsions using the *in vitro* model developed.
5. To conduct a comparison of behaviours of maternal milk, infant formulae and model systems.

The hypotheses of this research are:

1. The composition, structural, and physicochemical properties of milk affect the digestive behaviour of lipids under neonatal gastric conditions.
2. The chemical and biochemical environment in the gastric chyme (i.e. the presence of pepsin and lipase, and acidic pH) as well as the shear rate will influence the rate at which emulsions are digested.
3. The interfacial layer of different emulsions (commercial infant formulae, intralipid or lactoferrin-stabilized emulsions, and human breast milk), will affect the susceptibility of pepsin hydrolysis and lipase adsorption during the gastric digestion in infants.

The findings here will help researchers to understand the physicochemical properties of MFG in infant milk. The results will add to the current knowledge on the

digestion process of MFG in infants and its effects on health. Consequently, researchers will have a greater understanding of the differences between human milk and infant formula and use this to enhance the nutritional and physicochemical properties of the latter.

Chapter 2 Literature Review

2.1 Introduction

Milk is essential for the newborn of all mammalian species, but the composition, structure and chemistry of milk fat or lipids from different species vary considerably with such factors as diet, geographical location, stage of lactation, number of lactations, breed and season (Mansson, 2008). The milk fat is biologically emulsified into globules that are stabilised with a surrounding amphiphilic membrane. The core of the globules consists of triglycerides (>98%) while the membrane is predominantly composed of phospholipids, cholesterol, and proteins (Jensen et al., 1990, Malacarne et al., 2002).

How emulsion stability changes during the digestion is a key objective of this study. Digestion of milk fat is an interfacial process and depends on the consecutive action of several lipases. The partial hydrolysis of the milk fat globule core begins in the stomach by the gastric lipase and then continues into the duodenum, where the pancreatic lipase in conjunction with colipase and the bile salt-stimulated lipase endogenous to human milk complete the process initiated in the stomach (Hamosh et al., 1985b). The pancreatic development in the infant indicates that the action of colipase dependant pancreatic lipase is not as high as in the adult, this is in part due to the much lower levels of bile salts in the infant duodenum (Borgström and Patton, 2011).

This chapter provides an overview of the literature relevant to this thesis, and presents possible explanations on how the properties of milk fat globules are related to the digestion outcome in the infants. The aims of this literature review are:

1. To review current knowledge and research on the effects of physicochemical factors on milk emulsion stability.
2. To review the changes noted in milk fat globules and their membrane in the digestive system of the neonate.

2.2 Milk emulsion stability

From a molecular perspective milk is a complex food. It constitutes an important part of humans' diet, mainly because of its high nutritional value. In its natural state, milk is an o/w emulsion with the fat globules dispersed in the continuous serum phase (Matsumiya et al., 2010). The stability of the milk fat emulsion is influenced by the physical and chemical characteristics of milk products (Fox and McSweeney, 1998). The most frequent mechanisms of instability in emulsions are creaming, flocculation and coalescence (Figure 2.1). Moreover, the formation structures of emulsion destability, especially the flocculation and coalescence, may affect to the efficiency of gastric lipolysis and enhance the rate of digestion. For example, the surface area of fat droplet that is available for gastric lipase to access, the rate of fat transit through the stomach, and also the subsequently influence subsequent intestinal digestion, which these may enhance the lipid digestibility (Lentle and Janssen, 2011).

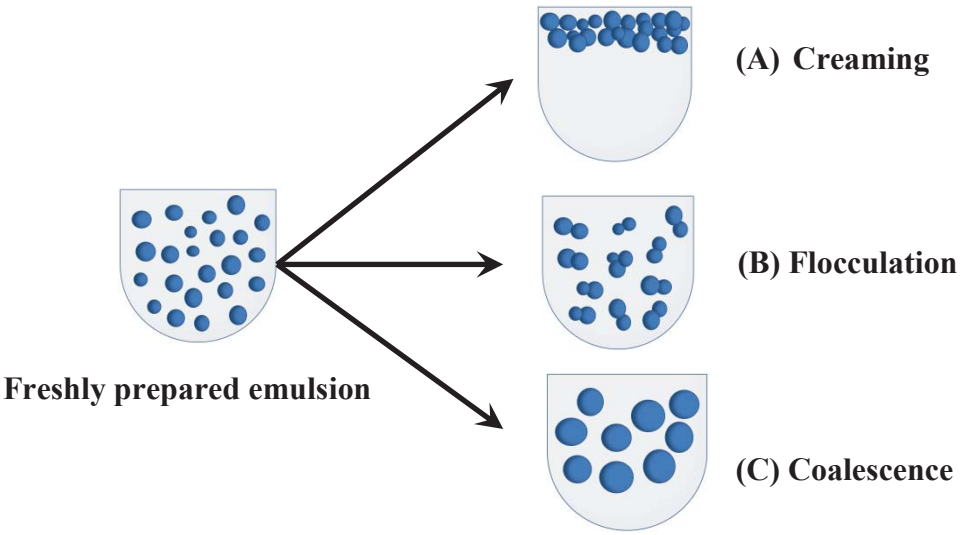


Figure 2.1 **Schematic representations of different types of emulsion destabilisation. [Adapted from (Martini and Marangoni, 2007, McClements, 2005)].**

2.2.1 Creaming

Creaming is referred to as the upward movement of droplets with a lower density than the continuous phase, that under gravity or accelerating forces form a droplet-concentrated layer on the top of the original emulsion (Figure 2.1A) (McClements, 2005). The rate of creaming can be described by Stokes' equation (equation 2.1):

$$v = \frac{2gr^2(\rho_2 - \rho_1)}{9\eta_1} \tag{2.1}$$

where, v = creaming velocity [the sign means the droplet moves upward (+) or downward (–)], g = local gravity or acceleration (~9.8 ms^{-2}), r = droplet radius, η_1 = shear viscosity of continuous phase, ρ = the density. The subscripts 1 and 2 refer to the continuous and dispersed phases, respectively (Osano, 2010).

Therefore, the equation shows that the rate of creaming depends on: i) the original size distribution as the creaming rate of bigger droplets is faster than for smaller droplets; ii)

the density difference between the droplets and the aqueous phase; iii) the rheology of the continuous phase (Keowmaneechai, 2002, Osano, 2010). This equation applies when there is no interaction between droplets in the infinite dilution or at very low droplet concentration.

From a digestive perspective, creaming or phase separation of an emulsion in the stomach, can result in a more rapid emptying of the non-creamed fractions from the stomach compared to an emulsion which retains a homogeneous distribution of fat (Marciani et al., 2003).

2.2.2 Flocculation

Flocculation occurs when two or more droplets come together forming an aggregate through weak physical interactions without losing their individual integrity (Figure 2.1B) (McClements, 1999, IUPAC, 2007). Usually, flocculation happens in the first step of emulsion destabilisation and can readily cause creaming and coalescence. Flocculation can accelerate the creaming rate, especially in a dilute emulsion, because of the increased effective particle size (Luyten et al., 1993). Increasing the attractive forces or decreasing the repulsive forces between the droplets can induce droplet aggregation. Flocculation can also affect the rheological properties of the emulsion. In dilute emulsions, ($\phi < 0.01$), where the mean distance between droplet surfaces is much greater than the average droplet diameter, flocculation increases the creaming rate because the effective size of the particles in the emulsion increases. In concentrated emulsions, ($\phi > 0.5$), where the mean distance between droplet surfaces is necessarily much smaller than the average droplet diameter, the movement that occurs prevents

droplet creaming as they form a three dimensional aggregated network of droplets (McClements, 1999, Dickinson, 2010).

The flocculation of fat globules occurs in the early stages of digestion, most notably for protein–stabilised emulsions. It can occur due to progressive hydrolysis of the interfacial protein layer which leads to detachment of hydrophilic and charged domains from the interface. This causes a reduction in stabilisation from charge repulsion, promoting hydrophobic interactions, which leads to the formation of large clumps increasing the aggregate particle size (Mackie and Macierzanka, 2010, Ye et al., 2010). Emulsion stability is also significantly affected by pH. Acidification also promotes flocculation, most noticeably in emulsions stabilised by proteins at conditions close to their isoelectric point (p*I*). The proteins adsorbed at the surface of individual droplets can flocculate due to the loss of charges compromising the steric stabilisation and resulting in aggregation. The main types of flocculation are shown in Figure 2.2; A) Bridging flocculation (droplets bridged by divalent ions or cross linked by polymers); B) Depletion flocculation (in the presence of an excess of non-adsorbed polymers or particles, the tiny sphere of polymer chains cannot get close to the particles thus the two particles happen to be near each other and causing particles to clump; and C) Electrostatic flocculation (loss of the surface charge of the droplet) (Keowmaneechai, 2002, Osano, 2010).

Under *in vitro* gastric conditions, protein stabilised emulsions may undergo extensive flocculation as a consequence of passing through the p*I*. In this flocculated state the emulsions are prone to coalescence as a consequence of the combined action of protease digestion of the interfacial layer (Golding and Wooster, 2010).

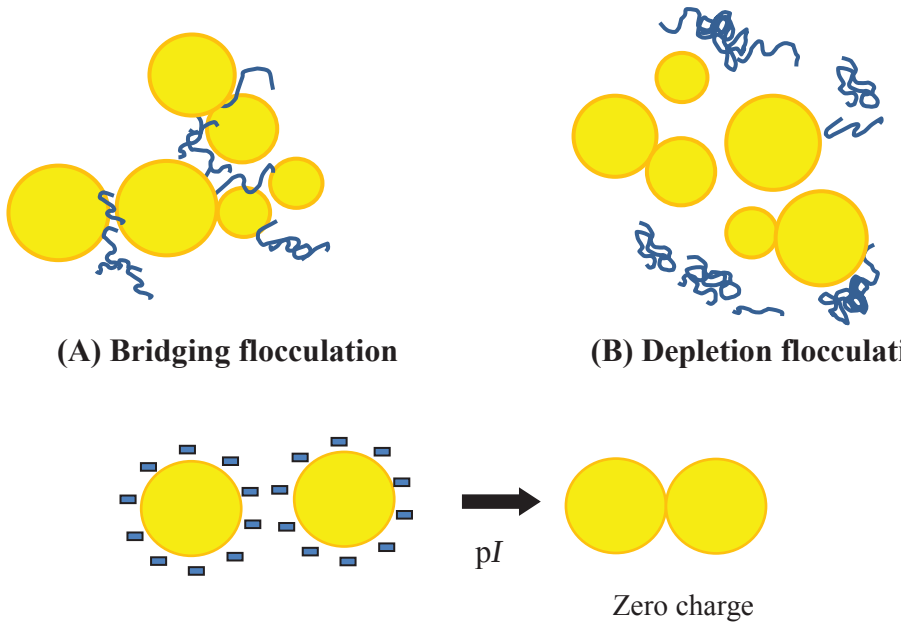


Figure 2.2 **Schematic representations of the flocculation of fat droplet.**

2.2.3 Coalescence

Coalescence occurs when two or more liquid droplets come close and merge together to form a single larger droplet (Figure 2.1C) (McClements, 1999) so the average droplet size increases. Coalescence can take place when the droplets are close together as the liquid film and interfacial membranes of the droplet are ruptured (Fox and McSweeney, 2006). The induced surface tension gradient leads to a more thermodynamically favourable state, when two oil droplets combine together to form a larger one decreasing the surface free energy of the system (Mahmoudi et al., 2012).

Droplet proximity and contact can be enhanced under conditions of flocculation, or as a consequence of applied shear. Coalescence more strongly depends on short-range forces. The rate of coalescence rises with increased probability of film rupture drainage. Factors that affect the rate of coalescence comprise the physical nature of the interfacial

surfactant film, size distribution of the droplet and phase volume ratio. Unlike weak flocculation and creaming, it is not possible to reverse coalescence by simple stirring. In addition, extensive droplet coalescence will bring about the formation of a layer of free oil on the top of an emulsion called oiling off which shows the breakdown of the emulsion (Keowmaneechai, 2002).

During digestion, flocculated fat globules may form large sized droplets by coalescence. As a consequence of hydrolysis, the integrity of interfacial protein layers is reduced due to proteolysis and increasing adsorption of surface active components (fatty acids, monoglycerides, bile salts) can take place. Droplets in which protein is orogenically displaced by fatty acids and monoacylglycerols will tend to coalesce, as these species have lower surface viscosity. Consequently, the original surface materials may not continue to dominate the behaviours of the fat globules when digestion time is longer (Ye et al., 2010). This effect and its relationship to the milk fat droplet stability are very important in this study. These instability mechanisms may take place at the same time in one system, and the incidence of one instability mechanism can encourage others. Thus, if a milk sample is undergoing flocculation, coalescence or separation, changes in the properties of the emulsion system can accordingly be related to the digestive behaviour of the emulsion in the neonate. In this way, better understanding of the differences in digestive properties of biological and manufactured milks can be achieved.

2.3 Structure and composition of fat globules in human milk and in formula feeds

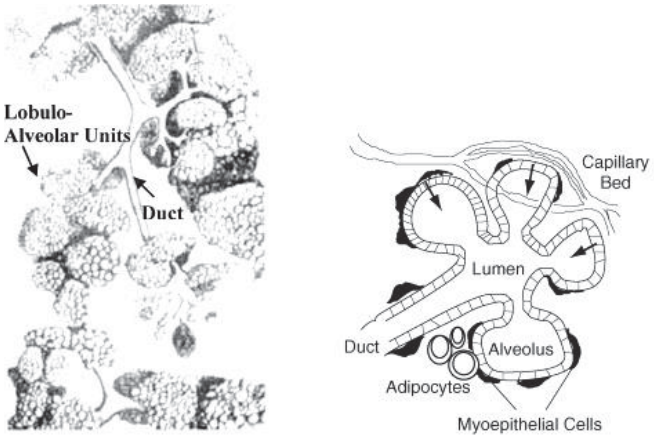
2.3.1 Human milk

The composition of human milk changes during lactation; fat is the main source of its energy, the others being lactose and proteins (Yu et al., 2007). The fatty acids in the lipid fraction of human breast milk can vary in concentration depending on feeding, from breast to breast, over the day's duration, the gestational age at birth, and between individuals. The main impact on the fatty acid profile of human milk is the maternal diet (Malbon, 2006).

2.3.1.1 Human milk secretion

The milk secreting structure is the mammary gland, the functional unit of which is the alveolus (Forsman and Schwertfeger, 2013) (Figure 2.3A). Alveoli are composed of a single layer of secretory epithelial cells that contract to bring about milk ejection, and also a connective tissue stroma that supports and separates the lobules. The stromal component also contains lymphatic vessels and becomes extensively vascularised during lactation to sustain the biosynthetic demands of alveolar epithelial cells (McManaman and Neville, 2005). In women who are not pregnant the gland and its duct system are inactive so the lobules and lobes are not developed. During pregnancy, alveoli proliferate at the ends of the ducts and are fully differentiated (Figure 2.3 B). In the lactating mammary gland, each lactiferous duct drains one lobe (Kierszenbaum and

Tres, 2012). Once lactation is completed, the gland reverts to the nonpregnant condition.



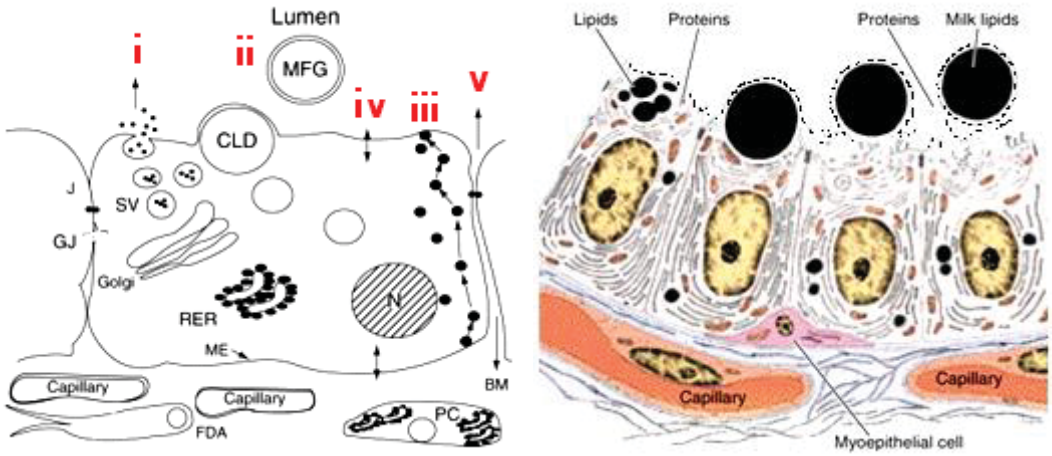
(A) **(B)**

Figure 2.3 **A section of human breast milk (A) and the cross-sectional diagram of the mammary gland (B). [From (McManaman and Neville, 2005)].**

The five pathways for milk secretion from the mammary gland by alveolar epithelial cells into the lumen are shown in Figure 2.4A and B.

These pathways transport the various components into the milk. In pathway I, milk proteins (i.e. casein and whey proteins), lactose, and other components are secreted by exocytosis (Figure 2.4Ai). Pathway II, is of the most interest in lipid delivery as this is how cytoplasmic lipid droplets (CLDs) form and are secreted via the apical membrane, this membrane coating the milk fat globule (MFG) (Figure 2.4Aii) The lipid droplets are assumed to originate from the rough endoplasmic reticulum (RER). Pathway III, represents the transition of proteins (i.e. immunoglobulins, hormones) and some macromolecules from the interstitial space called transcytosis (Figure 2.4Aiii). Pathway IV, the membrane transport pathway, shows a specific transporter of water, cell

cytoplasm and milk through the membrane cell (Figure 2.4Aiv). Pathway V, the paracellular transport pathway, shows direct exchange between the alveolar lumen and milk components (both low molecular weight substance and macromolecular solutes) via the interstitial space (Figure 2.4Av) (McManaman and Neville, 2003, Mather, 2011a).



(A) **(B)**

SV, secretory vesicle; RER, rough endoplasmic reticulum;
BM, basement membrane; N, nucleus;
PC, plasma cell; FDA, fat-depleted adipocyte;
GJ, gap junction; ME, myoepithelial cell;
CLD, cytoplasmic lipid droplet; MFG, milk fat globule;
J, junctional complex containing the tight and adherens junctions

Figure 2.4 **Diagram of a mammary epithelial cell showing pathways for milk secretion (A) (McManaman and Neville, 2005) and secreting cells from the mammary gland (B). [Retrieved from (http://intranet.tdmu.edu.ua)].**

In addition, secretion of lipid droplets and protein products (caseins) are found collocated the apical membrane (Monks and McManaman, 2013). As caseins are synthesised and packed in these vesicles, they self-aggregate into particles termed micelles. The inclusion of one specific type of casein (κ-casein) in this aggregate increases the solubility of the micelle so that milk proteins remain in dispersion after their release from the cell (Husvéth, 2011).

As milk lipids are released, a membrane derived from the cell membrane of the epithelial cell encases the lipid droplets (Husvéth, 2011). This membrane is considered to be a trilayer of polar lipids which is composed of a surface-active inner monolayer and outer bilayer. The surface-active inner monolyer membrane, made of proteins, phospholipids and cholesterol, is secreted from the endoplasmic reticulum (Keenan and Patton, 1995, Lopez and Ménard, 2011, Lopez, 2010). The outer bilayer is from the apical plasma membrane of the mammary epithelial cells. The components of this bilayer are not uniformly distributed, but rather have concentrations of different components such as membrane proteins, sphingomyelin rich phases and phases with other phospholipids.

Lopez & Menard (2011) defined two lipid phases: liquid-ordered (L*o*) and liquid-disordered (L*d*) phase as shown in Figure 2.5. The liquid-ordered phase has two regions; i) a symmetric bilayer composed of sphingomyelin (SM) and cholesterol in the two segments of the bilayer and ii) an asymmetric bilayer (SM only in the outer layer of the MFGM and cholesterol in both). The liquid-disordered phase contains glycerophospholipids: phosphatidylethanolamine, PE; phosphatidylcholine, PC; phosphatidylinositol, PI; phosphatidylserine, PS. In addition, the glycolipids and/or glycoproteins located in this outer bilayer also spread over the external membrane

surface (Lopez and Ménard, 2011) and assist in ensuring that the lipid droplet stays in the milk serum (Gallier et al., 2010, Lopez, 2010) helping to protect the droplet from coalescence.

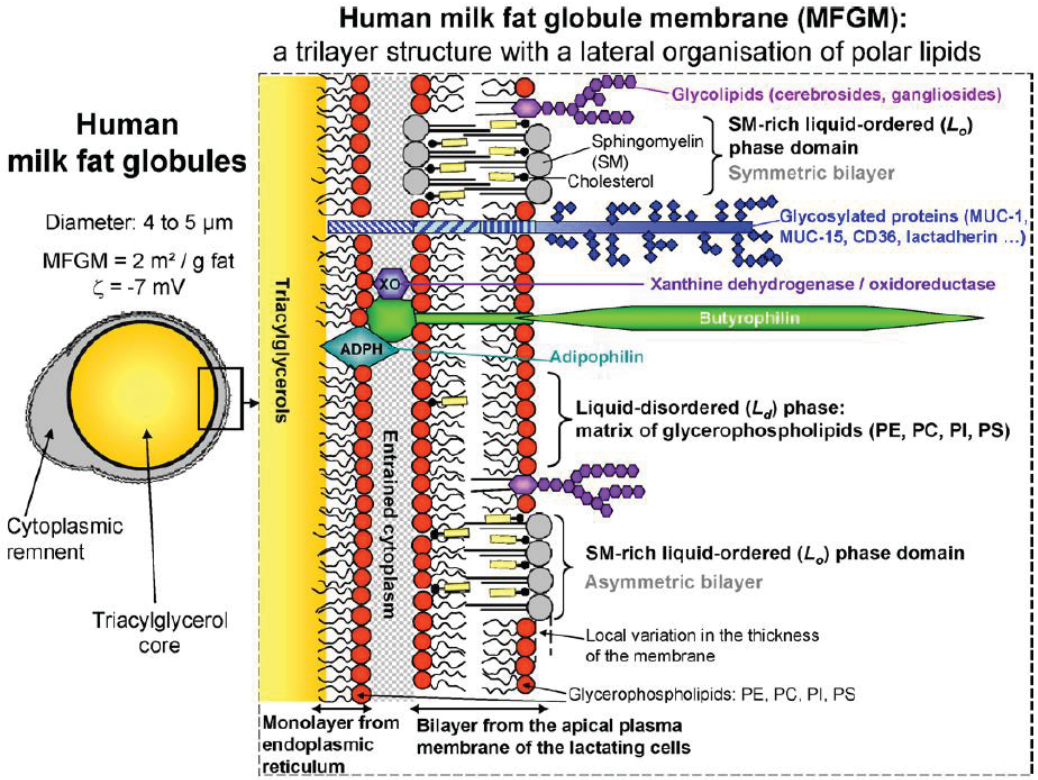


***Figure 2.5* Structure model of a human milk fat globule membrane. [From (Lopez and Ménard, 2011)].**

2.3.1.2 MFG of human milk

The milk fat globule (MFG) is a major nutritional component in milk which is formed throughout the mammary epithelial cell. It grows in size as it moves toward the apical cell membrane and is voided into the lumen of the alveoli of the milk secreting gland (Michalski et al., 2005b). The core of the globules consists of triacylglycerols which makes up about 98% to 99% of total milk fat. The number of carbon atoms in triacylglycerols varies with species. For instance, in human milk fat, the distribution

follows a typical unimodal pattern (maximum at 50–52 carbon atoms), while it follows a bimodal pattern in cows' milk (around 34 to 40 carbon atoms at the first maximum and 42 to 54 at the second peak) (Malacarne et al., 2002). Moreover, the globule size of human milk is correlated to the fat content; as the milk fat content increases, the volume of globules also increases, probably because of a limitation in production of MFGM (Mansson, 2008).

Milk fat has a complex composition. Although milk fat may have more than 400 different types of fatty acids (Wiking et al., 2009) the vast majority are at trace levels, with only around 20 making up the bulk of the composition. Fatty acids have chain lengths between 4 and 18 carbons and are either saturated, monounsaturated or polyunsaturated. In the form of bovine MFGs, the lipid component is typically composed of 98% triglycerides, 0.7% phospholipids and 0.5% cholesterol. However, in human milk fat, there is considerable variability in composition within and between women (Yu et al., 2007); with duration of lactation, time of day, and from the start of an individual feed until the finish (Forsyth, 1998). The changes of milk fat content and composition are most pronounced during early lactation (colostrums) and again during weaning (Hamosh et al., 1985a). In cow's milk, differences may occur due to animal origin (for example, related to genetics, breed and selection), stage of lactation, mastitis and luminal fermentation, or there may be feed-related factors such as fibre and energy intake, dietary fats, and seasonal and regional effects (Mansson, 2008).

2.3.1.3 MFGM of human milk

Most of the fat in milk is in the form of these globules that are surrounded by a membrane referred to as the "milk fat globule membrane" (MFGM) (Wade and Beattie, 1997).

The MFGM acts to stabilise the milk emulsion and facilitates ingestion and digestion of the fat by the suckling of the infant (Evers, 2004). The native MFGM acts as a natural emulsifying agent due to its amphiphilic nature (Vanderghem et al., 2010). It helps to stabilise the fat globules in the aqueous phase of milk (Keenan and Patton, 1995). The membrane is sufficient to prevent the fat globules from flocculation and coalescence (Cano-Ruiz and Richter, 1997). The estimated mass of the membrane of fat globules is 2–6% of the total fat globules (Singh, 2006). The average MFGM thickness is typically 10 nm but varies from about 10 to 20 nm (Walstra et al., 1999). The MFGM comprises a mixture of integral and peripheral proteins, enzymes, neutral lipids, phospholipids, glycoproteins, and other minor components surrounding the fat droplet.

The MFGM of both human and cow's milk are similar to each other in many respects. However, there are also certain defining differences between human and cow's milk. For example, in human milk, the external layers of the MFGM contain a group of glycoprotein filaments which are believed to enhance digestion by helping to bind lipase (Jensen et al., 1992, Jensen, 2002). However, in cow's milk the globules are coated by a thin protective film, with external layers consisted of proteins and phospholipids (Malacarne et al., 2002, Jensen et al., 1990).

Phospholipids found in the MFGM consist of a glycerol molecule with two fatty acids and a phosphate group linked to the third (OH) group of the glycerol molecules. The

main components in the phospholipid fractions of human milk are phosphatidylcholine (28.4%), phosphatidylethanolamine (19.3%) and sphingomyelin (37.5%). Smaller amounts of phosphatidylserine (8.8%) and inositol (6.1%), along with cerebrosides and gangliosides are also present (Forsyth, 1998). The MFGM of human milk contains less phosphatidyl choline, but more phosphatidyl ethanolamine and sphingomyelin than that of cows' milk (Malacarne et al., 2002, Keenan and Patton, 1995, Christie, 1995).

When milk is heated, phospholipids together with other components may move from the MFGM to the aqueous phase. However, it is uncertain whether this occurs due to heat treatment or during agitation when the membrane material desorbs because coalescence of fat globules may take place (Singh, 2006).

Phospholipids are exclusively synthesised *de novo* in the mammary gland and then located in the plasma membrane (Forsyth, 1998). Di- and monoacylglycerols, which are products of lipolysis, are present in the MFGM (Keenan and Mather, 2002). Other lipid components contained in MFGM are sterols and sterol esters. Cholesterol is the major sterol in MFGM, accounting for over 90% of the total (Keenan and Mather, 2002). Depending on the source, the MFGM contains between 25–60% of proteins (Danthine et al., 2000, Singh, 2006).

Despite their low concentration, MFGM proteins play an important role in various cellular processes and defence mechanisms in the newborn (Cavaletto et al., 2008). MFGM proteins account only for 1–4% of the total protein in milk. Proteins and phospholipids together account for over 90% of the membrane dry weight, but the relative proportions of lipids and proteins may vary widely (Singh, 2006). These proteins are located on both faces of the membrane layers.

Moreover, lipids are also found in the MFGM; triglycerides represent the major lipid component followed by diglycerides, monoglycerides, cholesterol and esters in MFGM in both cow and human milk (Table 2.1).

Table 2.1

Lipid composition of human and cow milk fat globule membrane.

Constituent class	**Human**[a]	**Cow**[b]
	% of total lipid	
Triacylglycerols	58	62
Diacylglycerols	8	9
Monoacylglycerols	0.6	Trace
Sterols	0.7	0.2 to 2
Sterol esters	Trace	0.1 to 0.3
Unesterified fatty acids	7.3	0.6 to 6
Hydrocarbons	Trace	1.2
Phospholipids	23	26 to 31
	% of total phospholipid	
Sphingomyelin	23	22
Phosphatidyl choline	30	36
Phosphatidyl ethanolamine	37	27
Phosphatidyl inositol	5	11
Phosphatidyl serine	1	4
Lysophosphatidyl choline	2	2

[a] Data complied from Jensen (1989).

[b] Data complied from Mather (2011b).

2.3.1.4 Human milk composition

a) Lipids

The properties of milk fat may influence its digestibility in the neonate. Key parameters such as fat content, the amount of globules, surface area and mean diameter of fat globule dispersions in human colostrums (1–5 days post-partum) and mature (>21 days post-partum) milks are shown in Table 2.2.

Table 2.2

Parameters of the fat globule dispersion in human colostrums and mature human milks. **[Adapted from (Jensen et al., 1995)].**

Parameter	Human milk average (+SD)	
	Colostrums	**Mature**
Fat content (g/100g)	2.3 (1.0)	3.3 (0.6)
Globules (approx. No./mL)	6×10^{10} (2×10^{10})	1.1×10^{10} (3×10^{9})
Surface area of 1 g fat in milk (m^2)	3.3 (0.5)	1.4 (0.1)
Volume/surface average diameter (μm)	1.5 (0.3)	4.0 (0.3)

Human mature milk has a higher fat content but less globules than human colostrum, so the surface area of fat in mature milk is less than that in colostrum milk. The colostrum (the milk produced in the first week after birth) is casein-free milk with a high proportion of the protein comprising immunoglobulins and MFGM proteins (Dewettinck et al., 2008).

b) Protein

Proteins in mammalian milk can be categorised into two major protein fractions: whey protein and casein, based on their solubility at pH 4.6. Casein is the protein that

coagulates and appears as curd in the presence of acid. Whey protein is present in the fluid remaining after the casein is removed. Additionally, some minor protein components are found in the MFGM (Huppertz et al., 2006). Casein is more difficult to digest than whey and may lead to internal gastrointestinal bleeding, and also iron deficiency (Huppertz et al., 2006, Carver, 2003).

i) Whey proteins

The main whey protein found in breast milk is α-lactalbumin (α-lac), which is an important source of the essential amino acids tryptophan and cysteine. Cows' milk contains less α-lac, its main whey protein being β-lactoglobulin which is absent in breast milk (Heine et al., 1991). In human milk, the protein α-lac is part of the lactose synthase, which is the enzyme in the mammary gland for lactose synthesis; it has a good nutritive value with a high lysine, cysteine and trptophan content. Alpha-lactalbumin and calcium bind in a 1:1 molar ratio, and may facilitate calcium absorption in the infant. The serum albumin in human milk does not appear to be synthesised in the mammary gland. The very small amounts of serum albumin, transferred into the milk from the blood, provide a further source of amino acids to the infant (Darragh, 1995).

ii) Caseins

There are differences between the cow's casein system and that in human milk. The major casein in human milk is β-casein (Packard, 1982). The α-casein, which is a major part of cow's casein is not present in human milk (Atkinson and Lonnerdal, 1989). Further infant formula improvements with respect to protein nutrition could be obtained by adapting the protein composition to that found in human milk. Two accepted models of a casein micelle are depicted in Figure 2.6. These micelles are composed of four

major caseins: α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein in a ratio of 3:1:3:1 (Figure 2.6B) (Dael et al., 2005, Carr, 1999).

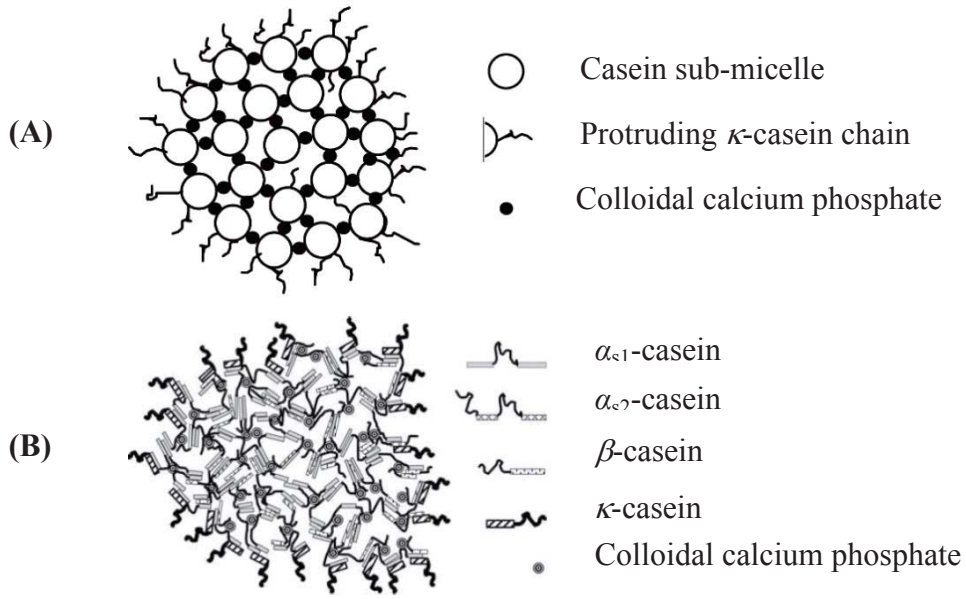


Figure 2.6 **The casein micelles; the sub-micelle model of the casein micelle (A), the dual-binding model of the casein micelle (B). [From (Anema, 2009)].**

Human milk casein micelles are smaller, sequester less calcium and phosphorus, and are hydrated to almost twice the extent of their cow's counterparts. In addition, human milk contains hormones, immune factors, growth factors, enzymes, and viable cells, most of which cannot practically be added to infant formulae. The comparison between types and amounts of protein (whey and casein proteins) and non-protein nitrogen of colostrums and mature milk is shown in Table 2.3 (Darragh and Lönnerdal, 2011).

Table 2.3

The types and amounts of protein and non-protein nitrogen in human colostrum and mature milk. **[From (Darragh and Lönnerdal, 2011)].**

Type	Colostrum (g 100 mL^{-1})	Mature milk (g 100 mL^{-1})
Whey proteins		
α-Lactalbumin	0.36	0.2–0.3
Serum albumin	0.04	0.03
Lactoferrin	0.35	0.1–0.3
Lysozyme		0.05
Secretory immunoglobulin A/immunoglobulin A	0.2	0.05–0.1
Immunoglobulin G	0.034	0.001
Immunoglobulin M	0.012	0.002
Casein proteins		
β-Casein	0.26	0.3–0.5
κ-Casein	0.12	0.1–0.3
Non-protein nitrogen (N)	48	50
Urea N	12.1	15–25
Creatine N		3.7
Creatinine N		3.5
Uric acid N	0.5	0.5
Glucosamine	14.2	4.7
α-Amino N	4.5	13
Ammonia N	0.2–0.8	0.2

Protein composition can vary considerably between species, e.g. cow's milk and human milk. Cow's milk is composed of 80% casein and 20% whey protein. (Fox and McSweeney, 1998, Walstra et al., 1999), but in human milk, caseins make up only 30–40% of total protein (Dael et al., 2005). Human milk is higher in whey and much lower in casein compared to cow's milk. The ratio in infant formula is therefore altered

to simulate human milk. There are also minor proteins such as immune proteins which are of importance, mainly as a defence against disease (Packard, 1982). The composition of human milk and cow's milk is shown in Table 2.4.

Table 2.4

Main differences in the composition of human and cows' milk casein and whey fractions. **[From (Wells, 1996)].**

	Human milk	**Cows' milk**
Whey ratio: casein	Changes throughout lactation Early lactation 90:10 Mature milk 60: 40 Late lactation 50: 50	20: 80
Whey	Mainly α-lactalbumin, lactoferrin and IgA	Mainly β-1actoglobulin, α-lactalbumin and IgG
Casein	Predominantly β-casein	Mixture of α_{s1}-, α_{s2}-, β- and κ- caseins
Non-protein nitrogen	25% of total nitrogen of which 50% is urea	5-6% of total nitrogen of which 48% is urea

2.3.2 Infant formulae

2.3.2.1 Milk processing

Infant formula is an oil-in-water emulsion containing protein, carbohydrate and other minor nutrients dispersed in water. In the manufacture of infant formula, homogenisation is an essential step as it prevents coalescence and creaming during storage. A thermal treatment is used to extend the shelf life and enhance the quality of

milk by reducing the initial microbial load preventing spoilage (Early, 1998, Raikos, 2010).

Homogenisation is a high-pressure mechanical treatment, where the fluid is forced through the homogenising nozzle. The homogenisation conditions of milk are usually 10–20 MPa at 55–65°C (Thiebaud et al., 2003). A two-stage homogenisation process is generally used to decrease the milk fat globules to a uniform size and shape (Montagne et al., 2009). The second stage valve operates at a lower pressure breaking flocs created in the first step and avoiding coalescence of newly formed fat droplets (Early, 1998). The size of milk fat globules highly decreases from 4 to 0.2–0.5 μm and the lipid interface area increases from 7 to 30 m^2 per g of fat (Lopez, 2005). In the processing of infant formulae, the emulsion droplet size is reduced to a mean size similar to standard homogenised cow's milk (0.4 μm).

Infant formula is processed either as ready–to–feed (liquid) or as powdered milk. Heat treatment of infant formula can vary; whereas standard pasteurisation (72°C for 15s) has little or no effect on the casein micelles of milk and milk quality, heating at higher temperatures can have an impact on the organoleptic and physical properties of milk, especially during storage. The preferred method in the food industry is an ultra–high–temperature (UHT) process which involves heating in the range of 135–150°C for a time span of 1 to 4 seconds (Packard, 1982) or the sterilisation at 120°C for 20 min (retort sterilisation) (Dalgleish, 1992). Moreover, powdered milk is achieved by spraying the milk concentrate as very fine droplets by a nozzle or a rotary atomiser and mixing with a stream of hot air at 180–220°C (Kim et al., 2009).

2.3.2.2 Fat droplets and their membranes in infant formulae

The fat fraction in most infant formulae consists of emulsified vegetable oils (Michalski et al., 2005b). The diameter of native fat globules in cow's milk varies between 0.1 to 15 μm, with most globules being in the range 1 to 8 μm (Walstra et al., 1999).

There are two effects involved in milk homogenisation; reduction in size of fat globules and interaction of homogenised fat with the protein fraction. The increase in surface area during homogenisation improves the capacity of fat to interact with casein and denatured whey proteins (Ciron et al., 2010). In the case of infant formulae manufactured only using vegetable oil, the species adsorbed at the interface are reduced to: whey proteins, caseins and phospholipids/monoglycerides.

A schematic representation of cow's native milk fat (A) and the homogenised milk fat globules (B) are shown in Figure 2.7. In homogenised milk, the globules are covered by proteins (casein and whey proteins) and remaining MFGM. A very similar mixed-whey dominated interfacial layer is expected in the processed infant formulae but without original MFGM material found in cow's milk, only phospholipids and monoglycerides added during processing.

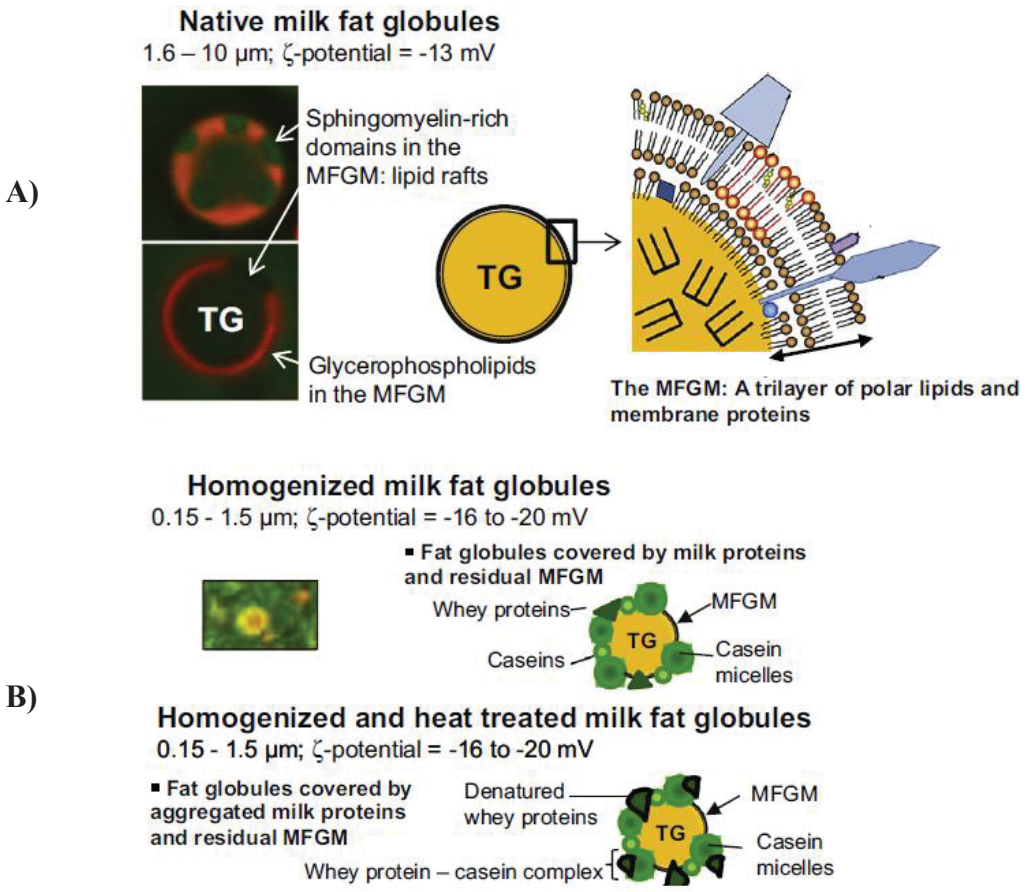


Figure 2.7 **The structure of native (A) and homogenised (B) cow's milk fat globules. [From (Garcia et al., 2014)].**

The heat treatment of infant formulae can cause denaturation of the globular proteins depending on temperature and time of the treatment. Interaction between adsorbed and non-adsorbed proteins as well as protein–protein interactions at the interface and at the aqueous phase may take place. New linkages between whey proteins and caseins as well as whey–whey interactions via intermolecular disulfide bonds are often the result of heat treatment processing. It has been shown that whey protein and κ–casein are more resistant to hydrolysis, so the potential for these protein aggregates to be attacked by human proteolytic enzymes is different from that of the native protein in maternal milk

(Almaas et al., 2006). Protein displacement phenomena may also take place as a consequence of heat treatment.

The physicochemical properties of the emulsion in formula feeds are important, as the changes that occur during processing and storage (Walstra et al., 1999) affect the subject behaviour in the digestive tract (Armand et al., 1999). There are certain compositional and structural differences between human breast milk and infant formula. For example, the fat droplets in human milk (diameter ~4 μm) are bigger than in infant formulae (diameter ~0.4 μm). Whereas the droplet size in formula feeds is mainly controlled by the homogenisation conditions, the main factors affecting the fat globule size in human milk are the stage of lactation, milk fat content, and hormones, such as prolactin and oxytocin (Michalski et al., 2005b, Ollivier-Bousquet, 2002).

In terms of emulsion stability, it should be noted that resistance to creaming is of little significance for biological milks, as a consequence of immediate consumption by the infant. However, for formula milks, notably those prepared as liquid compositions, droplet size is required to be reduced to ensure that creaming does not take place over the storage life of the product (<1 μm). This contrasts with the average diameter of milk fat globules of domestic mammals species i.e. cow, human, buffalo, goat, and ewe, in the range of 3 to 5 μm. Apart from particle size distribution, the structure of milk fat globules may be a key point of their functional properties. The MFG and their interfacial structure and the available fat surface area in milk are of primary importance for adequate lipolysis activity and digestion in the human infant (Michalski et al., 2005b).

Additionally, previous research has shown that the zeta (ζ)-potential value of the mature MFG of human milk (−7.8 mV) and cow's milk (−13.5 mV) are lower than the value of the commercial MFG of homogenised cow's milk (–20 mV) (Michalski et al., 2005b, Michalski et al., 2002). The ζ-potential is the quantification of the charge developed at the surface of particles. The amount of charges is related to the ionisation of charge groups (i.e. proteins at various pH's and the adsorption of ions at the interface of the droplets). This emphasises the differences in terms of the adsorbed components at the interface of raw and processed milk. These authors concluded that the particle size of fat droplets in infant formula might affect the efficient and rapid digestion in the digestive system of the newborns. Moreover, the ζ-potential may be an important factor in the functional properties and of nutritional significance for the infant.

Particles with a high ζ-potential of the same charge sign, either positive or negative, will repel each other. Conventionally, ζ-potential can be either positive or negative i.e. ≤–30 mV and ≥+30 mV, and both would be considered as high ζ-potential (Michalski et al., 2005b). Ultimately, charges at the interface of fat droplets may also play a role on the enzymatic activity during digestion.

It is important to note that when components of formula feeds are developed from milk of domestic animals, there are differences with regards to the profile and concentration of individual fatty acids; indeed the distribution of fatty acids on the backbone of glycerol appears to be unique to each species. These structural differences affect the bioavailability of individual fatty acids.

2.3.2.3 Infant formula composition

The composition of human milk is most often used as a guideline for the formulation of infant feeds. The aim in the design and development of infant formula is to achieve a similar digestive outcome and nutritional yield to that of human milk. For example, human milk contains, on average, 1.1% protein, 3.9% fat, 7.2% carbohydrates, but cows' milk, which is the base of infant formulae, contains 3.4% protein, 3.4% fat, and 4.8% carbohydrates. As such, cow's milk itself is not an appropriate substitute for human infant nutrition, however, its components can be reformulated to achieve an equivalent balance to human breast milk. In addition, 'optional' ingredients are added to more selective infant formulae with potential specific functional and nutritional benefits in accordance with regulatory requirements (Montagne et al., 2009) as shown in Table 2.5.

Table 2.5

Relative proportion of ingredients/components of human milk, cow's milk and milk based infant formulae (per 100 Cal). **[From (Hambidge and Krebs, 1991)].**

Nutrient	(Unit)	Minimum Level Recommemded[1]	Mature Human Milk	Cow's Milk (Mean)	Typical Commercial Formula
Protein	(g)	1.8 (see note 2)	1.3-1.6	5.1	2.3
Fat	(g)	3.3 (see note 3)	5	5.7	5.3
Carbohydrates	(g)	…	10.3	7.3	10.8
Linoleic acid	(mg)	300	560	125	2300
Vitamin A	(IU)	250	250	216	300
Vitamin D	(IU)	40	3	3	63
Vitamin E	(IU)	0.3 FT 0.7 LBW 1 g linoleic	0.3	0.1	2
Vitamin K	(µg)	4	2	5	9
Vitamin C	(µg)	8	7.8	2.3	8.1
Thiamin	(µg)	40	25	59	80
Riboflavin	(µg)	60	60	252	100
Niacin	(µg)	250	250	131	1200
Vitamin B6	(µg)	15 µg/g protein	15	66	63
Folic acid	(µg)	4	4	8	10
Panthothenic acid	(µg)	300	300	489	450
Vitamin B12	(µg)	0.15	0.15	0.56	0.25
Biotin	(µg)	1.5	1	3.1	2.5
Inositol	(mg)	4	20	20	5.5
Choline	(mg)	7	13	23	10
Calcium	(mg)	5	*50*	186	75
Phosphorus	(mg)	25	25	145	65
Magnesium	(mg)	6	6	20	8
Iron	(mg)	1	0.1	0.06	1.5 in fortified
Iodine	(µg)	5	4-9	7	10
Copper	(µg)	60	25-60	20	80
Zinc	(mg)	0.5	0.1-0.5	0.6	0.65
Manganese	(µg)	5	1.5	3	5-160
Sodium	(meq)	0.9	1	3.3	1.7
Potassium	(meq)	2.1	2.1	6	2.7
Chloride	(meq)	1.6	1.6	4.6	2.3
Osmolarity	(mosm)	…	11.3	40	16-18.4

[1]Committee on Nutrition, American Academy of Pediatrics.

[2]Protein of nutritional quality equal to casein.

[3]Includes 300 mg essential fatty acids.

Commercial formulae tend to show subtle variations in compositional design, depending on manufacturer specifications. The macro- and micronutrient compositions are relatively invariant, as they all imitate the nutritional profile of human milk and some are restricted by regulations. This can be demonstrated by comparing the concentrations of major components in infant formulae used in this study such as fat, protein, and emulsifiers; these are from the nutritional information and the ingredient list on their labels as shown in Table 2.6.

Table 2.6

The physicochemical characteristics of the different commercial milk samples that appeared on the label.

Samples		Fats		Proteins		Emulsifiers
		Ingredients	**Nutrients (g)**	**Ingredients**	**Nutrients (g)**	
Powder infant formulae	Anmum®	Vegetable oils (sunflower oleic, palm, coconut, soy, canola oils)	5.1 (per100 kcal)	Skim milk, whey protein	2.6 (per100 kcal)	Lecithin
	Enfamil® Premium Newborn	Vegetable oils (palm olein, coconut, soy, and high oleic sunflower oils), less than 1% of: Moetierella Alpina oil, Cryptheconium Cohnii oil	5.3 (per100 kcal)	Demineralised whey, whey, skim milk	2.1 (per100 kcal)	Soy lecithin
	Enfamil® Premium Infant	Vegetable oils (palm olein, coconut, soy, and high oleic sunflower oils), less than 1% of: Moetierella Alpina oil, Cryptheconium Cohnii oil	5.3 (per100 kcal)	Nonfat milk,whey protein concentrate	2.1 (per100 kcal)	Soy lecithin
	Enfamil® up & up	Vegetable oils (palm olein, coconut, soy, and high oleic sunflower oils), less than 1% of: Moetierella Alpina oil, Cryptheconium Cohnii oil	5.3 (per100 kcal)	Nonfat milk powder, whey protein concentrate [enriched in α-lactalbumin (α -protein)]	2.1 (per100 kcal)	Monoglycerides, soy lecithin

Table 2.6 (*Continued*).

Samples		Fats		Proteins		Emulsifiers
		Ingredients	**Nutrients (g)**	**Ingredients**	**Nutrients (g)**	
Powder infant formulae	Heinz® Nurture	Vegetable oils (sunflower, coconut, soy)	3.7 (per 100 mL) (=5.55g/100 kcal)	Skim milk, whey protein	1.5 (per 100 mL) (=2.25g/100 kcal)	Soybean lecithin
	Karicare®	Vegetable oils (contains soy oil)	3.4 (per 100 mL) (=5.1g/100 kcal)	Demineralised whey, whey, skim milk	1.4 (per 100 mL) (=2.1g/100 kcal)	Soy lecithin
	Mom to Mom®	Vegetable oils (palm olein, soy, coconut, and high oleic (safflower or sunflower) oil), less than 1% of: Moetierella Alpina oil, Crypthecon ium Cohnii oil	5.3 (per100 kcal)	Nonfat milk,whey protein concentrate	2.07 (per 100 kcal)	Monoglycerides, soy lecithin
	S–26® Gold	Vegetable oils (including soybean)	3.6 (per 100 mL) (=5.4g/100 kcal)	Nonfat milk powder, whey protein concentrate [enriched in α-lactalbumin (α -protein)]	1.3 g (per 100 mL) (=1.95g/100 kcal)	Monoglycerides, soybean lecithin
	Similac® Advance	High oleic safflower oil, soy oil, coconut oil, less than 2% of Crypthecon ium Cohnii oil, Moetierella Alpina oil	5.6 (per 100 kcal)	Nonfat milk, whey protein concentrate	2.07 (per 100 kcal)	Soy lecithin

Table 2.6 (*Continued*).

Samples		Fats		Proteins		Emulsifiers
		Ingredients	**Nutrients (g)**	**Ingredients**	**Nutrients (g)**	
Liquid infant formulae	S–26® Gold	Vegetable oils (including soybean)	3.6 (per100 mL) (=5.4g/100 kcal)	Nonfat milk, whey protein concentrate [enriched in α-lactalbumin (α-protein)]	1.3 g (per 100 mL) (=1.95g/100 kcal)	Monoglycerides, soybean lecithin
	Similac® Advance	High oleic safflower oil, coconut oil, less than 0.5% of Cryptheconium Cohnii oil, Moetierella Alpina oil	5.4 (per100 kcal)	Nonfat milk , whey protein concentrate	2.07 (per 100 kcal)	Soy lecithin, monoglycerides
	Enfamil® Premium Infant	Vegetable oils (palm olein, soy, coconut, and high oleic sunflower oils), Moetierella Alpina oil, Cryptheconium Cohnii oil	5.3 (per100 kcal)	Nonfat milk, reduced minerals whey	2.1 (per 100 kcal)	Mono- and diglycerides, soy lecithin

a) Milk and vegetable lipids

In infant formulae, the fat phase is dispersed in a solution of milk proteins, carbohydrate, minerals, vitamins and other nutrients (McSweeney, Healy, & Mulvihill, 2008). Fat is essential for the newborn, especially the premature infant, for several reasons; It provides more energy than other nutrients, it can be stored in the body in considerably larger amounts than carbohydrates and proteins, and it also functions as a vehicle for fat soluble vitamins assisting this absorption (Packard, 1982, Rogers et al., 1997). In infant formula milk, lipids provide 40–50% of the total daily energy intake.

Human breast milk fat is composed of palmitic (saturated), oleic, linoleic (ω-6), linolenic (ω-3), long chain polyunsaturated fatty acid (PUFA): arachidonic acid (ARA) and docosahexaenoic acid (DHA) (Fletcher et al., 2007). Cow's milk contains a higher proportion of saturated fatty acids than human milk and is lacking in linoleic acid and linolenic acid, polyunsaturated fatty acids that are essential for normal infant growth and development (Fletcher et al., 2007). Moreover, fats from cow's milk are not well absorbed by infants. Thus, to mimic human breast milk, most formulae have added a combination of vegetable oils such as palm, soy, corn, safflower, and sunflower to enhance the saturated, monounsaturated and polyunsaturated fractions to provide essential fatty acids like in human milk fat (Prosser et al., 2010). For example, soybean oil, oleo oil, and safflower oil are all polyunsaturated fats, generally used in formulae. Coconut oil provides a source of saturated fatty acids needed to balance the polyunsaturated (Packard, 1982), and palm olein is used to increase palmitic acid in the fat to 25%, similar to human milk

(Prosser et al., 2010). The fatty acid content of human milk fat and infant formulae is shown in Table 2.7.

Table 2.7

Fatty acid content of human milk fat compared with infant formulae and bovine milk (% of total fatty acids). **[From (Wells, 1996, Jensen, 2002)].**

		Human milk[a]	**Infant formulae[a]**	**Bovine milk[b]**
Saturated				
Caprylic	$C_{8:0}$	–	1.3 (0.7–1.5)	1–3
Capric	$C_{10:0}$	1.4 (1.2–1.6)	1.1 (0.9–1.3)	2–4
Lauric	$C_{12:0}$	5.4 (4.4–6.0)	9.4 (6.6–12.1)	2–5
Myristic	$C_{14:0}$	7.3 (6.9–7.9)	4.9 (4.3–6.6)	8–14
Palmitic	$C_{16:0}$	26.5 (26.0–27.0)	20.0 (14.0–26.3)	22–35
Stearic	$C_{18:0}$	9.5 (8.5–10.5)	4.9 (3.6–7.5)	9–14
Unsaturated				
Palmitoleic	$C_{16:1}$	4.0 (3.7–4.2)	0.4 (0.1–1.1)	1–3
Oleic	$C_{18:1}$	35.5 (34.5–36.0)	40.6 (28.7–53.0)	20–30
Eicosaenoic	$C_{20:1}$	0.5 (0.5–0.6)	0.3 (0.2–0.5)	–
Linoleic	$C_{18:2\ (n-6)}$	7.2 (6.4–7.7)	12.4 (8.8–18.9)	1–3
α -linolenic	$C_{18:3\ (n-3)}$	0.8 (0.7–0.9)	1.5 (0.7–2.2)	0.5–2

[a] Data complied from Wells (1996).

[b] Data complied from Jensen (2002).

Moreover, the essential fatty acids DHA and ARA for brain and retina development are normally added into infant formulae. While it seems that formula-fed infants would be missing out on these healthy fats that are found in breast milk, many studies have shown no benefits when DHA and ARA are added to the commercial milks (Uauy et al., 2003, Wright et al., 2006). Perhaps the types of DHA and ARA

normally added which originate from algae and fungi may have an impact, but little is known about this subject.

b) Protein

Whey–dominant infant formulae are considered suitable protein nutrition for infants from birth but provide no immune protection. Also, casein is more difficult for infants to digest than whey protein, being noticeably lower in concentration when compared to cows' milk (Carver, 2003). Therefore, careful tuning is needed when producing formula. Generally, whey is added to the cows' milk base to increase the whey to casein ratio. So-called whey–adapted infant formulae have been developed by reducing the proportion of casein from 80% as in cow's milk to 30–40% as present in human milk. The total protein content of starter infant formulae is typically 15–17 g/L, more than 8–12 g/L in mature human milk, but less than in cow's milk which is about 32 g/L (Dael et al., 2005).

New formulae are being developed and changes continue to be made to manufactured milks. These changes generally result in products with compositions and functions closer to human milk (Carver, 2003). However, it is important to note that whilst the protein in human milk is predominantly in the solution, a portion of the protein in formula milks can be located at the interface of the fat droplets as a consequence of formula processing.

Furthermore, the use of casein and whey protein protein hydrolysates in infant formulae (both partially or extensively) into smaller size than cow's milk base in order to promote the digestion (Koopman et al., 2009). The previuos study showed that the infant who fed hydrolysed protein formula showed faster gastric emptying

compared with non-hydrolysed protein (Berseth et al., 2009). Moreover, the hydrolysates protein may reduce the risk of cow's milk protein allergy (Maldonado et al., 1998). However, the efficacy of hydrolysates protein in infant formula in the allergy prevention is still controversial. Moreover, protein hydrolysates does not only affect digestibility and allergenicity of proteins but also affect the emulsion instability such as creaming and coalescence. Creaming is realted to the presence of large fat droplets after homogenization. Coalescence is correlated to the molecular weight distribution of the hydrolysates stabilizing the emulsions (van der Ven et al., 2001).

i) Whey proteins

Whey proteins consist of a diverse group of proteins whose major components are α-lactalbumin (α-lac), β-lactoglobulin (β-lg), serum albumin, immunoglobulins (IgA, IgG and IgM), lactoferrin and lactoperoxidase (Dael et al., 2005, Keowmaneechai, 2002). The physicochemical characteristics are shown in Table 2.8. Whey is rich in essential amino acids and is easily digested by the immature infant gut. The structure and properties of whey proteins depend upon pH, concentration and temperature. For instance, β-lg can exist as octamers at pH around its p*I*, but as dimers or monomers at pH above or below the p*I* (Dael et al., 2005).

Table 2.8

Characteristics of whey proteins in cow's milk. **[From (Keowmaneechai, 2002)].**

Protein	Isoelectric point	Molecular Weight	Denaturation temperature (°C)
α-lactalbumin	4.8–5.1	14,200	62
β-lactoglobulin	5.2	18,400	78
immunoglobulins	4.8–5.1	66,000	64
serum albumin	5.5–6.8	$15–96\times10^4$	42
lactoferrin	8.4–9.0	80–92,000	–
lactoperoxidase	–	89,000	–

ii) Caseins

The caseins are commonly the predominant milk proteins in many mammalian species (Carr, 1999), although human milk does not follow this generalised rule. Casein precipitates at pH 4.6, which provides a convenient means to separate it from whey protein. As previously indicated, most casein in milk is present in micelles which contribute to the white appearance of milk. Approximately 95% of the caseins are naturally self-assembled into these casein micelles which are spherical colloidal particles, about 50–500 nm (average 150 nm) in diameter and about 10^6 and 3×10^9 Da of molecular mass. They comprise 94% protein and 6% inorganic material collectively called colloidal calcium phosphate mainly calcium, phosphorus and magnesium. Casein micelles contain ~2 g water/g protein, and there are present in a quantity of 10^{14}–10^{16} micelles per mL milk (Fox and Brodkorb., 2008, Livney, 2010).

c) Emulsifiers

Emulsifiers are commonly added to formula milks to ensure emulsion stability. Emulsifiers possess both; hydrophilic and lipophilic fractions. The emulsifiers will coat the surface of oil droplets and effectively decrease the surface tension conferring at least short-term stability to the emulsion. Lecithin and monoglycerides are the common emulsifiers used in infant formulae generally recognised as safe by US Food and Drug Administration (Dickinson, 1993) and the Australia New Zealand Food Standards Council (ANZFSC) as shown in Table 2.6. The emulsifiers help proteins to stabilise the milk emulsion system because they make the emulsion more heat–resistant compared to purely protein–stabilised emulsions. Therefore emulsifiers are usually added to heat–sterilised products, especially those for newborn infants. Lecithin is a natural emulsifier that consists of a complex mixture of different polar phospho– and glycolipids and neutral lipids (triglycerides and sterine fatty acids). Lecithin is known to improve the heat stability of milk and wettability of instant milk powders (Sharma et al., 2012, Schubert, 1993). Monoglycerides may also be added to formulae as antifoaming agents, since excessive foaming of reconstituted milks is considered undesirable.

In general, the average fat globule size of whey protein–stabilised emulsions decreases as the proportion of lecithin or monoglycerides to protein in the emulsion is increased. As well as competing with proteins for space at the interface, emulsifiers can also interact with proteins adsorbed at the interface and with non-adsorbed proteins in the aqueous phase (McSweeney et al., 2008).

Theoretically, the amount of emulsifiers (lecithin and/or monoglyceride) added to these infant formulae is expected to be within a similar range, so this should not be a source of difference in the mean droplet size. Additionally, the interface is likely to be dominated by the excess proteins contained in the emulsions.

The nature of the emulsifier will impact the susceptibility of the lipid droplets to coalescence and breakup within the GI tract, thereby altering the total surface area of lipid exposed to lipase. In addition, the characteristics of the interfacial layer will impact the adsorption and activity of lipase at the oil/water interface. For example, lipase may not be able to adsorb to the lipid droplet surfaces if there is a sufficiently high concentration of a more surface active molecule present (Hur et al., 2009).

For infant formula, the milk fat globule has a mixed interface dependent on the type of proteins incorporated into the product. For example, some infant formulae have soy protein, milk proteins and also emulsifiers. The behaviour of the emulsion during digestion is affected by the adsorbed layer surrounding the lipid droplet (Malaki Nik et al., 2011). Proteins may undergo various conformational changes when adsorbed to the oil/water interface. These conformational changes can also affect the digestion process (Lesmes and McClements, 2012). As the emulsion goes through the human gastrointestinal tract, the interfacial layer of the lipid droplet is significantly modified by the various enzymes, change in pH, ionic strength and the presence of biosurfactants. Accordingly, the stability of an emulsion is dependent to a certain extent on the amount of pepsinolysis of the proteins at the interface (Malaki Nik et al., 2011).

2.4 Digestive system of infants

The digestive tract breaks down ingested food by mechanical and biochemical processes, to a state that such nutrients can be absorbed into the body. Fat digestion in infants is notably different from fat digestion in adults, especially as infants are principally fed human or formula milk, whilst adults consume lipids in a variety of compositional and structural states (with the exclusion of breast or formula milks). Fat digestion in the stomach is much more important in newborns than in adults (Borgström, 1974) because intestinal function is immature, resulting in reduced levels of pancreatic lipase and bile acid synthesis function (low bile salt levels), which may affect fat digestion (Liao et al., 1983). Lipid hydrolysis is related to: the chemical structure of lipid substrates; the physical state and physicochemical behaviour of the lipids; and the hydrolytic products (Bracco, 1994).

The digestion of fat is augmented by lipases which act in the infant gut. These lipases can be categorised into preduodenal, pancreatic and breast milk lipases (Manson and Weaver, 1997). Gastric and lingual lipases, which are preduodenal lipases, partially compensate for the low levels of pancreatic lipase and bile salts in infants which is only a fraction of that in older children and adults (Hamosh et al., 1981, Kempley et al., 2005).

The factors that influence the digestion and absorption of fat, include the type of lipase, bile salts, the pH of the gastrointestinal tract, the presence of other components (i.e. salts, calcium, monoacylglycerols (MAGs)), and the composition and structure of the fat globule and its membrane (Kontkanen et al., 2010). The stomach acts as a large reservoir where food is well mixed with enzymes through

movements (Hamosh et al., 1981). The enzymes facilitate the process of lipid digestion through the dispersion of fat droplets which are broken down into smaller droplets (Bracco, 1994) so digestion efficiency relies on the milk fat being emulsified.

The digestion of fats involves the enzymatic conversion of triglycerides into fatty acids and 2-monoglyceride by digestive lipases at the oil/water interface. Lipolysis is initiated in the stomach by the action of acid-stable gastric lipase and is continued in the duodenum by the dual action of gastric lipase and colipase-dependent pancreatic lipase in the presence of surface active bile salts. The structure of the emulsion has a considerable impact on lipolysis, with emulsion surface area being the key physicochemical factor affecting fat digestion.

2.4.1 The mouth

The first enzyme which hydrolyzes milk triglycerides (TAGs) is lingual lipase, secreted from the tongue. However, this lipase remains low in the newborn and rises only after weaning (Jensen et al., 1982). In the digestive system of the infant, the function of the mouth is to take in food and water and prepare them for chemical digestion and absorption as they pass through the gut. Swallowing acts are an autonomic reflex during the first three months. Milk is swallowed and passes through the esophagus into the stomach, where the majority of digestion takes place.

2.4.2 The stomach

The first step of dietary fat digestion occurs in the stomach. The infant stomach differs markedly from the adult's (Figure 2.8), as in the early stages of life it is

adapted principally for milk digestion. The newborn stomach can only hold about 60 to 90 millilitres of fluid. However, the capacity of the stomach increases as the baby grows (Table 2.9) (MacGregor, 2008).

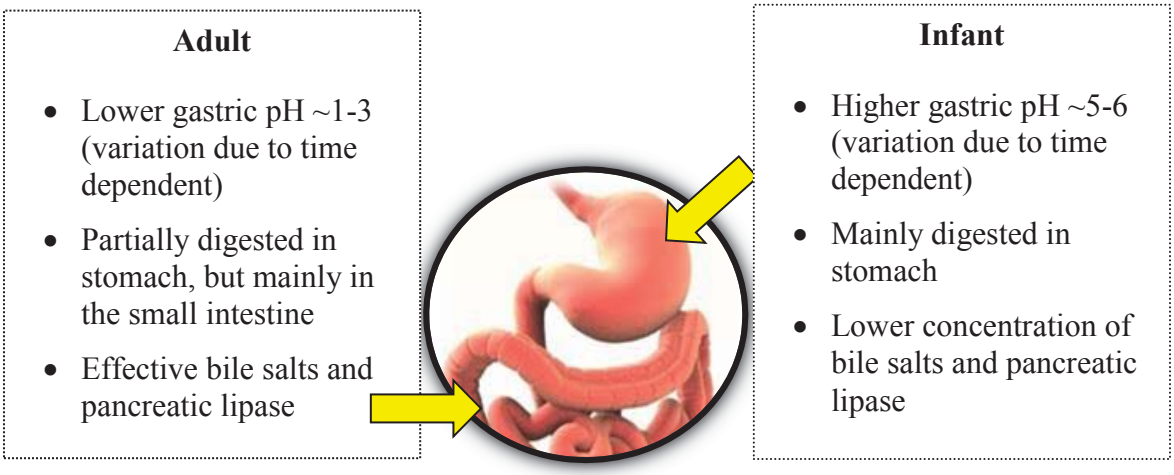


Figure 2.8 **The difference of the digestion properties between adult and infant.**

Table 2.9

Stomach capacities by age (mL). **[From (MacGregor, 2008)].**

Newborn	1 week	2–3 week	1 month	3 Months	1 year	2 years	10 years	16 years	Adult
10–20	30–90	75–100	90–150	150–200	210–360	500	750–900	1,500	2,000–3,000

The emptying time for the newborn's stomach is approximately two hours. In the stomach, contractions and peristaltic waves occur (three cycles/min) propelling the gastric content toward the pylorus. The content is then retropulsed from the closed pyrolus and this is known as the retropulsive jet that disperses oil droplets, as well as other solid particles (McClements and Decker, 2009). Significant physiological

differences based on pH and enzymatic activity between adults and infants may influence fat digestibility.

The gastric glands of the stomach include parietal cells which produce hydrochloric acid and intrinsic factor (glycoprotein). The chief cells in these glands secrete pepsinogen, which is changed into pepsin for breaking down proteins in the gastric juice (Lawrence, 1999, McCance and Huether, 2005). The components of gastric juice are mucus (salivary and gastric), gastric and lingual lipases, pepsins, urea, intrinsic factor and haptocorrin, H_2O, Na^-, Mg^{2+}, H^+, K^+, Cl^- and HPO_4^{2-}. Gastric lipolysis is essential to newborns fed with human milk, because the membrane of milk fat globules prevents the triglyceride from being hydrolysed by pancreatic lipase and bile salt-stimulated lipase (BSSL) in the intestine. The fatty acids released by human gastric lipase play an important role in triggering the activity of pancreatic lipase by changing the "quality" of the lipid-water interface (Bernback et al., 1989, Roman et al., 2007).

The various enzymes in the digestive tract of the newborn are shown in Figure 2.9. Gastric enzymes in infant stomach are proteolytic (pepsin, chymosin) and lipolytic (gastric lipase). Pancreatic enzymes in infant small intestine are proteolytic (trypsin, chymotrypsin, carboxypeptidases and elastase), lipolytic (colipase dependent lipase, bile salt dependent lipase and pancreatic lipase-related protein), and amylolytic (amylase). The summary of the characteristics of lipolytic activity in gastric aspirates of newborn infants is shown in Table 2.10.

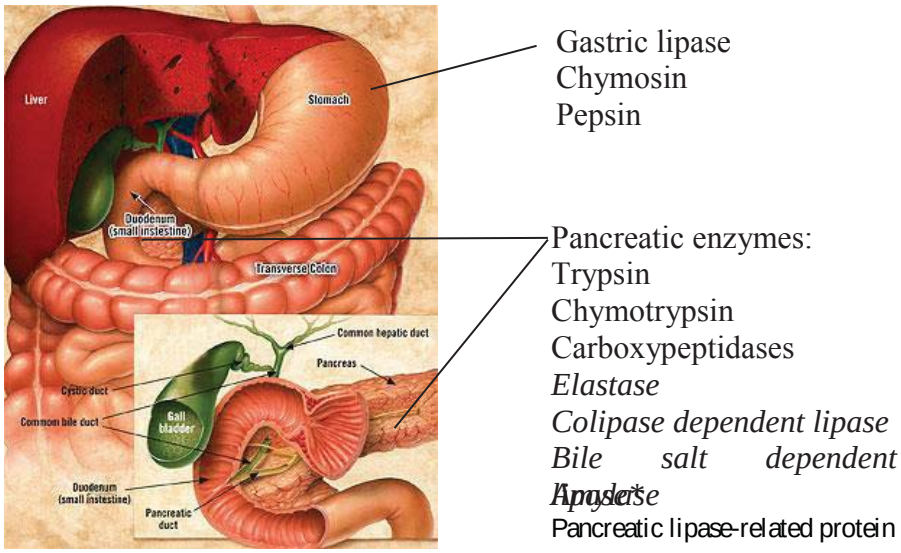


Figure 2.9 **Digestive enzymes in the digestive tract of the newborn: bold - high activity; regular - adequate activity; italics-low or trace level activity. *level of activity and/or function in the newborn infant unknown. [Adapted from (Hamosh, 1996)].**

Table 2.10

Characteristics of lipolytic activity in gastric aspirates of newborn infants. **[From (Jensen et al., 1982)].**

Factor	Specifics
Substrate specificity	a) Hydrolysis of triacylglycerols of various chain lengths (C_6-C_{18}). Activity 5 times higher on trioctanoylglycerol (C_8) than trioleoylglycerol ($C_{18:1}$). b) No activity on cholesteryl ester or phosphatidylcholine. Preferentially hydrolyzes primary ester, some s*n*-2
pH optimum	3.5–6.0
Subject to product inhibition	An excess of FFA inhibits the reaction but their removal simulates lipolysis.
Apparent mol wt of lipase	44–48,000 Daltons
Bile salts	Concentrations of 0.5–2.0 mM stimulate activity (20–30%) concentration of >2.0 mM inhibits.
Reaction products	Partial acylglycerols (DAG > MAG), FFA and glycerol

For an efficient fat digestion, the infant depends on mechanisms that are different from adults. Gastric lipolysis has also been shown to have greater significance in situations when pancreatic lipase is low in duodenal content, especially in premature and newborn infants (Figure 2.10) (Borgström and Patton, 2011).

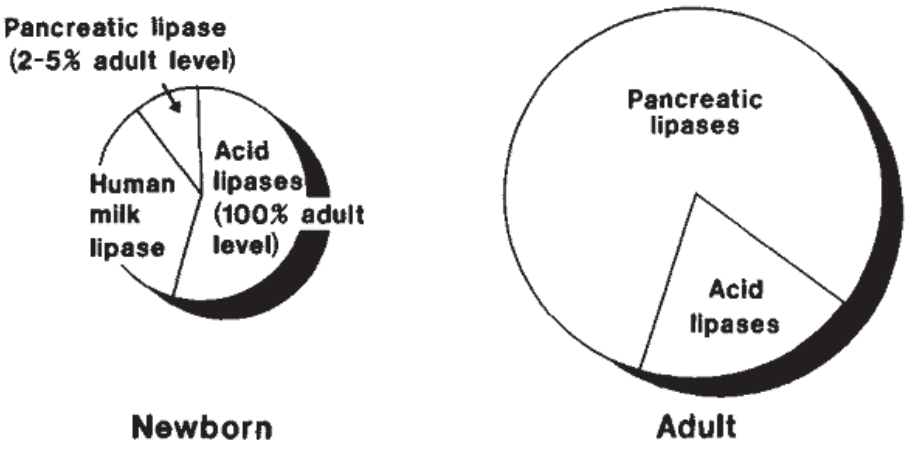


Figure 2.10 **Relative importance of different lipases for triglyceride digestion changes with age. [From (Borgström and Patton, 2011)].**

Fat digestion requires adequate lipase activity and bile salt concentrations, the former for the breakdown of triglycerides and the latter for emulsification of fat prior to and during lipolysis. The significant lipases for fat digestion in the newborn are listed in Table 2.11 and Table 2.12. Fat digestion begins in the stomach with the action of lingual or gastric lipase. Further digestion takes place in the small intestine through the action of pancreatic lipase and in the breast-fed infant of milk bile salt dependent lipase (BSDL) (Hamosh, 2006). At birth, both pancreatic lipase and carboxyl ester lipase are secreted by the pancreas in relatively low amounts (2%–5% of the adult level). Conversely, acid lipases are reported to be present at adult levels in newborns. The presence of human milk lipase (carboxyl ester lipase) during consumption of

breast milk partially compensates for low levels of pancreatic lipases (Borgström and Patton, 2011); however, of course, this enzyme is not present during consumption of formula milks.

Table 2.11

Lipases in the newborn and their contribution to fat digestion. **[From (Hamosh, 2006, Hamosh and Hamosh, 1996)].**

Lipase	**Site of action**	**Cofactors**	**Substrate**	**Contribution**	**Lipase selectivity**	**Products of digestion**
Gastric lipase	Stomach (pH 3.0–6.0)	None	Triglyceride	Moderate to high	*sn*-3	DG+FFA
Pancreatic						
Colipase-dependent lipase (CDL)	Intestine duodenum (pH ≥ 7.0)	Colipase, Bile Salts	Triglyceride, phospholipids	Low	*sn*-1, *sn*-3	MG+FFA
Carboxylester lipase (CEL)	Intestine duodenum (pH ≥ 7.0)	Bile salts	Triglyceride, other esters	Unknown to high	All positions	FFA (MG?)
Pancreastic lipase related proteins (PLRP and PRLP)	Intestine	?	Phospholipids, triglyceride	Unknown	?	?
Milk bile salt dependent lipase (BSDL)	Intestine (pH ≥ 7.0)	Bile salts	Triglyceride> other eaters	Moderate to high	All positions	FFA (MG?)

Table 2.12

Principal and potential lipases in the newborn. **[From (Hamosh and Hamosh, 1996)].**

Lipase	**Site of Action**	**Quantitative Contribution to overall fat digestion**
Gastric	Stomach	30–60%*
Pancreatic		
Colipase-dependent lipase (CDL)	Intestine (duodenum)	low
Carboxylester lipase (CEL)	Intestine (duodenum)	Low to adequate**
Pancreastic lipase related proteins (PLRP and PRLP)	Intestine	Not know
Milk bile salt dependent lipase (BSDL)	Intestine	Moderate to high***

*Studies in various species- rodents, carnivores; **High in newborn rat, very low expression in spite of high message in ferret; ***Moderate in human, cat, and dog. High in ferret.

2.4.2.1 Gastric lipase

In adults, the two major enzymes responsible for TAG digestion are the gastric lipase and the pancreatic lipase system (lipase, co-lipase and bile salts). The lipase in gastric aspirates is different from pancreatic lipase, being bile salt independent, however, it is similar to human and rat lingual lipase (Hamosh et al., 1981). Gastric lipase initiates lipolysis in the stomach by removing one fatty acid from the triglyceride molecule to produce a diglyceride molecule and a free fatty acid. Then the pancreatic lipase-colipase acts so the remaining outer fatty acid is removed from

the diglyceride to produce 2-monoglyceride (Figure 2.11) (Borgström and Patton, 2011).

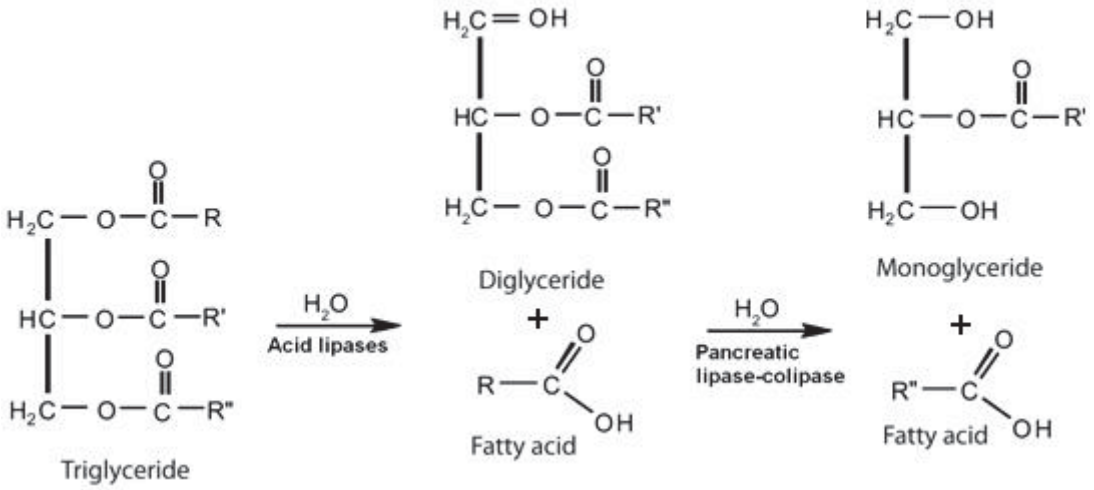


Figure 2.11 **Differing positional specificity of triglyceride lipases.**

Substrate selectivity is relevant to specific aspects of the digestion of newborn. Fatty acid and site selectivity of the gastric lipase results in the release of the *sn*–3 position fatty acid. This position locates the long–chain polyunsaturated fatty acids (LC–PUFA) which are released by the gastric lipase efficiently. The same location for medium–chain fatty acids (MCFA) in milk fat leads to their preferential release in the stomach, an observation that started the erroneous belief that gastric lipase is specific for MCFA (Hamosh, 1996). MCFA, an easily available energy source, are preferentially released. This indicates that these fatty acids are essential for infant development.

Gastric lipase is secreted (in high amounts already at birth and even in preterm infants (Atkinson and Lonnerdal, 1989)) in the gastric juice by the chief cells of fundic mucosa in the stomach (Mukherjee, 2003). The newborn infant has

considerable gastric lipase quantities which can compensate for the low pancreatic lipase activity of the neonate period (Hamosh, 1990b). Gastric lipases are glycoproteins with molecular weights of about 45,000 Da. These lipases have a broad pH optimum (2.5–7.0) and are stable down to pH 1.5 (Pearson and Hutton, 2005); the absence of requirements for specific cofactors or bile salts, the stability to pepsin and a low pH optimum (~2–4) enable these enzymes to act in the stomach (Hamosh, 1996, Hamosh et al., 1981). Gastric lipases are therefore able to survive in the stomach's acidic environment and will be active during feeding when the gastric pH rises to around 5.0. Secretion of gastric lipase is coupled with pepsin secretion by peptic cells in response to pentagastrin (a functional analogue of gastrin) (Pearson and Hutton, 2005). Gastric lipase is the predominant pre-duodenal lipase in humans; lingual lipase is present in trace amounts. This as opposed to other species like rodents, where lingual lipase is predominant (Mukherjee, 2003).

In the infant, the digestion of TAGs by gastric lipase in the stomach plays an important role in lipid digestion. Gastric lipases can penetrate into the MFG and initiate the digestive process (Hamosh, 1990a). Lipase reaction occurs at the interface between the aqueous and the oil phases (Reis et al., 2009). The enzyme binding to the surface of emulsified lipids is known as the interfacial activation (Miled et al., 2000).

Milk fat, the primary source of nourishment for infants, is primarily composed of MCT, an ideal substrate for acid lipases. Moreover, the pancreatic lipase system is not fully developed in the newborn (McClements and Decker, 2009). Whilst gastric lipase is suited to hydrolyse fat in the gastric environment, it can continue to be active in the proximal small intestine (Hamosh, 1990b). It is estimated that 30–60%

lipid hydrolysis take place in the stomach for infants, whilst only 15−30% takes place for adults. It is interesting to consider whether this variance is due to physiological or dietary changes during growth.

2.4.2.2 Pepsins

Protein consists of long chains of amino acids bound by peptide linkages. Enzymes attack peptide (–CONH-) linkages to break down proteins into amino acids. These enzymes can be categorised into two types: 1) Endopeptidases which split the peptide linkage in the interior of the molecule to break the long chain molecule into short chain peptides, and 2) Exopeptidases which split the terminal amino acids and break the short chain peptides into amino acids (Ghosh, 2006).

Pepsins are found in the gastric juice while the pancreatic juice consists of trypsin and chymotrypsin. They are all endopeptidases (Ghosh, 2006). Pepsins are a group of proteolytic enzymes secreted by the central cells as the proenzyme pepsinogen and activated by acid cleaving of terminal amino acid chain; hence the relationship between acid, pepsin and pepsinogen is not mutually exclusive (Newton, 2004). Pearson and Hutton (2005) stated that the optimum pH for pepsins on protein substrates is in the acidic range 1.9−3.6. At a pH greater than 5.0, limited pepsin is activated, and the rate of enzyme active increases with decreasing gastric pH. Pepsins have a broad specificity against large molecular peptides, preferentially cleaving proteins at phenylalanine, tyrosine, and leucine residues (Untersmayr and Jensen-Jarolim, 2008). They have been shown to be effective in hydrolysing soluble and insoluble proteins, and are able to hydrolyse interfacial proteins stabilising emulsion systems. However, it is interesting to note that the peptides produced as a

consequence of surface hydrolysis are not necessarily the same for an equivalent non adsorbed protein.

2.4.2.3 Gastric pH

In the adult, the pH of the stomach rises from a range of 2.0 to 2.5 before meals to 4.5 to 5.8 during and immediately after feeding, subsequently decreasing during digestion hence it is time dependent. In the newborn infant, the pH of the stomach is between 3.0 and 5.0 at 3 to 4 h after a meal and immediately preceding the next feeding; during and immediately after the meal, the pH can rise up to 5.5 (Hamosh, 1990b) or even up to 6.8 (Mason, 1962). In order to hydrolyse dietary triglycerides in the stomach the lipase in gastric aspirates would have to have an optimum pH compatible with the gastric milieu (Hamosh, 1990b). Therefore this research will be carried out at several pHs (2, 3.5, 4.5 and 5.5) to cover the effect of a pH range.

In newborn infants gastric acidity is influenced by several factors. Gastric acid secretion occurs even in the most immature infants, but immediately after birth the gastric pH may even be alkaline, with pH ranges from 6–8, due to residual alkaline amniotic fluids in the stomach (Freer and Lyon, 2005). The relatively alkaline milk consumed by the infant further decreases gastric acidity. pH then falls to a pH range of 1.5 to 3 within 24 to 48 hours after birth, due to increasing acid production, in order to facilitate digestion of protein by pepsin or destruction of ingested bacteria. Gastric acidity may also be influenced by milk feedings.

Early on Mason (1962) showed the changes in intraluminal pH during feeding of human milk or infant formula in 25 term infants in Figure 2.12.

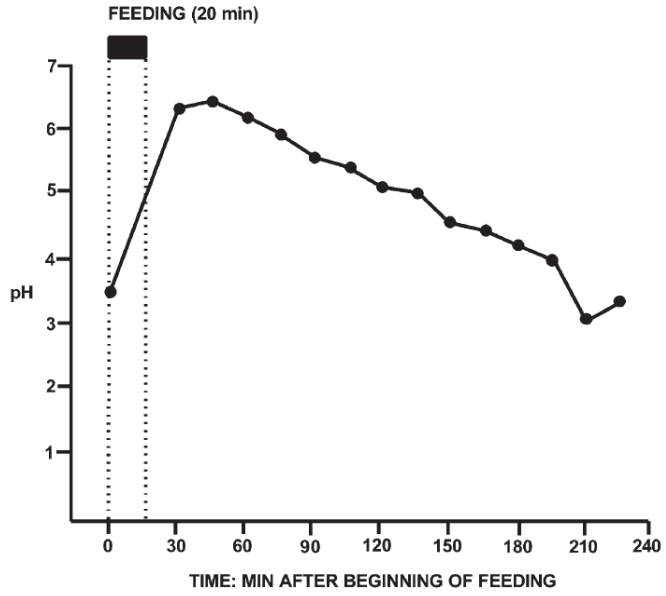


Figure 2.12 **Gastric pH during feeding, mean values of pH of the stomach contents of 25 newborn infants during the postprandial period. [From (Chatterton et al., 2004, Mason, 1962)].**

The mean gastric pH values reaches to 6.4 within 30 min after feeding, then the pH decreases subsequently with digestion time. This graph indicates that the pH in the stomach of newborn infants is far from the optimum pH of the gastric protease pepsin. The optimum pH of the gastric enzymes pepsin and gastricsin are found to be pH 2.0 and 3.0, respectively (Richmond et al., 1958). Hence, this should affect the digestion of milk protein as most of the protein would still be undigested during the first hour of digestion given this high pH. However, this alkaline pH at the beginning of digestion time might suit the lipase activity (pH 2.5–7) (Pearson and Hutton, 2005). Gastric pH reaches adult values by two years of age (Shields, 2003, MacGregor, 2008). An *in vivo* study of breast feeding has shown that ~90% of milk

protein was emptied at 3 h following ingestion from the stomach as compared to 1 h (Chatterton et al., 2004).

The rate of lipolysis is controlled, not by the amount of enzyme, but by the amount of interfacial area available for lipase binding (Lundin et al., 2008). There may be two reasons for lipase's greater importance in the newborn: 1) the sudden change from the high carbohydrate diet of the fetus to the high fat diet of the newborn and, 2) the immaturity of exocrine pancreatic function (Shi and Burn, 2004). The development of pepsin and gastric lipase is shown in Table 2.13. The pepsin and lipase activity and output in the human stomach comparing newborns and adults is shown in Table 2.14.

Table 2.13

Development of gastric digestive enzymes: pepsin and gastric lipase. **[Adapted from (Hamosh and Hamosh, 1999)].**

Age	**Human**	
	Pepsin	**Lipase**
10–20 wk	2.0	4.0–5.0
25–35 wk human	ND	60–100
Newborn 1 wk	ND	100
Newborn 4 wk	18	100

Data are % of adult activity; ND = not determined.

Table 2.14

Comparison of pepsin and lipase activity and output in the human stomach between infant and adult. **[From (Hamosh and Hamosh, 1999)].**

	Enzyme activity*		Enzyme output**	
	Pepsin	**Lipase**	**Pepsin**	**Lipase**
Infant	125	10.0	597	25.0
Adult	600	5.7	3352	23.0

*Enzyme specific units per mL gastric aspirate; **units $\times$vol·kg^{-1} body weight.

Due to lipid composition, droplet size and milk globule surface layers, the digestion of cow's milk and infant formula milk differ from that of human milk. This has implications for non breast-fed babies. In addition to droplet size, the structure and composition of the MFGM has an influence on the metabolic rate of dietary TAGs. Previous studies have reviewed that the digestion rate of human milk fat globules with a native membrane is faster than that of much smaller homogenised fat globules of infant formula with modified fat globule membranes (Kontkanen et al., 2010, Michalski and Januel, 2006). This presents something of a paradox, in that the formula emulsions present a considerably larger surface area for lipase adsorption than breast milk. The implication here is that the structural variance between formula and biological milks may play an important role in the digestive discrepancy of these two systems. This particular research hypothesis forms the basis of this study, and the research plan has accordingly been designed to determine how emulsion design, notably in terms of interfacial composition, influences the subsequent digestive behaviour of biological and manufactured milks, most specifically during the gastric stage of digestion.

2.4.3 The small intestine

The small intestine in newborns is about 250 to 300 centimetres compared to 600 to 800 centimetres in adults. It can be subdivided into the duodenum, jejunum, and ileum. The duodenum ranges from 7.5 to 10.0 centimetres in length and about 1.0 to 1.5 centimetres width. The rest of the small intestine composes of the jejunum which constitutes the proximal two–fifths whereas the ileum is three–fifths (Crelin, 1973). The enzyme values in duodenal juice of infants are significantly lower than those of older children (Manson et al., 1999). The chime from the stomach is mixed in the duodenum with digestive juices from three sources: the pancreas, the liver and the intestinal mucosa itself (Rumsey, 2005). The pH gradually increases from 4–5 at the duodenum to 7–8 in the ileum (MacGregor, 2008). The passage of nutrients through the small intestine is relatively slower for infants than for adults.

In the first three months, the infant's pancreatic juice contains little lipase which limits the baby's capacity to convert fat into fatty acids and glycerol. Moreover, specific long–chain polyunsaturated fatty acids are present in breast milk, which are necessary for brain development. During the first six months, only small amounts of pancreatic amylase are produced to digest more complex food. If foods are too difficult to breakdown, the lack of the relevant digestive enzymes allows material to pass undigested to the colon (MacGregor, 2008).

As mentioned earlier in this chapter, in adults, pancreatic lipase is the main enzyme involved in the digestion of TAGs in the small intestine (Caro et al., 2004) resulting in products which can be absorbed into the intestinal wall. This enzyme is inactive at the concentration of bile salts necessary for the formation of micelles or the "critical

micelle concentration". A protein cofactor or colipase is required to overcome the inhibition. Colipase apparently recognises and binds to a TAG molecule in the presence of bile salts, and then lipase binds to this complex (Jensen et al., 1982). In infants, pancreas and liver functions are not optimal and the reduced availability of pancreatic lipase and bile salts could and apparently does curb the digestion and absorption of dietary fats in the intestine. The problem of low absorbability of infant formulae is intensified by the resistance of fat droplets to the action of pancreatic lipase in the absence of prior exposure to lingual lipase and bile salt–stimulated lipase (BSSL) (Jensen et al., 1982).

In conclusion, the neonatal period is crucial for the digestion and absorption of fatty acids, not only for energy, but also for neural and retinal development. Human milk fat is especially constituted to suit human babies such as by the inclusion of a lipase (which may compensate for the "immature" pancreatic exocrine function found in the first weeks of life) (Manson et al., 1999), the droplet size of MFG, and the specific milk composition. However, many babies cannot be breastfed or a wet nurse may not be an option for reasons of safety, cost or mother's convenience. Therefore an alternative milk that suits babies' needs and fat requirements is needed. There is a great deal known about the fat digestion in adults, but little is known about the fat digestion in neonates, even though there are important differences between adult and infant digestion. This gap in knowledge provides the context for this research study.

Chapter 3 Materials and Methods

3.1 Materials

3.1.1 Human breast milk

Breast milk was obtained from volunteers in Palmerston North, New Zealand. There was no direct collection from the subjects. All the expressed milk was surplus to the mothers' requirements and would otherwise have been discarded. The milk samples were collected in a container with mothers using a breast pump or expressing by hand if preferred. The collected breast milk was stored in the fridge (4°C) in sterilised containers and it was immediately transported to the laboratory. This validation sub-study focused on the breast milk of women over the age of 18 years old, who had no serious illness during their pregnancy (see Appendix C).

Ethics

This breast milk project was reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 13/08 (see Appendix C).

3.1.2 Commercial infant formulae

Commercial infant formulae were classified into two categories: ready-to-use liquid infant formulae and powder infant formulae. The three liquid infant formulae included S–26® Gold (UHT liquid milk), Similac® Advance (UHT liquid milk), and Enfamil® Premium Infant (retorted liquid milk). The nine powder infant formulae included Anmum® Infacare, Enfamil® Premium Newborn, Enfamil® Premium

Infant, Enfamil® up & up, Heinz® Nurture Gold Starter1, Karicare®, Mom to Mom®, S–26® gold and Similac® Advance. All milks were appropriate for feeding normal full-term newborn infants during the first six months of life.

All commercial infant formulae were purchased at a supermarket. The milks were stated to contain ~3.6% fat and ~1.4% protein and to comply with the US Federal Regulations. The principal protein sources were stated as non-fat (cows') milk and mineral reduced whey. The liquid milk samples were used as obtained. The powdered samples were prepared by dissolving them in deionised water according to the manufacturers' instruction. Hence, the ratio of powder: water (w/v) used to make up the samples and provide ~1.4% protein content in each case were: 14.8% (w/v) (Karicare®), 14.67% (w/v) (Anmum® and Enfamil® Premium Infant), 14.50% (w/v) (Enfamil® premium Newborn, Heinz® and Similac®), 14.33% (w/v) (Enfamil® up & up and Mom to Mom®) and 14.17% (w/v) (S–26® Gold).

3.1.3 Raw cows' milk

Samples of raw cow milk were collected from the Fonterra Research and Development Centre, Palmerston North, New Zealand and used as obtained. They were kept at 4°C in a 60 mL clear vial and treated with 0.02% (w/w) sodium azide, added as preservative. They were stirred for 1 hour at room temperature pending use.

3.1.4 Intralipid®

Intralipid®, a lecithin–stabilised emulsion of soya oil suitable for intravenous administration to human subjects was purchased from Fresenius Kabi Australia Pty

Limited, New South Wales, Australia. The solution contained 20% (w/v) triglyceride, 1.2% (w/v) phospholipid and 2.2% (w/v) glycerol. As such 500 mL Intralipid® contained 100 g soya oil, 6 g egg lecithin, and 11 g glycerol, titrated with sodium hydroxide q.s. to pH 6.0 to 9.0. Osmolality was 350 mOsm/kg. The energy content was 4200 kJ (1000 kcal) per 500 mL. The material was stable when stored below 25°C.

3.1.5 Lactoferrin

Bovine lactoferrin powder was supplied from the Fonterra Research and Development Centre, Palmerston North, New Zealand. The powder contained 99.7% protein, of which 95.8% was lactoferrin. The powder contained 0.3% moisture and 0.8% ash. The iron content of the powder was 130 ppm.

3.1.6 Enzymes

For the simulated gastric fluid (SGF), porcine gastric pepsin (EC 3.4.23.1; P7000, activity of 800–2500 U/mg) was purchased from Sigma-Aldrich Pty Ltd., St. Louis, MO, USA. Fungal lipase (Enzidase® FL8000; activity of 80 U/mg) was obtained from Zymus International Limited, Auckland, New Zealand. The fungal lipase used in this experiment was a purified food grade lipase powder derived from a selected strain of *Rhizopus oryzae* (ATCC 1996) and was characterised by its ability to hydrolyze triglycerides. It was determined as being effective up to a temperature of about 50°C, with an optimum temperature of ~40°C. Moreover, it was stable over a wide pH range, from about 2.0–10.0, with an optimum pH of about 6.5 (Ahmed and Traistaru, 2009). Calf pregastric lipase (Biocatalysts) was a gift from the Salkat

Laboratory. Its optimum pH was within the range 5.7–6.4 and its optimum temperature was within 37–48°C (Kremer et al., 1997). Sodium chloride (NaCl) was purchased from LabServe (Biolab (Aust) Ltd).

3.1.7 Walstra solution A

Walstra solution A used in the emulsion particle size measurements comprised 0.375% (w/v) disodium ethylene diamine tetra-acetate (EDTA) and 0.125% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20). It was dissolved in water and adjusted to pH 10 with NaOH. Tween 20 is able to displace proteins from the interface of oil droplets and EDTA can disaggregate casein micelles, hence this solution is able to break up any flocs formed to distinguish between droplet flocculation and coalescence. The alkaline pH of 10 inhibits the pepsin activity and inhibits ongoing proteolysis and also prevents any further change in particle size following sampling

3.1.8 Chemicals

This study used RO water and deionised water (Milli-Q water, Millipore, Bedford, MA, USA) throughout the experiments. All the chemicals used were purchased from BDH Chemicals (BDH Ltd, Poole, England) or Sigma Chemical Co. (St. Louis, MO, USA), unless specified otherwise. Analytical grade chemicals were used.

3.2 Methods

3.2.1 Composition of simulated gastric fluid (SGF)

The choice of lipase for use in the *in vitro* simulations of gastric digestion was restricted owing to shortages of suitably standardised human gastric lipase (HGL). Furthermore, the lack of definitive understanding of the kinetics of the binding and cleaving sites of this enzyme, and of the pH microclimate within the active sites, creates difficulties in choosing a lipase from an alternative source that has matching kinetics and a similar optimal pH range of 4.0–6.0 (Frayn, 2010).

Fungal lipase is well characterised (Hiol et al., 2000), exhibiting similar regio-specific hydrolysis of triglyceride substrates to that of HGL and is acid stable. Like human lipase it possesses an α/β hydrolase fold covered by an amphiphilic lid which becomes accessible only after location of the enzyme on the oil water interface (Brzozowski et al., 1991). Suitably standardised supplies are more readily available and have been used in other *in vitro* models (Golding et al., 2011, van Aken et al., 2011). However, the 'optimal' pH of hydrolysis by *Rhizopus oryzae* lipase is 7.5 and the enzyme is said to be most stable in the range pH 4.5–7.5 (Hiol et al., 2000). These values are some distance from the lower of the two pK_a's of porcine gastric lipase which is stated to be 3.5 (Campos and Sancho, 2003) and lies outside of the reported pH range of activity of human gastric lipase (pH 4–6) (Aloulou and Carrière, 2008). Whilst there has been considerable debate in regard to other fungal lipases as to whether the reported pK_a's and range of optimal activity reflect the local pH at the active site rather than that of the bulk solution (Poulsen et al., 2005), it is

likely that significant lipolysis does not occur when ambient pH lies in the very acidic range e.g. between pH 2 and 3.

Pregastric calf lipase is mammalian in origin and has a somewhat lower optimal pH than that of *Rhizopus* lipase, with the maximum rate of hydrolysis occuring at pH 6.5. The enzyme exhibits specificity for the *sn*-1 or -3 positions in the triglyceride (Manuel, 1999). Ruminant lipases generally have optimal pH ranges around 5.6–6.5 extending to below 5.5 in the young (Lai et al., 1997).

Given the dichotomy in the kinetics and optimal pH ranges of the two enzymes, it was elected to conduct the bulk of studies with the *Rhizopus oryzae* enzyme and to check critical results with pregastric calf lipase. There was good equivalence for analyses when experimental data for the two lipases were compared.

3.2.2 Stimulated gastric fluid preparation

Simulated gastric fluid (SGF) was prepared by dissolving 200 mg of sodium chloride (NaCl) and 1.3 mL of 1M hydrochloric acid (HCl) in 80 mL of Milli-Q water. The pH of the solution was adjusted to 2, 3.5, 4.5 or 5.5 with 1 M sodium bicarbonate solution ($NaHCO_3$) or 1 M HCL solution and the total volume made up to 100 mL with Milli-Q water. Four hundred and fifty mg of dry powdered porcine pepsin (800–2500 units/mg protein) and/or 20 mg of dry powdered *Rhizopus oryzea* lipase equivalent to 1,600 IU (80 U/mg) were added to this solution and dispersed by constant stirring at 37°C, at 10 rpm for 10 minutes. The pH of the digesta was continuously monitored during *in vitro* digestion and was maintained at the selected value by appropriate drop-wise additions of bicarbonate or HCl solution. A further

simulation was conducted under otherwise identical conditions substituting the *Rhizopus oryzea* lipase with pregastric calf gastric lipase in the simulated gastric fluid.

Other researchers have used different amounts of pepsin/mL gastric juice in the *in vitro* digestion models, such as 0.4 mg/mL (Golding et al., 2011), 1 mg/mL (Versantvoort et al., 2004), 1.25 mg/mL pepsin (Ruby et al., 1993), 3.2 mg/mL (Sarkar et al., 2009) and 5 mg/mL (Versantvoort et al., 2005). Based on verbal discussion with an expert currently working on infant digestion (T. J. Wooster, personal communication, July 29, 2010) a choice of 4.5 mg/mL was chosen as being representative of stomach digestion in infants. Similarly, the choice of 0.2 mg/mL of lipase is based on previous work with adult digestion models as this is also considered appropriate for infant *in vitro* studies (T. J. Wooster, personal communication, July 29, 2010). The rate of lipolysis is not absolutely dictated by the amount of lipase present, but by the lipases' ability to access to the interface of the emulsified droplet (Golding et al., 2011).

3.2.3 *In vitro* gastric digestion

Liquid formula and other emulsions containing 3.6% (w/v) fat (as shown on the label), were used as substrates. Each emulsion was mixed with SGF (milk: SGF ratio = 20:50 v/v). The pH of the mixture was maintained at either pH 2.0, 3.5, 4.5 or 5.5 so as to span the physiological range of post prandial variation in pH. The specific pH was maintained by the appropriate ongoing drop-wise addition of either 1 M HCl or 1M $NaHCO_3$. The mixture was incubated in a 400 mL beaker at 37°C for 2h while stirring by using a bar (7 cm long and 1 cm diameter) and maintained at a specific

rpm i.e. either 0 rpm, 10, 20, 50 or 100 rpm on a magnetic stirrer (2mag magnetic motion, bioMIXdrive, John Morris Scientific). A 1 mL subsample of digestate was removed from the beaker every 15 minutes. The size of the stir bar was chosen to match the size of the container and thus to distribute mixing across the bulk of the fluid.

It is noteworthy that gastric contractile activity is incompletely developed in neonates (Chen et al., 1997) with antral peristalsis being reduced and irregular (30% of that in adults). The shear rate in the infant's stomach is likely to be lower than in the adult stomach. Therefore, the lowest of the range of stirring speeds (10 rpm - which was the lowest practicable speed, given the limitations of the stirrer) was used in the bulk of the experiments as it most closely approached the magnitude of shear generated during contractile activity in the infant stomach. The maximum shear rate developed at the tip of the stirring bar when it was revolving at 10 rpm was 16.25 s^{-1}. The linear velocity of retropulsion in the human adult stomach was calculated to be around 7.5 mms^{-1} (Pal et al., 2004) and the shear rate is 0.7 s^{-1} (Lentle and Janssen, 2011, Lentle et al., 2010). Thus the velocity at a stirring speed of 10 rpm in this experiment was somewhat greater than that in the adult stomach.

A series of *in vitro* digestion experiments were conducted for 120 minutes at pH 3.5, each with a different stirring speed i.e. 0 rpm, 10 rpm, 20 rpm, 50 rpm and 100 rpm and hence maximum shear rate i.e. 0, 16.25, 32.5, 81.25, 162.5 s^{-1}, respectively.

3.2.4 Emulsion characterisation

The physicochemical properties of MFG in different milk samples were analysed at the Food Characterisation Laboratory at Massey University and Fonterra Research and Development Centre.

3.2.4.1 Size distribution

Emulsions were measured using the light scattering of static laser light in a Malvern MasterSizer 2000 Hydro MU (Malvern Instruments Ltd, Malvern Worcestershire, UK). This laser diffraction technique is suitable for measuring the particle size of emulsion samples and is able to determine particle sizes ranging from 0.02 to 2000 μm. The characterisation of emulsion was analysed under high dilution conditions by dispersion within a water-filled tank of Mastersizer. The main components of the instrumentation are shown in Figure 3.1. This instrument consists of the three parts.

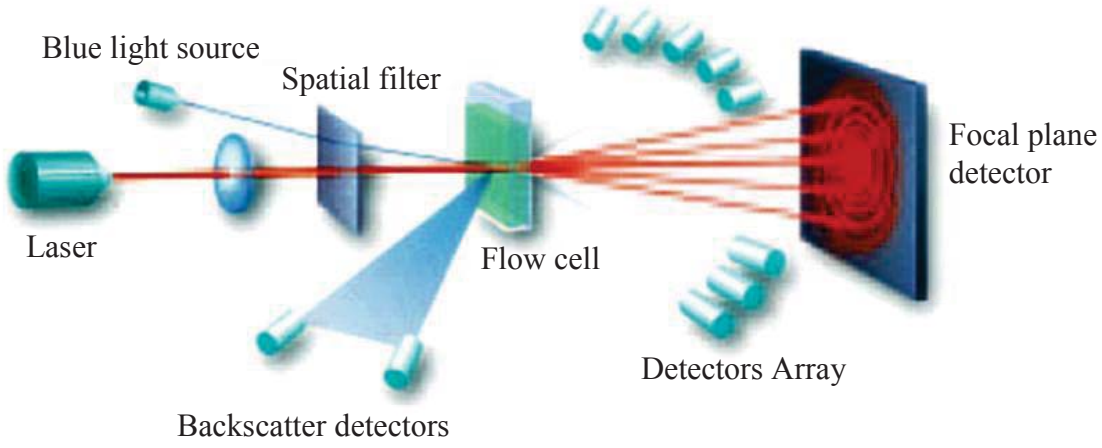


Figure 3.1 **Main components of the instrumentations. Laser light scattering technique. [From (MasterSizer 2000)].**

i) Light source: a red laser beam generated by a mixture of helium–neon (He–Ne). Gas laser is used as an emitter of coherent intense light of fixed wavelength ($\lambda = 633$ nm). This instrument also uses a single wavelength (monochromatic) blue light source ($\lambda = 466$ nm) to improve resolution, so the very small signals from submicron size particles can be detected. Therefore, this wide-angle dual wavelength detection system (or the combination of the two light sources, blue light and red laser) is designed for measuring a wide range of particle sizes, between the lower (0.02 μm) and higher (2000 μm) ends. When the particles are passed through a focused laser beam, the intensity of the scatter light at an angle, that is around the sample and inversely related to their size, is measured and recorded by a series of photosensitive detectors. The wide angle scattering measurements are related to smaller particles while the narrow angle scattering measurements are sensitive to large particles.

ii) A measurement cell and dispersion unit: the measurement cell is situated in the path of the laser beam and it is in here where the light is scattered by the particles. The sample dispersion is pumped continuously from the wet sample dispersion unit which was designed for stirring the dispersant and ensuring the sample circulates through the measurement cell by pumping (Storti and Balsamo, 2010).

iii) An array of fixed-angle detectors located around the sample, measure the intensity of the scattered light as a function of scattering angle. The light scattering adsorbed by the material is showed as the %obscuration and also indicates the amount of sample which has been added into the dispersant. The scattering pattern is recorded by the detectors and sent to a computer. A calculation of particle size

distribution is based on theoretical models by fitting the experimental data (*i.e.* the proportion of scattered light falling on each detector element in the array) to data obtained from theoretical mathematical analysis.

The parameters for particle size used in this experiment are defined below:

i) The *volume mean diameter* (*De Broucker mean*), which is mathematically termed d_{43}. The d_{43} parameter refers to the sphere of equivalent volume for the real sized particle (of arbitrary shape). This parameter is used as a sensitive mean diameter value for monitoring changes in droplet-size distribution diversity. d_{43} is defined by equation 3.1:

$$d_{43} = \frac{\sum_{i} n_i d_i^4}{\sum_{i} n_i d_i^3} \tag{3.1}$$

where n_i is the number of droplets of diameter d_i.

ii) The *surface area mean diameter* (*Sauter mean*) or d_{32} refers to the specific surface area for the real sized particle. This parameter is used as a sensitive mean diameter to identicate the presence of fine particulates in the size distribution. d_{32} is defined by equaion 3.2:

$$d_{32} = \frac{\sum_{i} n_i d_i^3}{\sum_{i} n_i d_i^2} \tag{3.2}$$

Values of d_{32} are mostly useful for considering changes in the average area of particle surfaces since the parameter is proportional to the inverse total surface area of oil droplets (A_s) as shown in quation 3.3:

$$A_s = \frac{6V}{d_{32}} \tag{3.3}$$

where V is the total volume of oil droplets.

Calculation of each droplet distribution was used to quantify the degree of coalescence. The refractive index of the fat phase was set at 1.456 and that of the aqueous phase at 1.33 giving a ratio of 1.095. The droplet size distributions were determined after dilution of the aliquot with sufficient RO water to achieve a requisite obscuration value of 15%. Each aliquot was analysed in triplicates.

The effects of various shear rates and enzyme composition on droplet size and emulsion stability were explored by comparing the droplet size distributions obtained with and without dilution with a dissociating agent (Walstra solution A). For each sample of digestate, droplet sizes distributions were determined after 1:9 dilution with water or with Walstra solution A. By comparing these two, the changes in particle size due to flocculation can be distinguished from those due to coalescence.

3.2.4.2 Microstructure characterisation

Emulsion microstructure was visualised by confocal scanning laser microscopy (CSLM). Aliquots from each sample of digestate were stained with 5 μl of 1% (w/v) aqueous solution of Nile blue (to dye the fat) and a 5 μl 1% (w/v) aqueous solution of Fast green FCF (to dye the protein). An aliquot portion of each subsample was placed on a concave confocal microscope slide (Sail, Sailing Medical-Lab Industries Co. Ltd, China) with a cavity and covered by a cover slip (0.17 mm thick).

Confocal images of emulsions were obtained using a Leica Microsystem CSLM, a motorised z-focus, and a 40 or 63× oil immersion objective lens (Leica SP5 DM6000B, Heidelberg, Germany) operating in fluorescent mode. The oil phase was imaged in stained slides with argon laser light at 488 nm and the protein with helium/neon laser light at 633 nm. The images were subsequently captured and processed in Adobe Photoshop CS version 8.0 (Adobe Systems Incorporated, San Jose, CA).

The most important application of confocal microscopy is to obtain optical sections of the sample at different depths in a non-destructive way. This technique uses multiple mirrors (typically two or three scanning linearly along the x x−y plane to scan the laser across the sample. A schematic representation of a typical CLSM is given in Figure 3.2.

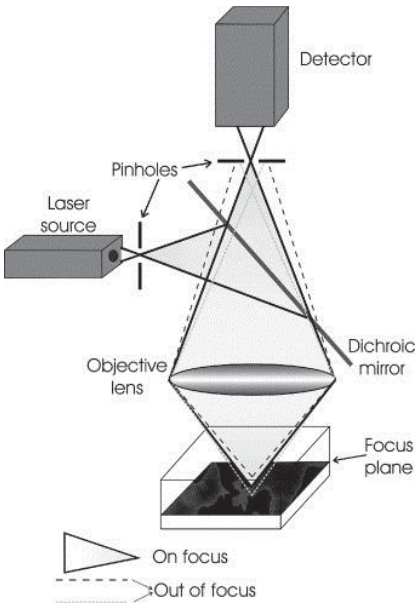


Figure 3.2 **The principle of the confocal microscope. [From (Menéndez et al., 2001)].**

The laser light beam is deflected by the dichroic mirror and focused onto a small spot on the sample in the x−y direction. The fluorescence in the sample is excited and then emits light at a lower wavelength, which passes through the objective lens, and through the dichroic mirror (also called a "dichromatic mirror"), and is focused down to a spot surrounded by a pinhole as two cones of light above and below the focal plane (Yao and Wang, 2005, Matia-Merino, 2003). All the light not coming from this point is eliminated. The detected light passing the image pinhole is received by the detector (called photomultiplier). In a conventional microscope, there is no pinhole and all of the light collected by the objective lens will be detected (Menéndez et al., 2001, Dean, 1998).

In CLSM the pinhole blocks all fluorescence light originating from regions above or below the in-focus plane. Therefore, only the light from the illumination volume is detected, and this is referred to as optical sectioning. Three-dimensional microstructures can be obtained by gathering optical sections at different depths in the specimen by adjusting the specimen in an axial direction (Loren et al., 2007).

3.2.4.3 Droplet charge

The ζ-potential value was determined with a Malvern Zetasizer Nano ZS model ZEN 3600 (Malvern Instruments Ltd, Malvern Worcestershire, UK). This Zetasizer employed a combination of laser doppler velocimetry (LDV) and phase analysis of scattered light (M3-PALS) to measure particle electrophoretic mobility. The surface charges of the emulsion droplets were quantified by determining the electrical potential just outside the Stern plane of the charged surface (Figure 3.3A).

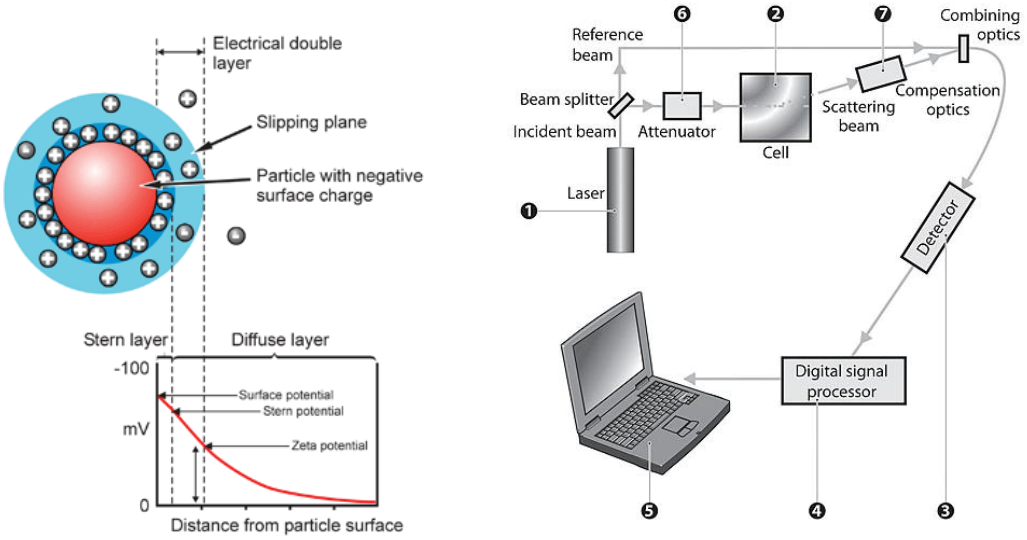


Figure 3.3 **Schematic representation of ζ-potential (A). Optical configuration of the Zetasizer Nano series for ζ-potential measurements (B). [From (Zetasizer Nano series technical note)].**

The main components of zetasizer are illustrated in Figure 3.3B. A laser provides a light source which then splits to give an incident and reference beam to the particles within the sample cell. Then any particles that move through the measured volume cause the intensity of light detected to fluctuate. The frequency of the light that fluctuates is related to the particle speed. The scattered light angle is detected and recorded to a computer.

The ζ-potential is related to particle electrophoresis. When an electrical field is applied across the sample, the charged droplets move towards the oppositely charged electrode. The viscous forces exerted by the surrounding liquid tend to prevent this movement and when the equilibrium is reached, between the opposing forces, the droplets move with a constant velocity (v), which is referred to as electrophoretic mobility. This micro-electrophoresis system is a capillary cell with electrodes at

either end, to which a potential is applied. The droplets accelerate towards the electrode, at a certain velocity, which is measured by the frequency shift of the incident laser beam. The measured velocity (U_E) was converted to ζ value by the Henry equation (equation 3.4):

$$U_E = \frac{2\varepsilon\zeta f(\kappa a)}{3\eta} \tag{3.4}$$

where, ε =dielectric constant, η = viscosity, and $f(\kappa a)$ = Henry's function. The $f(\kappa a)$ value is 1.5, referred to as the Smoluchowski approximation, which is used for aqueous media.

Aliquots of digestate were diluted with Milli-Q water and their volumes subsequently adjusted with either 1 M HCl or 1M $NaHCO_3$ solution (chosen to match the pH of the sample) to give an oil droplet concentration of approximately 0.01% (v/v). One millilitre of the sample was injected into one of the ports of a universal folded capillary cell (DTS 1060, Malvern Instruments Ltd., Malvern, Worcestershire, UK) equipped with platinum electrodes and any air bubbles were removed before inserting the stopper. The ζ-potential measurements were undertaken at five sequential readings (at 20°C) and data were reported as means and standard deviations.

This ζ-potential value (unit in mV) may change by several mechanisms such as the dissociation of ionogenic groups in the particle surface and the differential adsorption of solution ions into the surface region. The net charge at the particle surface affects the ion distribution in the nearby region, increasing the concentration of counterions close to the surface. Thus, an electrical double layer is formed in the

region of the oil/water interface. In an electric field, as in microelectrophoresis, each particle and its most closely associated ions move through the solution as a unit, and the potential at the surface of shear between this unit and the surrounding medium. When a layer of macromolecules is adsorbed on the particle's surface, it shifts the shear plane further from the surface and alters the ζ-potential.

3.2.4.4 Statistical analysis

All experiments were analysed in either three (for droplet size) or five (for droplet charge) measurements carried out on two freshly samples, unless otherwise specified. Results are reported as the calculated means and standard deviations.

Chapter 4 Characteristics and physicochemical properties of different types of milks

4.1 Abstract

For all infant mammals, including humans, nutritional lipids are provided by the milk fat globule (MFG). Whilst this is an essential nutritional component in milk, it is well known that there are compositional differences between native milk (human breast milk and raw cow milk), and commercial infant formulae (liquid and powder). These differences can potentially affect the rate of digestion in each type of milk, with previous human studies indicating that infants digest formula more slowly than breast milk (Splinter and Schreiner, 1999). In considering fat digestion, the core of the MFG contains triacylglycerols, and is surrounded by a milk fat globule membrane (MFGM), which is primarily comprised of phospholipids and proteins (Singh, 2006). The MFGM acts as an emulsifier and protects the MFG from coalescence and degradation (Dewettinck et al., 2008). Infant formulae, in turn, may comprise a number of other amphiphilic species including: phospholipids, monoglycerides and milk proteins (Montagne et al., 2009). In this respect, the interfacial composition of commercial formulae is less well defined, potentially varying not only as a consequence of composition, but also due to processing conditions. Therefore, it is necessary to first define the physicochemical properties of milk and formula emulsions (i.e. structure, size distribution, and ζ-potential), in order to understand the effect of milk fat globules on the digestion of different types of milk.

The findings showed native milk (raw cow milk, human milk) to have markedly larger droplet size (d_{43}) compared to the powder formulae and liquid formula, respectively. The ζ-potential of liquid formulae was greater than that of powder formulae and breast milk. In addition, the size distribution and ζ-potential varied among the different brands of powder formula depending on their ingredients and manufacture process. In order to enable the visualisation of the structure of MFG emulsion, confocal microscopy was used. Confocal microscopy results were in agreement with particle sizing data, indicating a smaller droplet size distribution for liquid formula milks. Visualisation of droplets also showed the adsorption of protein at the interface, with some formula milks showing extensive protein coverage. Findings from this study can be considered in relation to differences in digestibility of infant milks, both human and formula, that have been observed in prior human trials (Leung and Sauve, 2003).

4.2 Introduction

Milk fat globules are a major component in human milk, and are surrounded by a milk fat globule membrane, composed of phospholipids, cholesterol, glycoproteins and enzymes. The membrane acts as an emulsifier and protects the fat globule from coalescence and degradation (Dewettinck et al., 2008). Human studies have shown that infants digest infant formulae more slowly than human milk (Splinter and Schreiner, 1999), A possible hypothesis is that the composition of fat globule, and the membranes may affect the efficiency of the enzymes and cofactors in the gastrointestinal tract.

Comparison of the casein: whey protein composition of human and cow milks reveals that cow's milk (80:20) has much higher casein content than human milk (40:60). For infant formulae to mimic human milk the gold standard for infant formula composition proteins need to be adjusted: whey proteins, which can easily adsorb at oil/water interfaces, are added in the aqueous phase after casein has been removed from liquid milk products so that infant formulae closer resemble breast milk. In terms of legislation, it is stated that infant formulae contain, per 100 kcal of intake, the following (FSANZ, 2011): (a) not less than 1.8 g and not more than 4 g protein of nutritional quality equivalent to that of casein or greater quantity of other protein in proportion to its nutritional quality. In New Zealand, infant formula contains per 100 kcal of intake the following: (a) not less than 1.9 g and not more than 2.9 g protein; (b) not less than 4.4 g and not more than 6.3 g fat.

The physicochemical properties of protein–stabilised emulsions may vary: their stability depending largely on interactions between the emulsion droplets and environmental conditions that affect these interactions (McClements, 1999). Previous studies show that the structure of fat globules and their membrane may be a key point affecting the functional properties of milk in the newborn (Michalski et al., 2005b, Ye et al., 2010). In order to understand the instability of milk emulsions, it is necessary to know these physical and chemical parameters. This chapter aims to investigate the physicochemical properties such as structure, size distribution, droplet charge, and protein composition of different milk samples relevant to this study.

4.3 Materials and methods

4.3.1 Materials

4.3.1.1 Human breast milk

The method of breast milk collection and sampling has been described in Chapter 3. Ethics approval was obtained from the Massey University Human Ethics Committee. Samples were transferred into sterilised containers and stored at 4°C before used within one day.

4.3.1.2 Cows' milk

Raw cows' milk was provided by Fonterra Research and Development Centre, Palmerston North, New Zealand. The raw milk was stored at 4°C before measurement and prepared as described in Chapter 3.

4.3.1.3 Commercial infant formulae

Commercial infant formulae were classified into two categories: ready-to-use liquid infant formulae and powder infant formulae. Three liquid infant formulae; S–26® Gold (UHT liquid milk), Similac® Advance (UHT liquid milk), and Enfamil® Premium Infant (retorted liquid milk) and 8 powder infant formulae including Anmum® Infacare, Enfamil® Premium Newborn, Enfamil® Premium Infant, Enfamil® up & up, Heinz® Nurture Gold Starter1, Karicare®, Mom to Mom®, S–26® Gold and Similac® Advance were used in this experiment and prepared as described

in Chapter 3. The details of the compositions of commercial infant formulae are given in Table 2.6.

4.3.1.4 Reagents

Wastra solution A was used to break up any flocs formed. 0.375% (w/v) EDTA and 0.125% (v/v) Tween20 were dissolved in water and adjusted to pH 10 with NaOH as described in Chapter 3.

The analytical grade chemicals were purchased from BDH Chemicals (BDH Ltd, Poole, England). Chemical was used. Both reverse osmosis (RO) water and Milli-Q water (water purified by treatment with a Milli-Q apparatus, Millipore Corp.) were used.

4.3.2 Methods

4.3.2.1 Droplet size distribution measurement

The size distribution of particles in emulsion samples was measured using a MasterSizer 2000 Hydro MU following the method as described in Chapter 3.

4.3.2.2 Microstructure characterisation

The microstructure evaluation under Confocal Scanning Laser Microscopy (CSLM) was used as described in Chapter 3.

4.3.2.3 Surface charge

The surface charge quantified by the ζ-potential of droplets was measured with a Malvern ZetaSizer Nano ZS (ZEN 3600) instrument (Malvern Instruments Ltd, Malvern Worcestershire, UK) following the method described in Chapter 3.

4.4 Results

4.4.1 Droplet size distribution

The study compared the fat globule size distributions of different milk samples. The milk samples analysed were: human breast milk, raw cow milk, powder formulae and liquid formulae. Particle size distributions for these tested milks are shown in Figure 4.1. Native milk (human breast milk and cows' milk) has clearly larger mean droplet size compared to processed milk (infant formulae). Additionally, human breast milk d_{43} (~5 μm) presents smaller mean droplet size compared to raw cow's milk d_{43} (~9 μm). Native cow's milk fat globule size distribution normally ranges between 0.2 and 15 μm (Michalski et al., 2002), confirming that the distributions shown here are within this range.

There are some differences among the fat globule size distributions of the various powder infant formulae brands. The mean particle diameters (d_{43}) of all these commercial powders (S–26®, Karicare®, Heinz®, Enfamil® Premium Newborn, Enfamil® Premium Infant, Enfamil® Up & Up, Anmum®, Similac® and Mom to Mom®) are in the range of 0.3–3.1 μm, with a monomodal and/or bimodal particle size distributions. Some powder infant formulae such as Anmum® and S–26® (Figure

4.1) showed a slight bimodal distribution with a first peak at ~0.3 μm and a second smaller peak observed around 10 μm. This second peak is likely to be a greater amount of poorly homogenised fat globules (~10 μm). Commercial liquid infant formulae, either UHT (S–26®, Similac®) or retort milk (Enfamil® Premium Infant), all presented similar mean droplet size (d_{43} ~0.3 μm) with the former showing slightly higher d_{32} (~0.25 μm) versus 0.18 μm in UHT milks (Table 4.1).

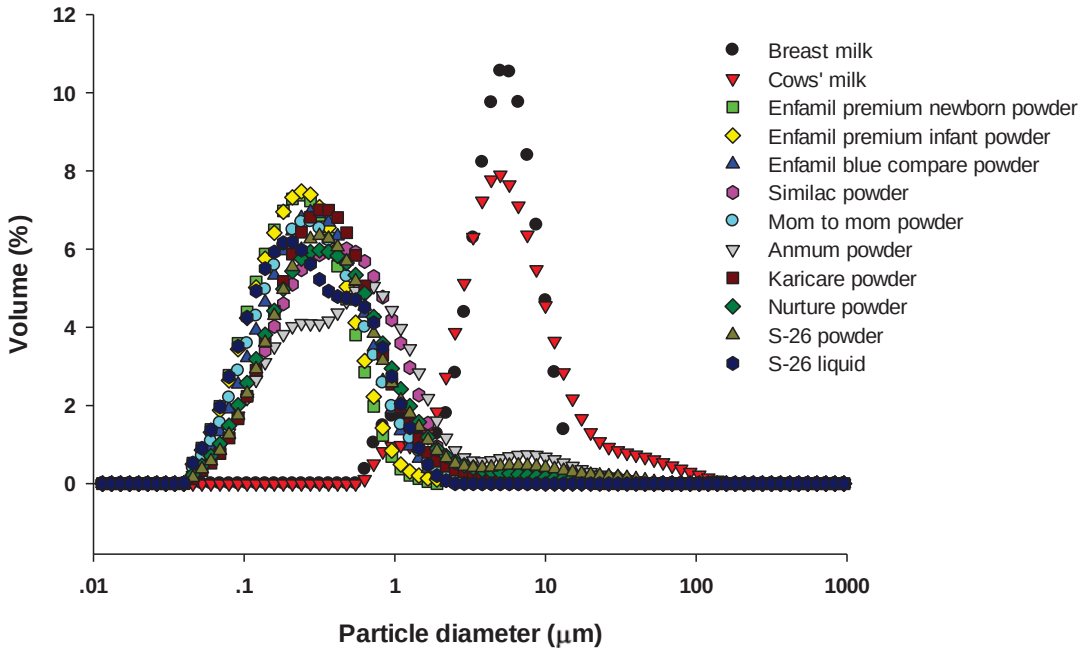


Figure 4.1 **The size distribution of milk fat globules of different milk samples.**

Table 4.1

The physicochemical characteristics of the different milk samples.

Samples	Brands	pH	d_{43} (μm)	d_{32} (μm)	Specific surface area (m^2/g fat)	ζ-potential (mV)
Native milk	Cow's milk	6.64	8.753±0.208 [a]	3.880±0.010 [a]	1.533±0.006 [l]	–35.2±0.4 [b,c]
	Human breast milk	7.23	4.991±0.002 [b]	3.269±0.002 [b]	1.970±0.000 [l]	–20.7±0.7 [a]
Powder infant formulae	Heinz® nurture	6.78	3.164±0.530 [c]	0.261±0.001 [c]	22.733±0.058 [j]	–33.3±1.2 [b]
	Anmum®	7.05	1.397±0.043 [d]	0.285±0.003 [c]	20.833±0.153 [k]	–34.8±3.2 [b,c,d]
	S–26® Gold	7.23	1.156±0.033 [d]	0.251±0.002 [c]	25.633±0.115 [h,i]	–36.3±2.3 [b,c,d,e]
	Karicare®	6.94	0.693±0.031 [e]	0.219±0.001 [c]	27.133±0.153 [g]	–35.1±1.0 [b,c]
	Similac® Advance	7.23	0.488±0.001 [f]	0.256±0.001 [c]	25.133±0.115 [i]	–36.6±2.0 [c,d,e,f]
	Enfamil Premium Newborn	7.05	0.471±0.019 [f,g]	0.172±0.001 [d]	37.467±0.058 [a]	–38.5±1.8 [f]
	Enfamil® Up & Up	7.33	0.441±0.002 [f,g]	0.205±0.001 [c]	31.433±0.115 [f]	–36.6±2.1 [c,d,e,f]
	Mom to Mom®	7.31	0.431±0.002 [g]	0.195±0.000 [c]	33.033±0.058 [e]	–35.8±1.9 [b,c,d,e]
	Enfamil® Premium Infant	7.10	0.324±0.001 [j]	0.174±0.000 [c,d]	36.967±0.058 [b]	–37.0±2.0 [c,d,e,f]
Liquid infant formulae	Enfamil® Premium Infant (Retort)	6.79	0.371±0.000 [h]	0.249±0.002 [c]	25.833±0.153 [h]	–37.3±0.2 [e,f]
	S–26® Gold (UHT)	6.96	0.338±0.000 [i]	0.180±0.001 [c]	35.900±0.000 [c]	–37.1±1.1 [d,e,f]
	Similac® Advance (UHT)	7.00	0.319±0.001 [k]	0.185±0.001 [c]	34.833±0.058 [d]	–37.3±1.0 [d,e,f]

Means are expressed ± SD. Values in the same column carrying different superscripts are significantly different ($p< 0.05$) by Tukey's Test.

4.4.2 Confocal microscopy

The comparisons of the fat globule size distributions by volume obtained using light scattering (Mastersizer) and the corresponding CSLM images are shown in Table 4.2. The microstructure of biological milks (human and cow's milk) clearly showed high density protein region (in green colour) with distinctive fat droplets (in red colour).

The CSLM images of human breast milk and raw cows' milk sample shows greater fat globule sizes than powder and liquid formulae. Confocal imaging of milk is less effective in representing the droplet size distribution of fat droplets compared to light scattering analysis (Figure 4.1 and Table 4.1). However, large big fat globules can be easily visualised even if are not always picked up in the light scattering measurement. The resolution of the instrument does not effectively visualise droplets in the first quartile of the distribution, this is typically <0.2 μm. However, droplets of size typically >0.5 μm are more clearly visualised. A common feature of infant formula confocal images is the prevalence of protein that appears associated or bound to the fat droplets. Structural variations of the protein phase are observed between samples, with powder compositions showing more extensive protein aggregation compared to liquid compositions. Liquid formulae showed very fine emulsion whereas big oil droplets were very distinctive in powder formulae.

Table 4.2

Comparison of the fat globule obtained using the Mastersizer and CSLM for different types of milk samples. Samples were stained with Nile blue (for fat) and Fast green (for protein). (Scale bar: 20 µm).

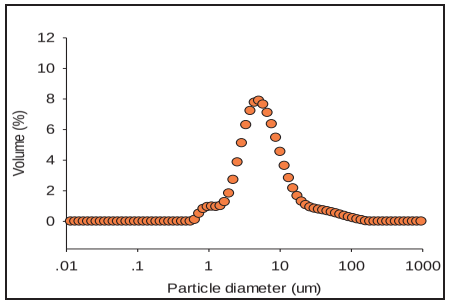
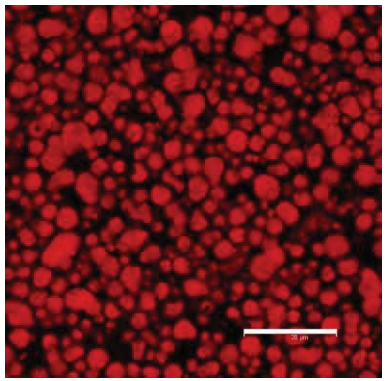
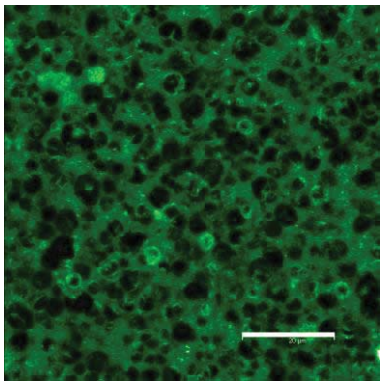
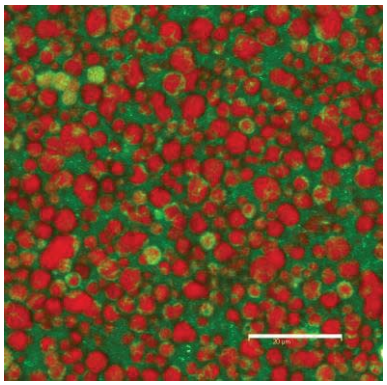
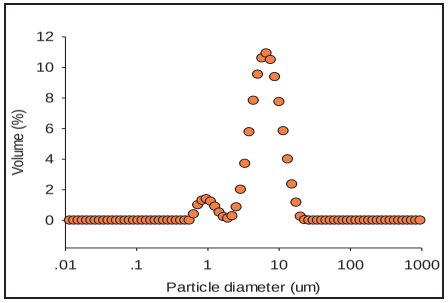
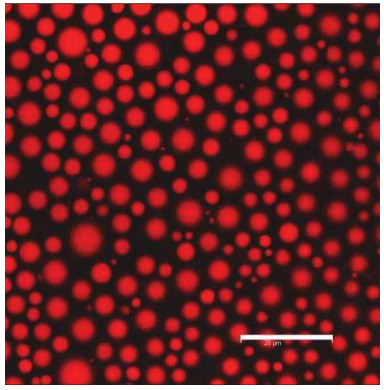
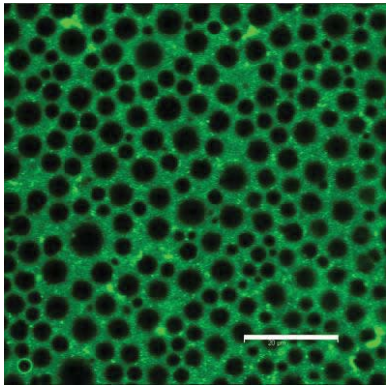
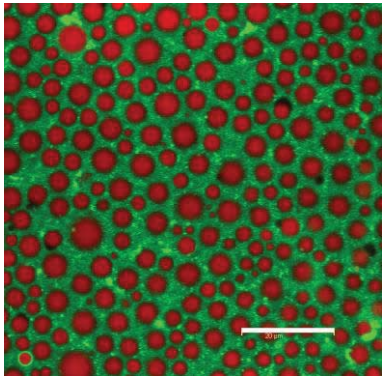
Type of milk sample	Characteristic		
	(A) Lipid phase	(B) Protein phase	(C) Lipid and protein phase
1) Raw cows' milk 			
2) Breast milk 			

Table 4.2 (*Continued*).

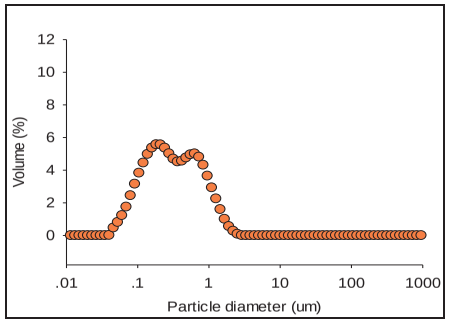
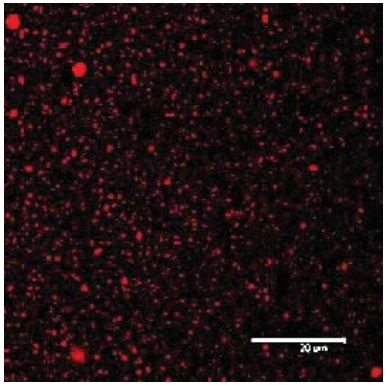
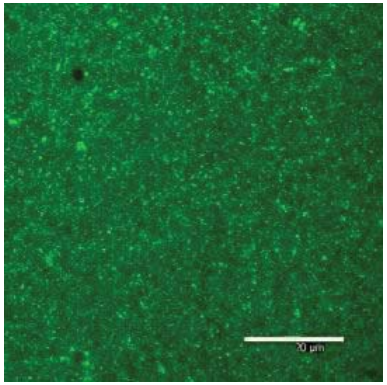
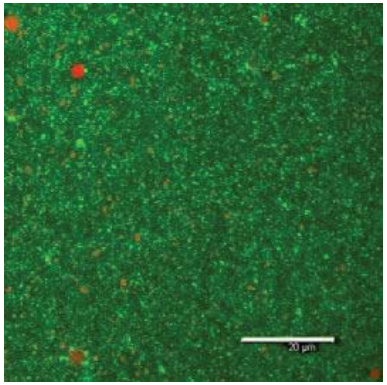
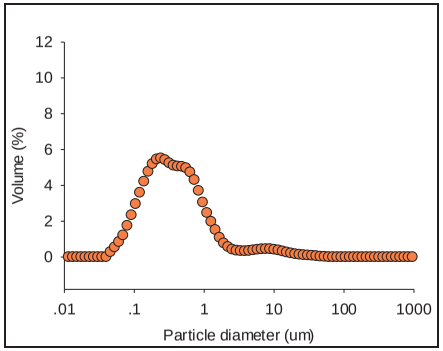
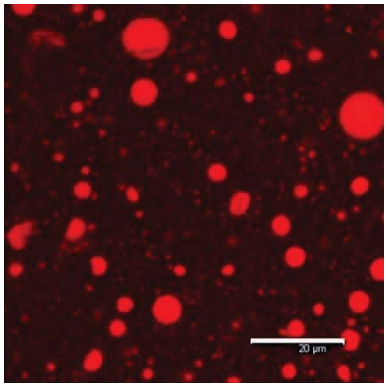
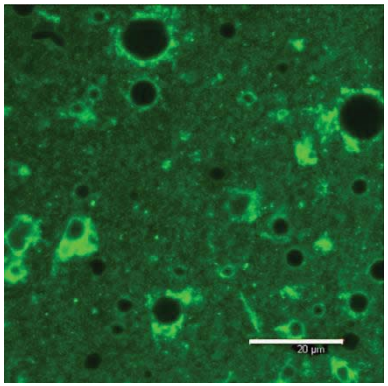
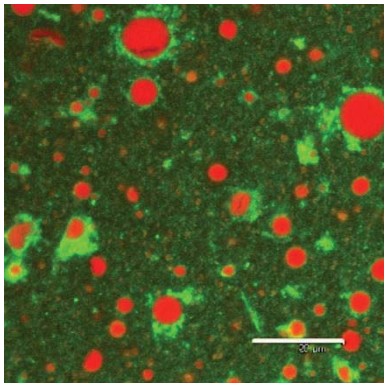
Type of milk sample	Characteristic		
	(A) Lipid phase	(B) Protein phase	(C) Lipid and protein phase
3) S–26® (liquid, UHT) 			
4) S–26® (powder) 			

Table 4.2 (*Continued*).

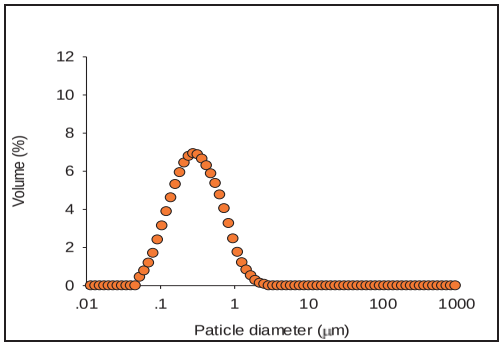
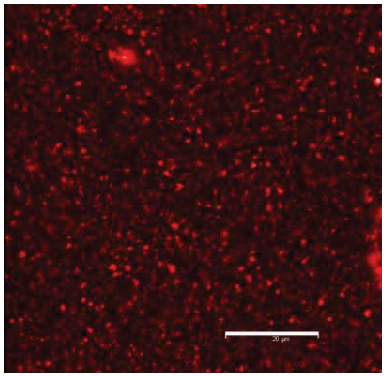
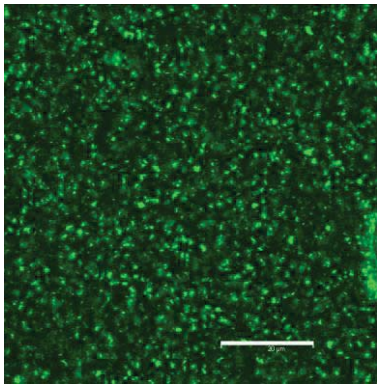
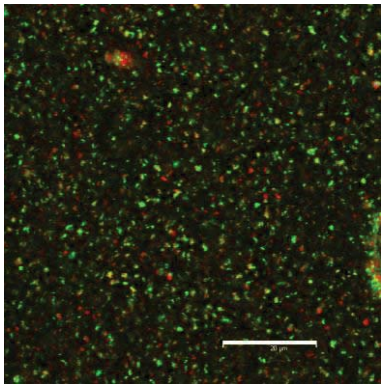
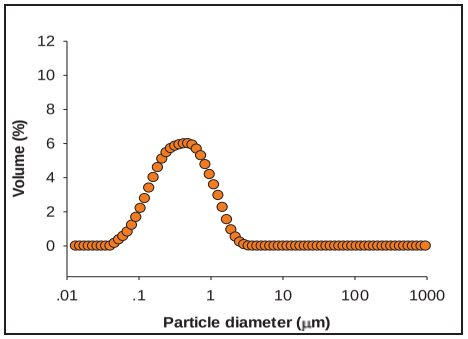
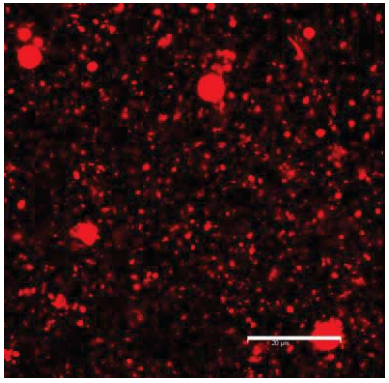
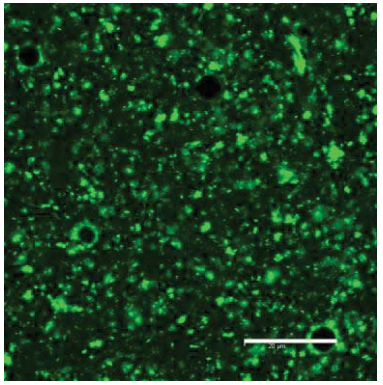
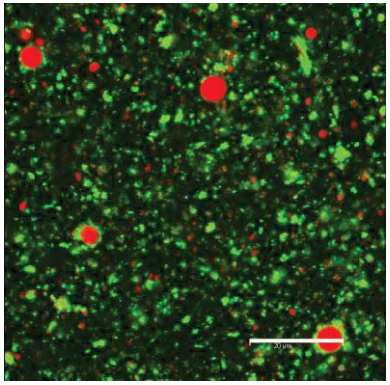
Type of milk sample	Characteristic		
	(A) Lipid phase	(B) Protein phase	(C) Lipid and protein phase
5) Similac® (liquid, UHT) 			
6) Similac® (powder) 			

Table 4.2 (*Continued*).

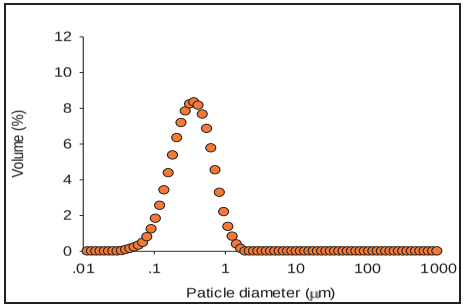
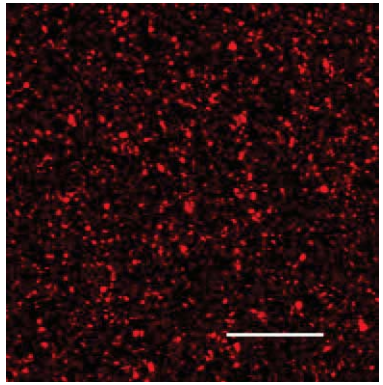
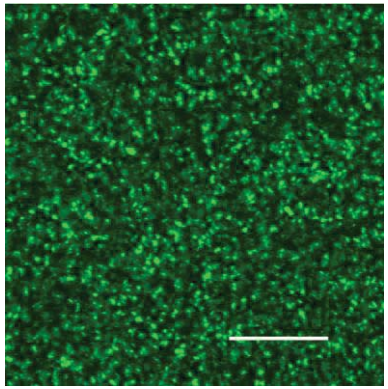
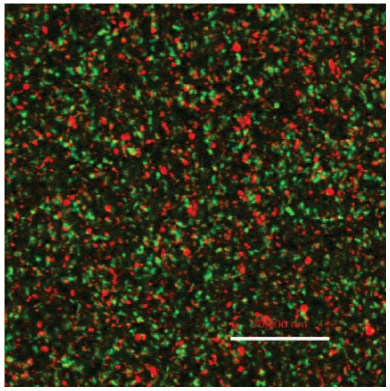
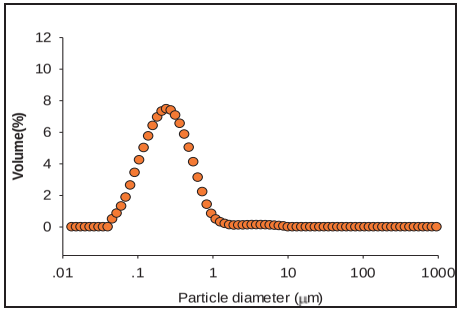
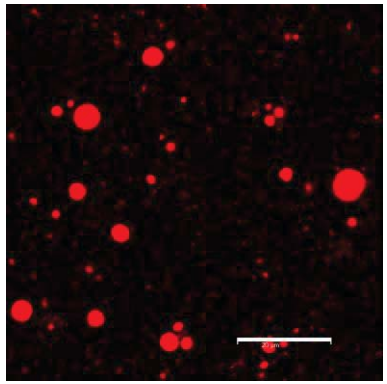
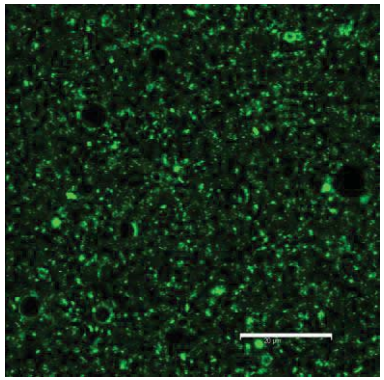
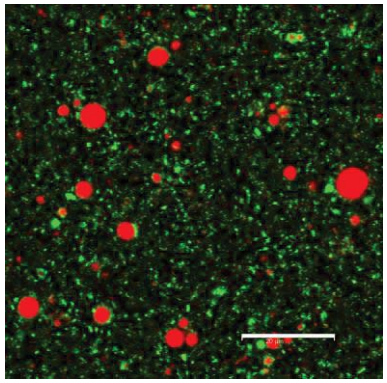
Type of milk sample	Characteristic		
	(A) Lipid phase	(B) Protein phase	(C) Lipid and protein phase
7) Enfamil® Premium Infant (liquid, retort) 			
8) Enfamil® Premium Infant (powder) 			

Table 4.2 (*Continued*).

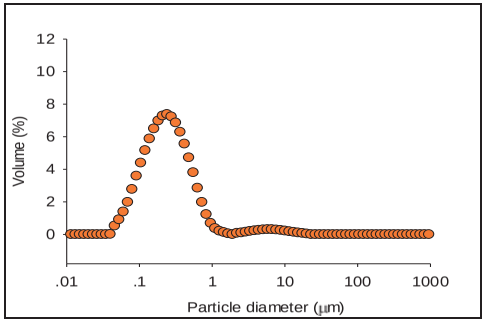
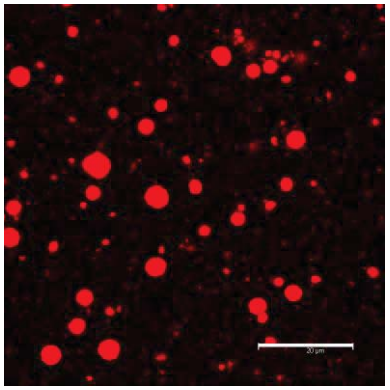
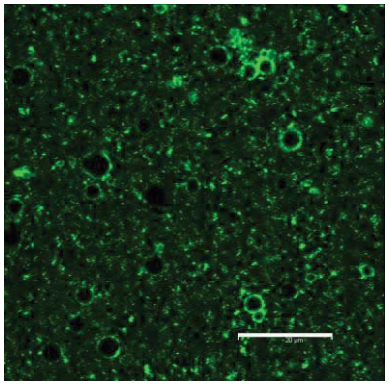
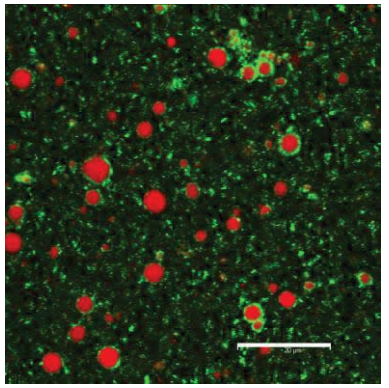
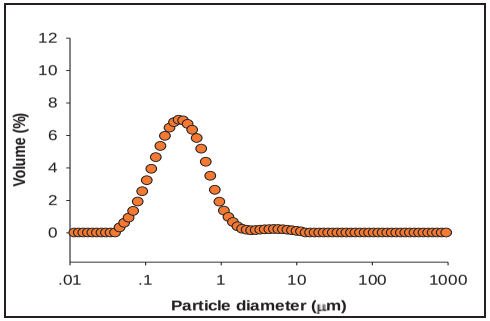
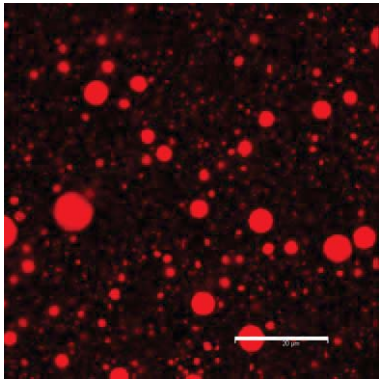
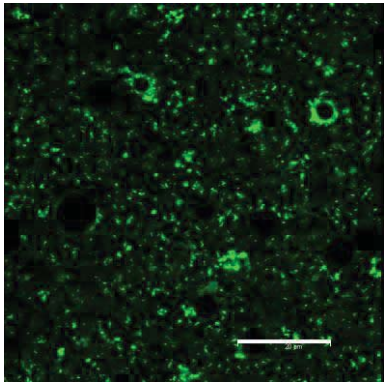
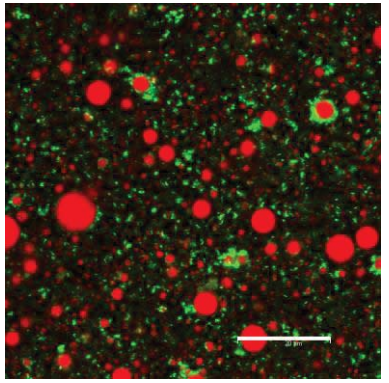
Type of milk sample	Characteristic		
	(A) Lipid phase	(B) Protein phase	(C) Lipid and protein phase
9) Enfamil® Premium Newborn (powder) 			
10) Enfamil® Up & Up (powder) 			

Table 4.2 (*Continued*).

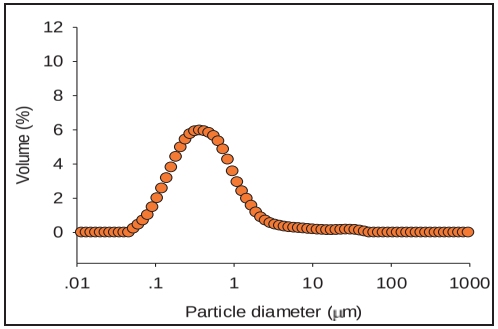
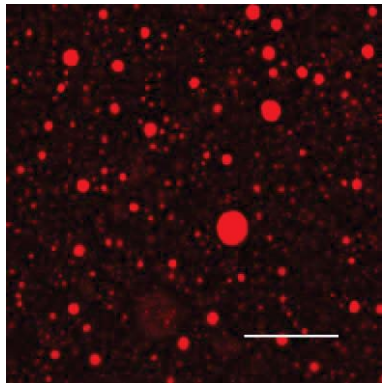
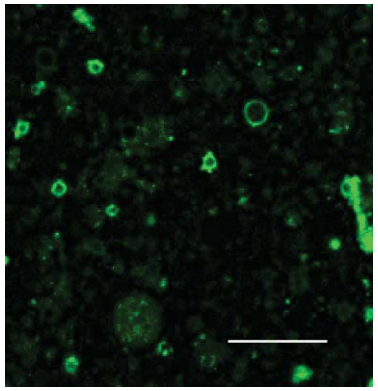
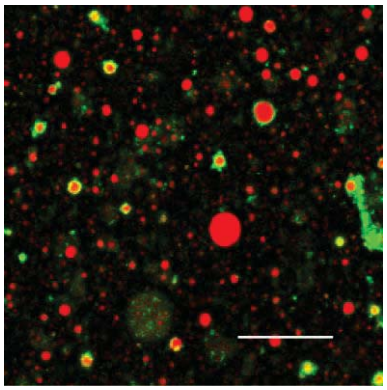
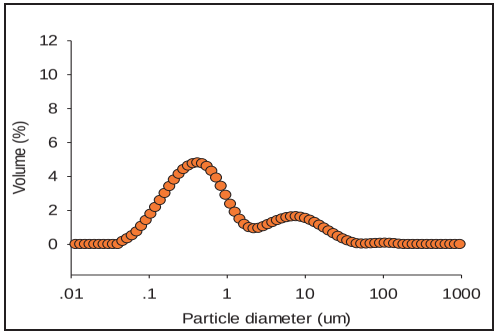
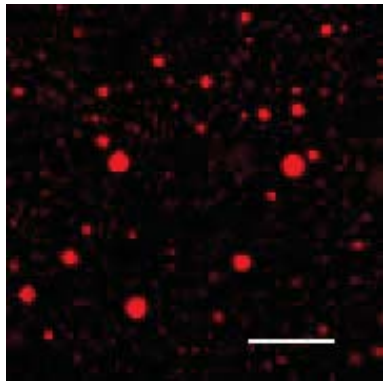
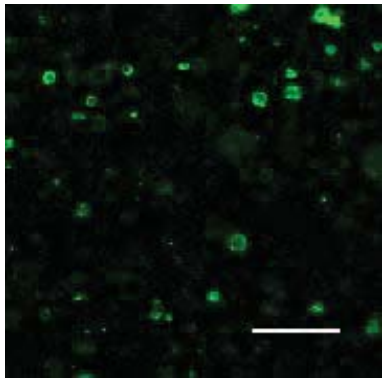
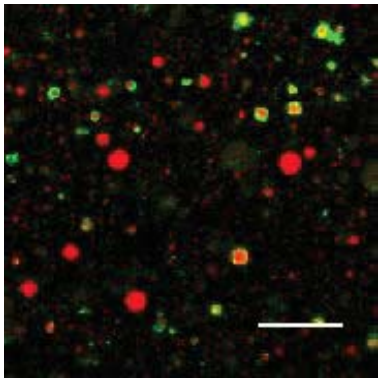
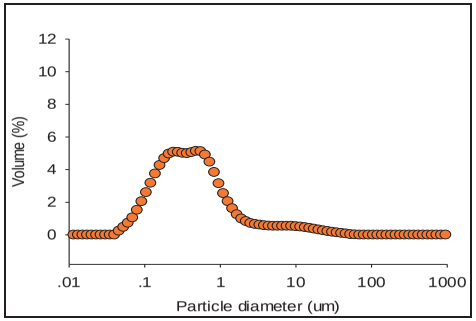
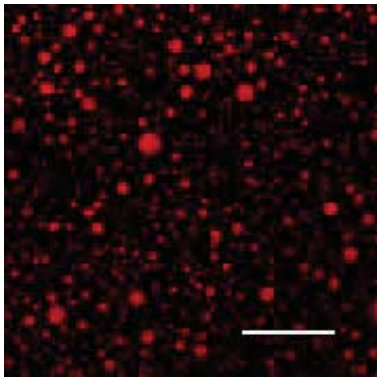
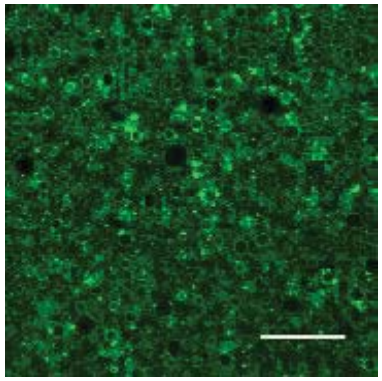
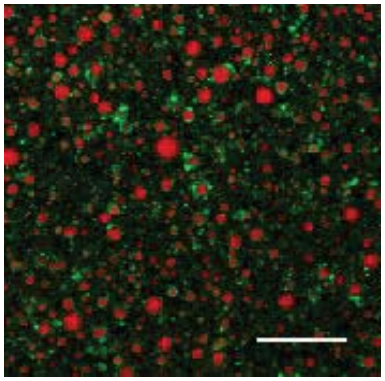
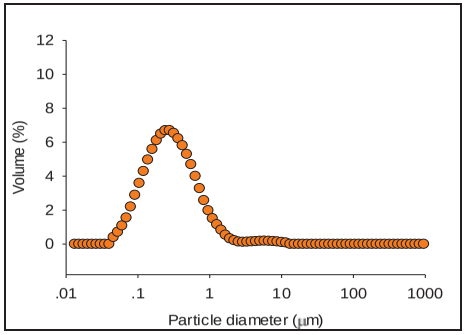
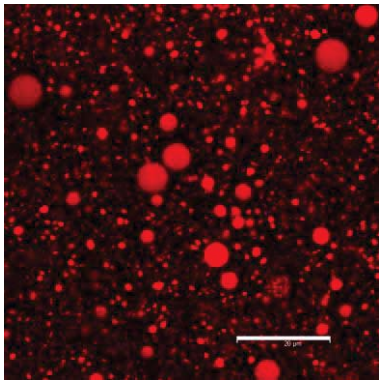
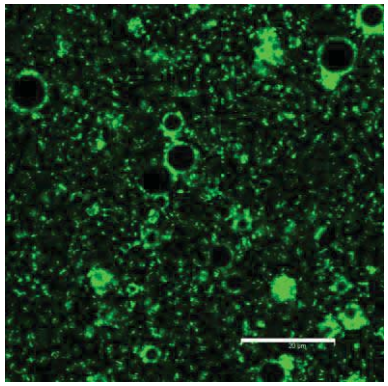
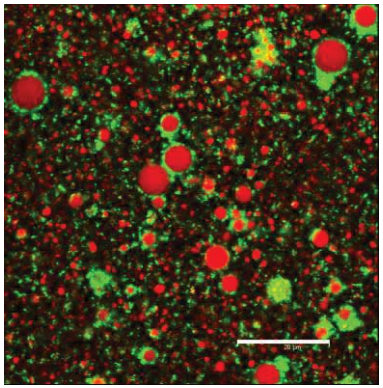
Type of milk sample	Characteristic		
	(A) Lipid phase	(B) Protein phase	(C) Lipid and protein phase
11) Heinz® Nurture (powder) 			
12) Anmum® (powder) 			

Table 4.2 (*Continued*).

Type of milk sample	Characteristic		
	(A) Lipid phase	(B) Protein phase	(C) Lipid and protein phase
13) Karicare® (powder) 			
14) Mom to Mom® (powder) 			

4.4.3 Surface charge

In order to know the effect of different media and medium concentration on the ζ-potential value, comparison of tris and $CaCl_2$, NaCl, tap water, Milli-Q water, milk serum and Ca-imidazole used as diluting media for the liquid formula milk were studied. The results are shown in Figure 4.2. Traditionally, Ca-imidazole buffer is used as diluting medium because it mimics the ionic environment in milk (Paul Van der et al., 2004). However, in this study Mill-Q water was chosen as the medium to quantify the actual surface charge of the droplets, avoiding any screening of charges in the presence of ions to be able to compare more effectively between samples. The emulsion surface potentials of different milk samples using Milli-Q water (1:100) as a medium are shown in Figure 4.3. In this study, it was decided to dilute the milk samples with Milli-Q water prior to measuring the ζ-potential.

The ζ-potential of MFG represents the surface charge of the droplets and it varies with the protein/phospholipids native to the membrane. At the natural pH of milk samples, the ζ-potential values are all negative (Table 4.1 and Figure 4.3). The ζ-potential of human milk fat globules was determined as –20 mV whereas the ζ-potential of raw cows' milk, powder infant formulae and liquid formulae are between –33 and –38 mV. The ζ-potential data were statistically analysed and also tabulated in Table 4.1. These ζ-potential results agree well with those reported by Michalski et al. (2002). These authors showed that the absolute ζ-potential value of raw cow milk fat droplets was –13.5 mV when measured at 25°C on milk diluted in

the imidazole buffer at pH 7.0 (Michalski et al., 2002). Whereas, the mature human breast MFG was −7.8 mV (Michalski et al., 2005b).

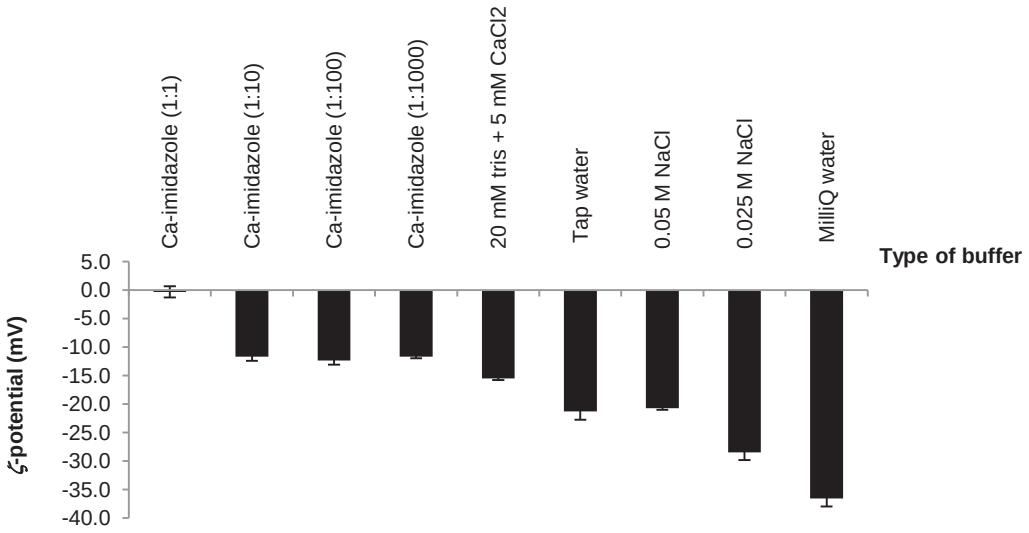


Figure 4.2 **The ζ-potential of fat globules in liquid infant milk emulsions using different buffers. The error bars represent the standard deviation of five repeat measurements.**

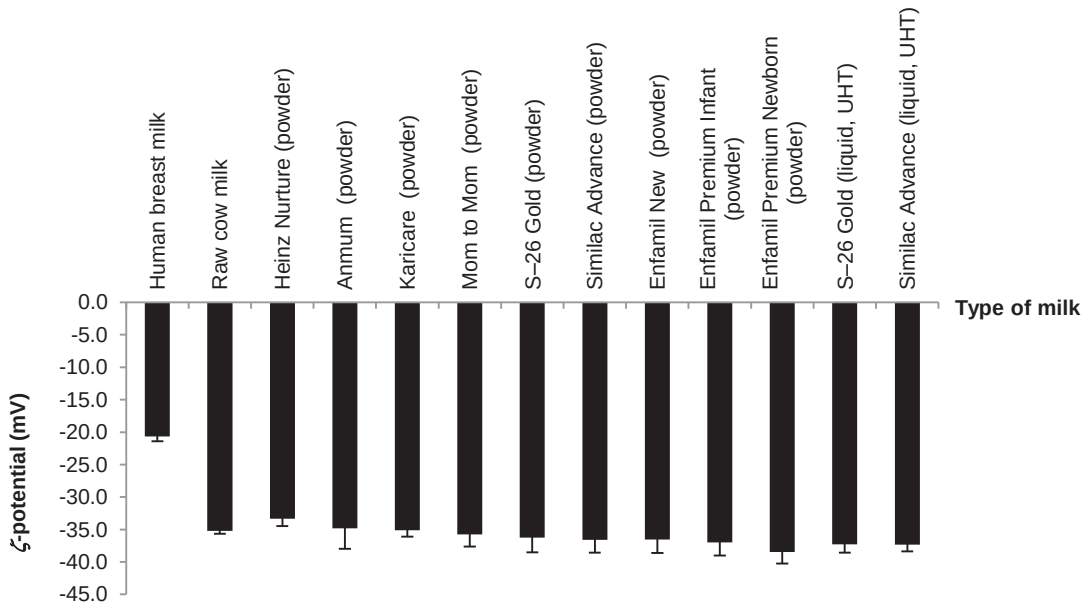


Figure 4.3 **The ζ-potential of fat globules in varies types of milk using Milli-Q water as a medium.** The error bars represent the standard deviation of five repeat measurements.

4.5 Discussion

This current research compared the physicochemical properties between native milk (human milk and cow milk) and processed milk (liquid and powder infant formulae). For human breast milk, mature milk in the range of 1–3 months postpartum was investigated. Infant formulae for normal full-term newborn infants during the first six months of life were compared.

Biological milk fat is secreted from the alveolar epithelial cells of the mammary gland as milk fat globules (MFGs). These MFGs are composed of a lipid core made up to 95% or more of hydrophobic triacylglycerols (TAGs). This is surrounded by a membrane layer, comprised of amphipathic compounds (phospholipids, proteins glycolipids bipolar substances, enzymes and cholesterol) forming an emulsion–stabilising membrane (Abrahamse et al., 2012, Jensen et al., 1992). The membrane of breast milk derived from apical plasma membrane of the epithelial cells, helps to maintain milk lipids in a dispersed state and prevents coalescence (Valivullah et al., 1988). In addition, some trace elements, enzymes, hormone and growth factors are found in the crescents of human milks but these are completely absent in infant formulae. On the other hand, most of the infant formula manufacturers use a mixture of vegetable oils in place of milk fat to provide the same overall fatty acid profile found in human milk. Fat droplets in infant formulae consist of a triglyceride core surrounded predominantly by surface active protein and phospholipids (Oosting et al., 2012, Michalski et al., 2005b). The above accounts for the main differences observed here between maternal and infant formulae of droplet size and ζ-potential value.

The particle size of the fat droplets present in milk is important in defining properties, especially emulsion stability. Particle size could thus be one of the key factors that regulate fat digestion. Comparisons of the MFGs of human and infant formula showed that biological milks (human and cows' milk) have a bigger droplet size than the processed infant formula milks (both powder and liquid formulae) (Table 4.2). The significant variation in size between biological and processed formula milks is due to the fact that the fat droplets in infant formulae are not native MFGs as they are produced by the emulsification of vegetable oils and passed through the homogenisation process so that the droplet size is significantly reduced and the specific surface area is larger (Michalski, 2013). However, although breast milk lipids have a bigger droplet size, they are reported as being more efficiently digested and absorbed by the infant than infant formula lipids (Michalski, 2013).

Armand et al. (1999) found that a fine emulsion underwent a higher degree of lipolysis than a coarse emulsion. The rate and extent of gastric lipolysis is dependent on the available surface area of the triglyceride for the lipase to act, and this is determined not only by the initial fat content but also by the droplet size (van Aken et al., 2011); the smaller the droplet size, the larger the surface area available for the lipase to act on. However, the composition of the fat droplet membrane is also important since lipases must pass through this membrane to gain access to the TAG core. As described above the researchers have found that the much bigger MFGs in human milk which are surrounded by a native MFGM have more efficient gastric lipolysis than the smaller fat droplets present in homogenised infant formula (Armand et al., 1999, Favé et al., 2004). Possible differences in terms of fat digestion

between the infant formulae may be expected given the microstructure variation found here. These differences are the focus of following chapters.

Comparing the two native milks (human and cow's milk), this current study showed that mature human breast milk had smaller droplet size than cow's milk (Table 4.1 and Figure 4.1). The difference in fat droplet size of biological milks depends on many factors such as the species, the maturity of mammary gland, and the stage of lactation. Moreover, some researchers had stated that the diameter of mature human MFGs increases with advancing lactation which is opposite to bovine MFGs (Michalski et al., 2005b, Rüegg and Blanc, 1981, Mulder and Walstra, 1974).

Zeta-potential is a function of the surface charge of the particle, which depends on the adsorbed layer at the interface and the nature and composition of the surrounding medium in which the particle is suspended. The ζ-potential depends strongly on pH, conductivity and concentration of components. All ζ-potential results in this current study are negative. Human breast milk had the lower ζ-value compared to bovine milk and infant formulae. This implies that the electrostatic repulsion forces between fat droplets of human milk were the weakest. Michalski (2013) described that a lower ζ-potential value of human milk could be due to the present of glycoproteinaceous filaments at external membrane of human MFG surface which these filaments are absence in cow's milk. In cow's milk, the external membrane are proteins and phospholipids (Jensen et al., 1990).

The particles in formula emulsions possessed greater negative charge than the biological milks, which is considered a consequence of protein adsorption during homogenisation as mentioned above. The negative surface charge generates

electrostatic repulsion keeping the emulsion droplets apart and preventing them from aggregating. Milk emulsions, especially liquid infant formulae had all good electrostatic stability. The emulsifier type used in the infant formulae may be crucial for stabilisation of the fat droplets by influencing the ζ-potential value. Moreover, the emulsifier might protect the triglyceride surface against enzymatic lipolysis as the type of polar lipid at the surface of MFG modifies the TAGs hydrolysis by gastric lipase depending on the biding strength of the enzyme onto the surface (Michalski, 2013). As stated earlier, the area of the triglyceride surface available to the lipase may also affect the rate and extent of gastric lipolysis (van Aken et al., 2011).

Commonly used emulsifiers in infant formulae include lecithin, monoglycerides and/or diglycerides. McSweeney, Healy and Mulvihill (2008) have studied the concentration effect of monoglycerides or lecithin, used as emulsifiers on the ζ-potential of oil droplets in model infant formula emulsions. They found that when the concentration of monoglycerides increased the ζ-potential became less negative due to the displacement of protein molecules from the interface of the fat droplet by monoglycerides. On the other hand, lecithin had no effect on the ζ-potential value because the negative charge of lecithin may displace or interact with protein at the interface. As it can be seen from the ingredient lists of commercial infant formulae in this study (Table 2.6) the most used emulsifier is lecithin. The ζ-potential value (Table 4.1) measured in infant formulae showed no drastic differences among the samples.

In processed milk, the fat droplet size distributions among the infant formulae both liquid and powder varied. The different heat processing of liquid infant formulae, UHT and retort showed no obvious differences in terms of droplet size (d_{43}), being in the range of 0.32–0.37 μm. However, some powder formulae had bigger droplet size, presenting a bimodal size distribution, probably due to processing. The microstructure of different infant formula milks clearly showed very fine emulsions for the liquid formulae versus coarser emulsions for powder formulae. The differences in the extensive protein adsorption at the interface of fat droplets were observed (Table 4.2). The fat membranes in infant formulae are new membranes formed from whey proteins and caseins during processing or from phospholipids and proteins, depending on the formulation (Michalski et al., 2005b). In the aqueous phase of the emulsion system non adsorbed protein is present.

Infant formula uses vegetable oils instead of milk fat and is subjected to severe mechanical processes. The variation of fat globule size can be due to different processing conditions during blending, pasteurising, homogenising, and spray drying. The homogenisation is the extreme process where fat globules are subjected to such high shear stress, thus component redistribution and interactions such as protein–protein, protein–lipid and protein–lipid–mineral may occur (Guo et al., 1998, Guo et al., 1996, Hendricks et al., 1994). Interactive forces such as electrostatic, steric, and bridging between droplets can induce the aggregation and coalescence of fat droplets (Britten and Giroux, 1993). Some proteins such as denatured whey proteins can become clustered during pasteurisation or sterilisation and precipitated with micelles during processing. Also casein micelles and denatured whey protein clusters cover small fat globules during homogenisation. Powdered

milks which undergo a drying process after homogenisation showed secondary 10 μm particles probably due to a high degree of whey protein aggregation and more damaged globules compared to liquid milks as shown in the micrographs of powder formulae in Table 4.2. The differences in fat globule size distribution among brands (Figure 4.1 and Table 4.1) is likely due to variations in the manufacturing of the processed milk powder, especially homogenisation pressures conditions, heat treatment and the use of different ingredients in their formulations.

In powder infant formulae, the observed bimodal peak maybe the result of the spray-drying process. Thus, the fat globules are disrupted during spray drying and then (i) caseins/heat denatured whey proteins complexes adsorb onto the new expanded lipid droplet interface (Michalski and Januel, 2006) or (ii) the fat globules are attached to each other by casein micelle–denatured whey aggregates and a short-chain-like structure is formed (Guo et al., 1998). All this results in the formation of larger particles affecting the surface protein coverage, given the incidence of coalescence of "uncovered fat" occurring at the surface of powder particles (Ye et al., 2007). Moreover, newly uncovered surface on the fat globules may come into contact at the air-water surface of the drying droplet, with the result of some fat spreading easily on the powder particles surface (Ye et al., 2007, Walstra, 1995). The amount of damage fat in the powder samples may have an impact on the digestion in the infant.

4.6 Conclusions

The fat globules in infant formulae, based on the same 3.6% fat content as the human breast milk, are smaller than the native milk (4 μm) with a uniformly diameter ~0.4 μm due to the homogenisation process. Infant formulae emulsions present 10 times

more surface area than native milk. The membrane of infant formulae is covered by milk proteins instead of a native milk fat globule membrane as in native milk. There is a different between liquid versus powder infant formulae in terms of size and microstructure. The droplet charge (ζ-potential) represents the different between biological milks and process milks due to the differences in characteristics, composition of the membranes and type of emulsifiers at the surface of oil droplets. This membrane stabilises fat droplets in the continuous phase and protect TAGs against lipolysis, also it is of primary importance for sufficient the digestion.

Chapter 5 Factors influencing the dynamics of emulsion structure during neonatal gastric digestion in an *in vitro* model[1]

5.1 Abstract

Findings are presented of the chemical, electrochemical and physical changes in the composition of a formula milk following digestion in an *in vitro* system that replicated the shear rates that would normally be encountered in the infant stomach. These changes were examined over a range of shear rates and pH levels that would be encountered in stomach lumen postprandially, and in the presence and absence of proteolytic and lipolytic enzymes. Digestion was conducted either with a fungal lipase that is commonly used in *ex vivo* systems (*Rhizopus oryzae*) or a mammalian gastric lipase (calf pregastric lipase). Prior to digestion the fat droplets in the formula milk were considerably smaller than those in human breast milk, and were stabilised by an adsorbed layer of milk protein. Droplet size increased progressively due to coalescence during *in vitro* digestion at pHs between 3.5 and 4.5 when both lipase and protease were present, but not when either enzyme was omitted or when pH levels were outside this range. Coalescence was augmented by shear, notably at rates above the normal physiological range. The stability and structural properties of formula emulsions appear to be influenced by disruption of the proteinaceous oil/water interface during digestion, being most susceptible to the concerted activity of pepsin and gastric lipase over a limited range of pH. Given that the onset of secretion of pepsin, lipase and hydrochloric acid does not occur synchronously in the developing infant stomach, inappropriately formulated milks may lower digestive

[1]Part of the contents presented in this chapter has been published as a paper: 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*, 162-172

efficiency. The fidelity of *in vitro* systems does not appear to be compromised by the use of fungal lipases but may be compromised by the use of inappropriately high stirring rates.

5.2 Introduction

It is essential that neonatal digestion is efficient, so as to optimise growth and development. However, the principal energostatic nutrient changes from carbohydrate in the foetus to fat in the neonate (Hamosh et al., 1981). The latter is digested and assimilated from the emulsion of fat in an aqueous phase that is maternal milk. The aqueous phase contains various dissolved and suspended nutrients which include casein micelles, whey proteins, lactose, minerals and vitamins (Brans et al., 2004, Raikos et al., 2009) that may interact with the oil/water interface and influence digestion.

The processes by which infants digest fat differs from those in adults: the levels of bile in the intestinal lumen are lower, owing to immaturity of the liver (de Belle et al., 1979, Norman et al., 1972), as are the levels of pancreatic lipase and co-lipase (Zoppi et al., 1972). Although small intestinal lipolysis is aided by bile salt activated lipase that is secreted in maternal milk (BSAL) (Baba et al., 1991, Olivecrona and Bengtsson, 1984), it is considered that there is greater reliance on gastric lipolysis to achieve effective lipid digestion.

Whilst the composition and structure of breast milk is best suited to infant nutrition (Thompkinson and Kharb, 2007), infant formula is a necessary alternative in situations where breast milk is not available. As demonstrated in the previous

chapter, it is, however, difficult to replicate the lipid structure and composition of the fat emulsion in human milk. The blending of a number of vegetable oils in suitable proportions can produce a fatty acid profile similar to that of human milk, however, the efficiencies with which such formulated emulsions are digested and assimilated critically differ from those of human breast milk (Andersson et al., 2007, Splinter and Schreiner, 1999). This is thought to result from differences in the physical structure of the formulated emulsion from those of the milk fat globule (MFG)

The ordered structure of the MFGM, and hence the stability of the emulsion, depends upon the conditions in the aqueous phase. These conditions change with time after ingestion (Chatterton et al., 2004, Mason, 1962) and with the age (Chatterton et al., 2004) and maturity (Mason, 1962, Mitchell et al., 2001) of the infant. It is likely that evolution has optimised the physical structure and chemical characteristics of human milk for efficient digestion under these changing conditions. The lack of such optimal characteristics in formula feeds may account for the lower rate at which milk fat globules are hydrolysed compared to those in human milk (Armand et al., 1996). Correspondingly it is desirable to formulate synthetic milks with similar characteristics and to compare their behaviour with that of human milk under these varying conditions.

The inconsistency of the chemical conditions within the infant gastric lumen after feeding and lack of forceful mixing in the human stomach are well described, yet little has been done to test formulated feeds under these conditions. The postprandial temporal profile of infant gastric pH was first described nearly 50 years ago (Mason, 1962). It is known that the gastric pH is relatively high in early neonatal life (Ahn

and Kim, 1963, Christensen et al., 1988, Ebers et al., 1956). Furthermore, it is recognised that the lumen pH rises postprandially above the optimum for peptic proteolysis (1.9–3.6) (Pearson and Hutton, 2005) i.e. above the lower (pH 3.5) of the two pK_a values governing the action of this enzyme (Campos and Sancho, 2003) and remains above these values for around 120 min (Mason, 1962). Consequently, the temporal profiles of gastric pH in neonates are far from the optimum pH of pepsin during this period (Untersmayr and Jensen-Jarolim, 2008). Conversely, the pH range over which human gastric lipase is active is controversial. Whilst the pK_a of the ser-his-asp catalytic 'lid' site of this and other lipolytic enzymes is thought to be approximately 7.0, optimal activity is nevertheless maintained over the pH range of 4.0–6.0 (Armand et al., 1996), either as result of a the enzymes ability to initially associate with the oil/water interface at a separate site with a lower pK_a (Aloulou and Carrière, 2008, Chahinian et al., 2005), or as a result of local pH microenvironment being maintained at the active site (Poulsen et al., 2005).

After a formulated milk has been consumed, the changing temporal profile of gastric pH may differently affect lipolytic and proteolytic activity, so that proteinaceous elements at the interface are digested at a different time to that when triacylglycerol (TAG) is hydrolysed. Again, it is not known whether gastric lipase and pepsin preferentially locate on the oil/water interface in close proximity to each other, or can only locate separately, similarly it is not known whether any such co-location is sensitive to shear.

This chapter investigates the effects of lipolysis, proteolysis, shear and pH on the components of synthetic emulsion structures in formula milks in an *in vitro* system

under an array of conditions that reflect the varying physical and chemical environment that develops post-prandially within the lumen of the infant stomach.

5.3 Materials and methods

5.3.1 Materials

5.3.1.1 Infant formulae

Infant formulae were stated to contain about 3.6% fat and 1.4% protein. The principal protein sources were stated as non-fat (cows') milk and mineral reduced whey. The fat phase was stated to contain vegetable oils with soy lecithin, monoglycerides and/or diglycerides as emulsifiers (details mentioned in Chapter 3).

5.3.1.2 Enzymes

Porcine pepsin (porcine, 800–2500 U/mg), fungal lipase extracted from *Rhizopus oryzae* (80 U/mg) and calf pregastric lipase (Biocatalysts) were used (details mentioned in Chapter 3).

5.3.1.3 Walstra solution A

Wastra solution A was used to break up any flocs formed. To prepared, 0.375% (w/v) EDTA and 0.125% (v/v) Tween20 dissolved in water and adjusted to pH 10 with NaOH as described in Chapter 3.

5.3.2 Methods

5.3.2.1 *In vitro* digestion of the infant formulae

Each commercial formula emulsion was prepared as described in Chapter 3, then mixed with SGF (milk: SGF ratio = 20:50 v/v). The pH of the mixture was maintained at either pH 2.0, 3.5, 4.5 or 5.5. The specific pH was maintained by appropriate ongoing drop-wise addition of either 1 M HCl or 1 M $NaHCO_3$. The mixture was incubated at 37°C for 2 h at a specific rpm i.e. either 0 rpm, 10, 20, 50 or 100 rpm and shear rate i.e. 0, 16.25, 32.5, 81.25, 162.5 s^{-1}, respectively. A 1 mL sample of digestate was removed from the beaker every 15 min (details mentioned in Chapter 3).

5.3.2.2 Determination of emulsion-SGF mixtures

The effects of the various shear rates and enzymes on droplet stability and size were explored by comparing the droplet size distributions. Characterisation of droplet structure was examined by confocal microscopy. The charge on the fat globules was quantified by determining the electrical potential termed the ζ-potential, as described in Chapter 3. Protein composition and protein interfacial coverage of emulsion droplets was determined using the depletion method as described in section 5.3.2.3.

5.3.2.3 Protein analysis

The protein composition of infant formulae was analysed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This technique separates proteins according to their electrophoretic mobility. SDS-PAGE was conducted with

a 4% (w/v) stacking gel and a 16% (w/v) separating gel mounted on a vertical mini-PROTEAN II slab cell system (Bio-Rad Laboratories, Richmond, CA, USA). Electrophoresis was conducted at a constant voltage of 210 V, 70 mA and 6.50 W for approximately 1.5 h. The gel was stained with Amido black, dried and subsequently scanned.

For reducing SDS-PAGE, 0.05% of β–mercaptoethanol was added as a reducing agent into the emulsion-sample buffer mixtures and heated to near boiling (95-100°C) for 5 min to reduce the disulfide linkages thus protein band was unfolded (Sarkar, 2010).

In order to determine the quantity of the unadsorbed protein (present in the continuous phase) on the emulsions subsamples following the various treatments, they were centrifuged for 40 min at 45,000 g and 20°C (Sorvall RC5C, DuPont Co., Wilmington, DE, USA). The subnatant was carefully removed using a syringe, filtered sequentially through 0.45 and 0.22 μm filters (Millipore Corp., Bedford, MA, USA), and examined using SDS-PAGE (Sarkar, 2010) (sample: sample buffer = 400 μl: 800 μl, with 10 μl of sample being loaded).

The protein composition at the interface of the emulsion droplets (adsorbed protein) was also determined by analysing the cream. The cream layer obtained by centrifugation of the emulsions (as mentioned above) was carefully removed, dispersed in Milli-Q water and again centrifuged at 45,000 g for 40 min at 20°C. The cream layer was collected carefully and a certain amount of cream was spread on to a filter paper (Whatman No. 42, Whatman International Ltd, Maidstone, Kent, UK)

and air-dried. The dried cream was then mixed with SDS buffer in glass vials and heated in a boiling water bath for 5 min with the addition of 0.05% β-mercaptoethanol (sample: sample buffer = 100 µg:1000 µL, with 10 µL of sample being loaded) using SDS-PAGE (Srinivasan et al., 2000). The final surface coverage (Γ) was calculated by equation 5.1.

$$\Gamma\,(\mathrm{mg\ m^{-2}}) = \frac{(C_{ads} \times 1000)}{SSA \times f_v \times \rho_{oil}} \tag{5.1}$$

where C_{ads} is the number of grams of protein adsorbed to the surface of the oil droplets in 100 mL of emulsion. SSA is the specific surface area, is expressed in m^2 of surface per gram of the oil phase. f_v is the oil volume in mL per 100 mL of emulsion, and ρ_{oil} is the oil density.

5.4 Results

5.4.1 Protein analysis of commercial liquid infant formula

5.4.1.1 Protein composition of liquid formula

This study used reduced–sodium dodecyl sulphate polyacrylamide gel electrophoresis (reduced SDS–PAGE) to examine protein compositions by analyzing the relative abundance of casein, α–lactalbumin, and β–lactoglobulin in the different milk samples.

Comparisons of milks between using reduced (Figure 5.1) and non–reduced SDS–PAGE (no boiling and no reducing agent) (Figure 5.2), showed that the reducing

system was better for analysis compared to the non-reduced form in SDS–PAGE The presence of *β*–mercaptoethanol as the reducing agent denatures the proteins by reducing disulfide linkages leading to greater amounts of the whey protein bands. A dilution ratio of 200:800 (sample: buffer) showed clear results. These gels confirm the greater proportion of the whey fraction in liquid formula, specifically for the bands corresponding to *β*-lactoglobulin. Also in the reduced gels the three fractions of *α*-, *β*-, and *κ*-caseins were clearly separated. This just emphasises the fact that infant formula is a whey protein dominated emulsion and a mixed protein interfacial layer is expected at the interface, also probably dominated by higher quantities of *β*-lactoglobulin. .

Additionally, the results from non–reduced SDS–PAGE showed that formula milks were found to have increased amounts of minor whey protein or high molecular weight protein fractions accumulated on the top of the gel (Figure 5.2), suggesting a degree of protein aggregation as a consequence perhaps of processing.

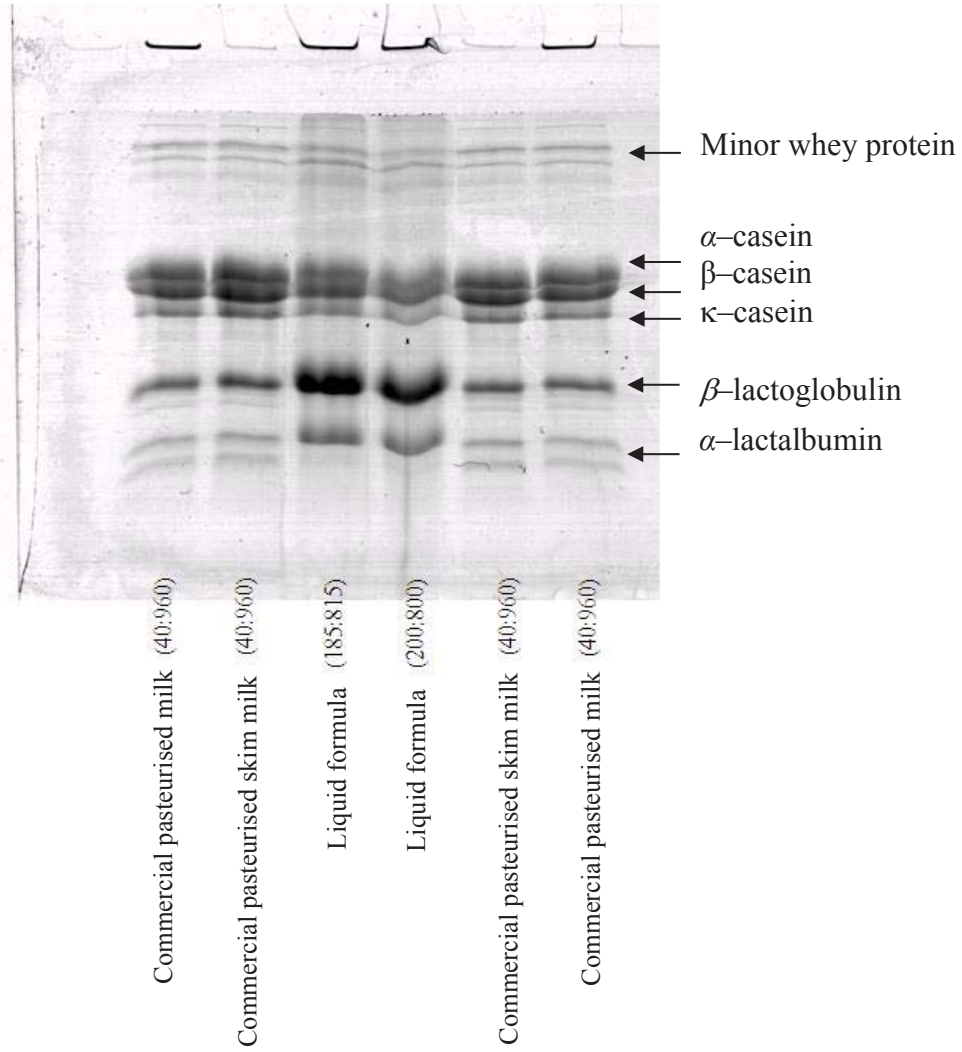


Figure 5.1 **SDS–PAGE (reduced) patterns of different concentration of liquid infant milk emulsions (S–26®).**

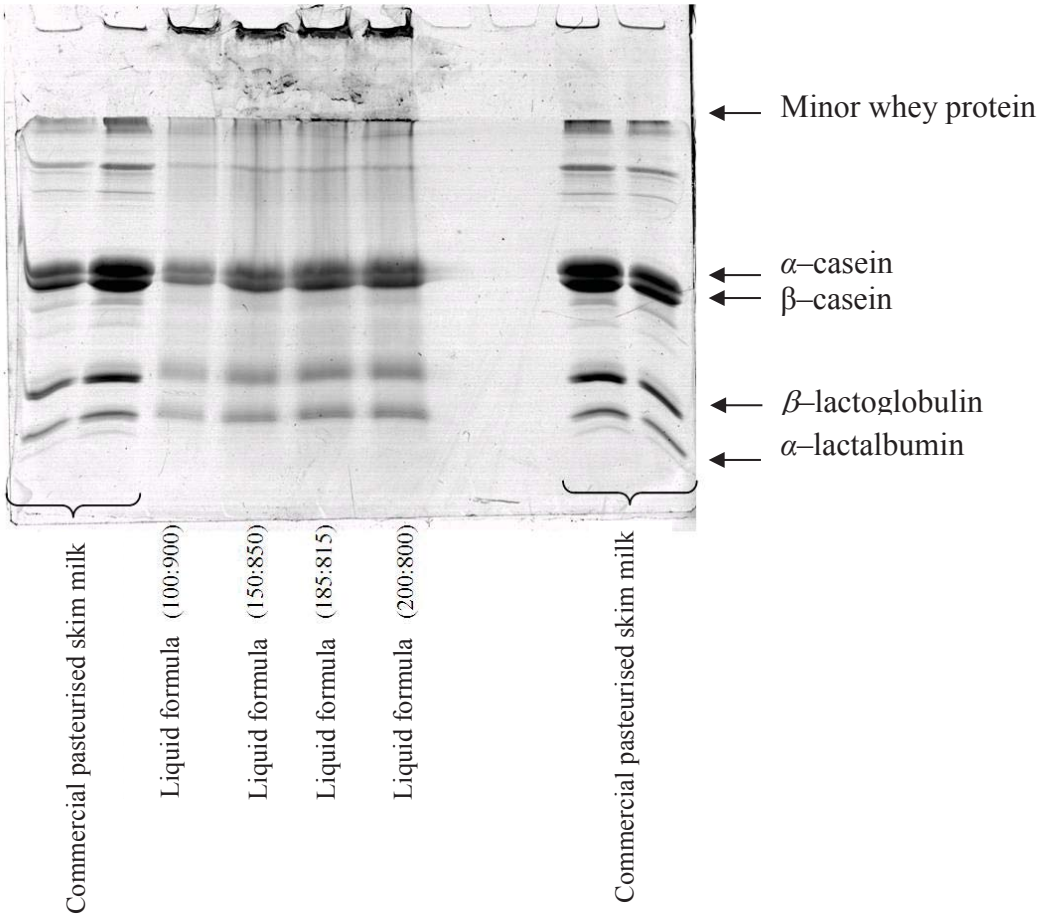


Figure 5.2 **SDS–PAGE (non–reduced) patterns of different concentration of liquid infant milk emulsions (S–26®).**

5.4.1.2 Protein surface coverage of liquid infant milk

The fat content of liquid formula was 3.6% (w/w) and SSA was 35.9 m^2 /g. The total N content of cream phase in infant formulae was 0.961. The density of cream = 0.9. The total N conversion factor is 6.38. Based on equation (5.1) the protein surface load calculation on the liquid infant milk emulsion droplets was determined as 1.9 mg m^{-2}. However, the SDS-PAGE gels of the cream layer did not show a clear separation of the bands therefore the results are not shown here.

The protein surface coverage value of liquid formulae (S–26®) indicates that the interface of oil droplet is comprised of adsorbed protein, this finding is in agreement with confocal micrographs that show extensive coverage by protein (green colour) in Chapter 4.

5.4.2 Droplet size distribution

The sizes of the liquid infant milk emulsion droplets in samples of digesta that had been diluted with the dissociating Walstra A solution were generally smaller than in those (Figure 5.3B) that had been diluted with water (Figure 5.3A) and the distribution of sizes in the former tended to be more unimodal. However, the extent and timing of these differences varied with the pH at which the *in vitro* digestion had taken place, and with the speed at which the digestate was stirred.

Comparisons of shifts in the sizes of emulsion droplets over time in samples diluted with water (Figure 5.3A) with those diluted with Walstra A solution (Figure 5.3B) indicated that increasing flocculation and coalescence occurred during *in vitro* digestion at all pH values except at pH 2 where initial flocculation during the first 15 min of digestion was followed by progressive dissociation of floc structures back towards the initial droplet distribution. Analysis with Walstra solution A showed that no significant coalescence had occurred at pH 2 (d_{43} changes from 0.410 to 0.531 μm). Coalescence was most rapid in onset and greatest in extent and duration at a pH of 4.5, with flocculation being evident after 5 min and coalescence after 30 min with an on-going increase in droplet size continuing for the entire 120 min period of *in*

vitro digestion. At pH 3.5 and at pH 5.5 the onset of flocculation was slower, with a corresponding delay in onset of coalescence.

Comparisons of the temporal profiles of droplet size distributions during *in vitro* digestion (at pH 3.5) over a range of different stirring rates (Figure 5.4) indicated that increasing the shear rate significantly increased the measured droplet size and accelerated the rate at which droplets coalesced. Further, at stirring speed above 10 rpm discrete additional peaks of particles of around 100 microns diameter were formed.

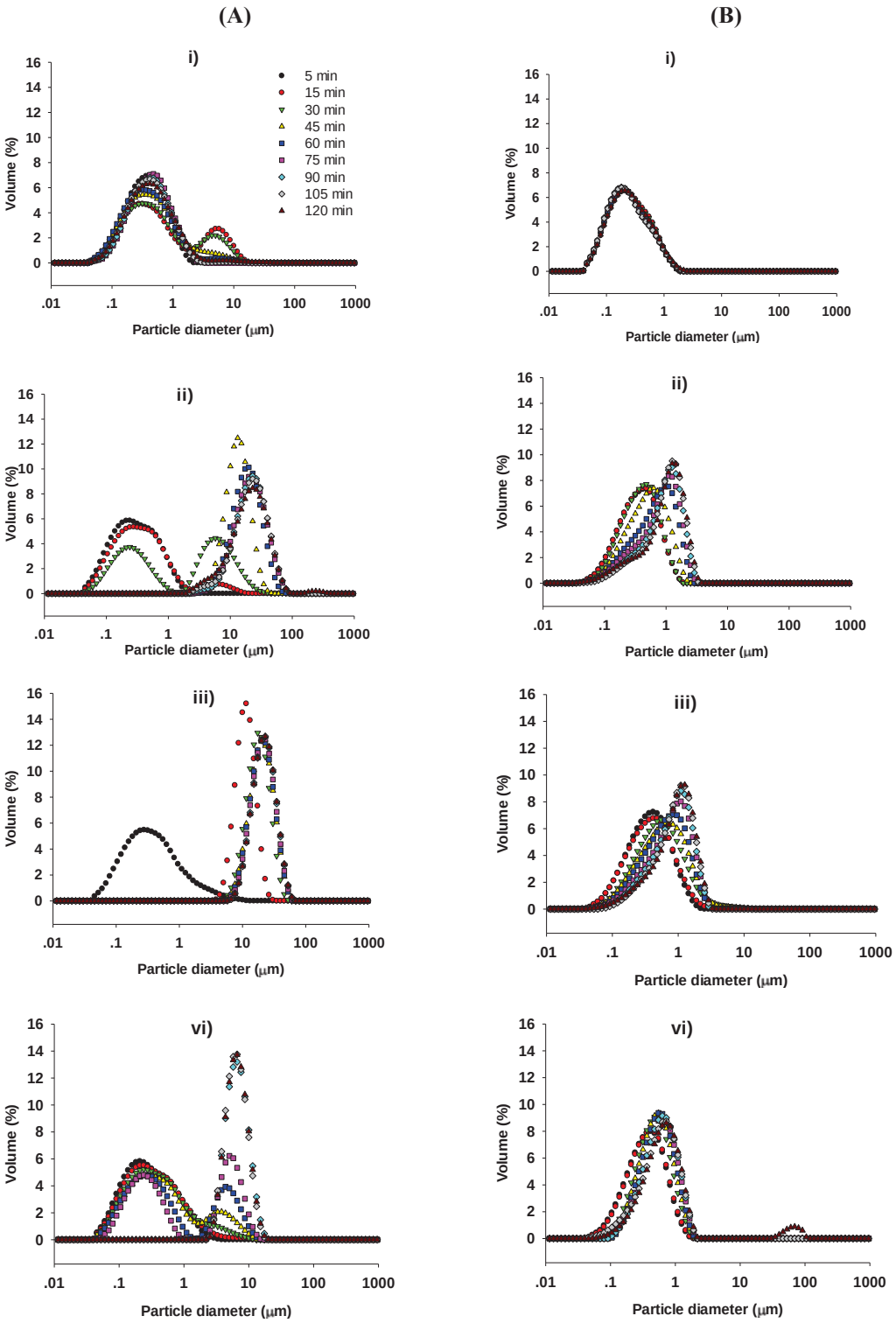


Figure 5.3 **Changes in droplet size distribution of liquid infant milk emulsions during digestion with SGF with pepsin and fungal lipase, at pH 2 (i), pH 3.5 (ii), pH 4.5 (iii), and pH 5.5 (iv), at 37°C, 10 rpm, for 120 min. Analysed without Walstra solution A (A) or with Walstra solution A (B).**

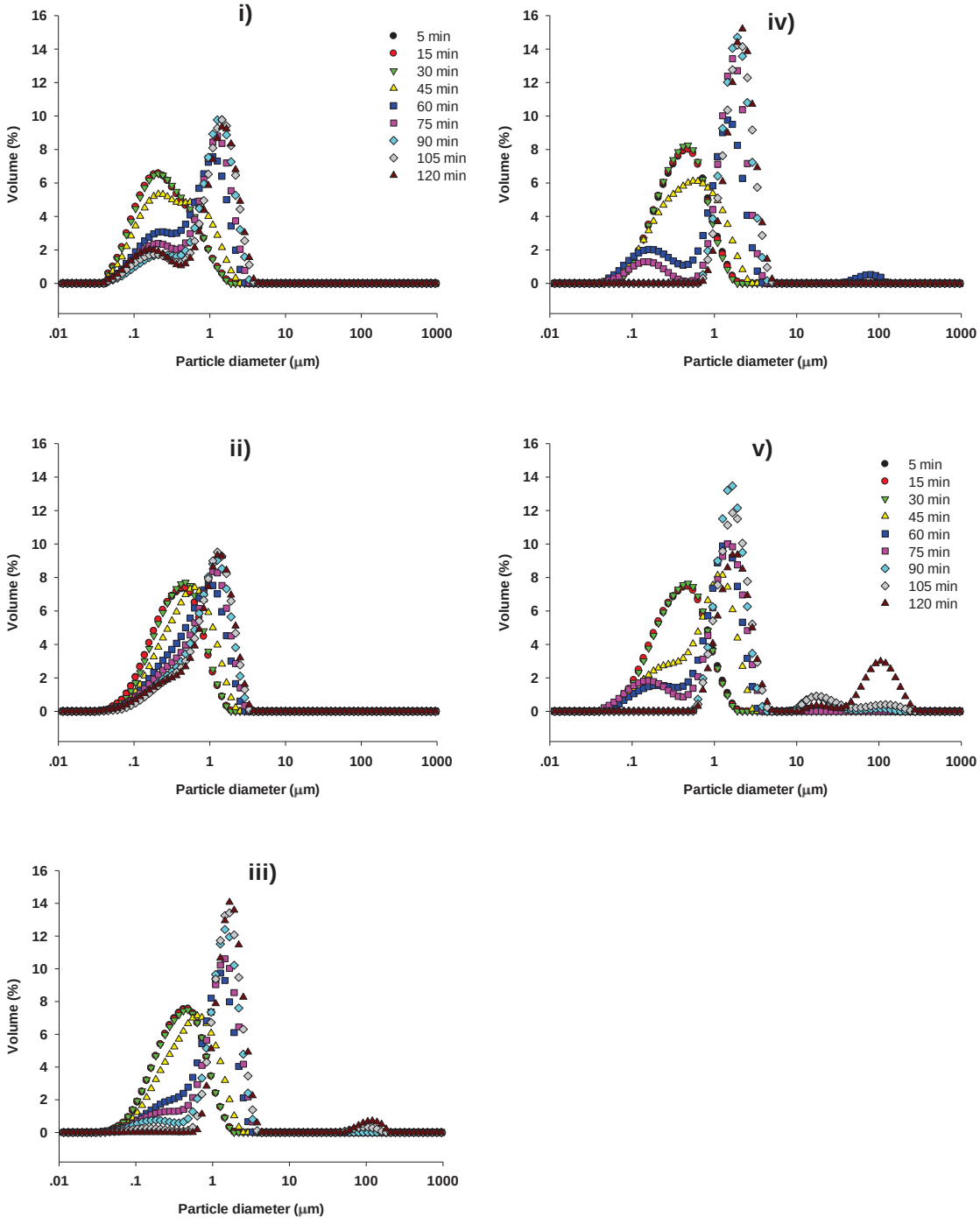


Figure 5.4 **Changes in droplet size distributions of liquid infant milk emulsions during digestion with SGF with pepsin and fungal lipase at pH 3.5, at 37°C, for 120 min at various stirring speed: 0 rpm (i), 10 rpm (ii), 20 rpm (iii), 50 rpm (iv) and 100 rpm (v). Analysed with Walstra solution A.**

The changes in the temporal sequences of droplet size distributions during *in vitro* digestion when both protease and lipase were present were compared with those in which one of the two principal enzymes had been omitted. These comparisons showed that the extent to which the emulsion systems coalesced during *in vitro* digestion was either reduced or eliminated when either the protease or lipase were omitted from the *in vitro* system (Figure 5.5 and Figure 5.3B). As such, the droplet size distributions in the principal peaks from Walstra-treated digestate after digestion at pH 2, 3.5, 4.5 or 5.5 in the absence of lipase i.e. with pepsin alone (Figure 5.5A) all remained unimodal. There was, however, some slight variation in droplet size with the pH at which digestion was conducted. At pH 3.5 and to a lesser extent pH 4.5, only minimal variations in size were detected. This behaviour was not seen at pH 2 or pH 5.5. In the absence of pepsin (Figure 5.5B), no significant change in droplet size was observed during the course of digestion, irrespective of pH.

Pregastric calf lipase has the similar kinetic properties to human gastric lipase so it can be used as a model for simulated gastric studies related to human neonates (Bernbäck et al., 1987). However, fungal lipase has more commercial available and reproducible compare to pregastric lipase. The combination of pregastric lipase and pepsin (Figure 5.6A), and pregastric lipase alone (Figure 5.6B) were compared to validate the outcomes based on *in vitro* digestion with fungal lipases.

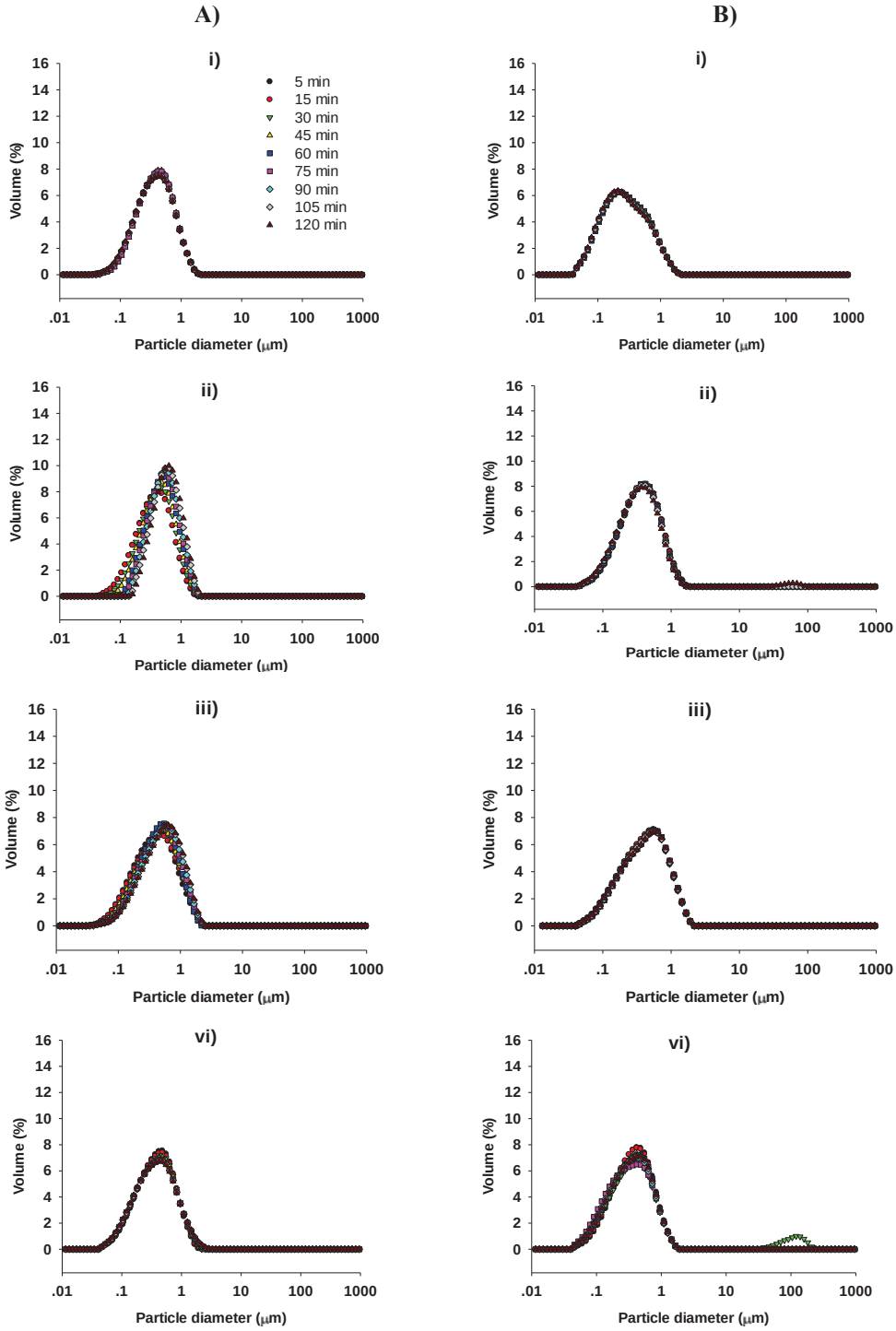


Figure 5.5 **Changes in droplet size distribution of emulsions during digestion with SGF with pepsin (A) or with fungal lipase (B) at pH 2 (i), pH 3.5 (ii), pH 4.5 (iii), and pH 5.5 (iv), at 37°C, 10 rpm, for 120 min. Analysed with Walstra solution A.**

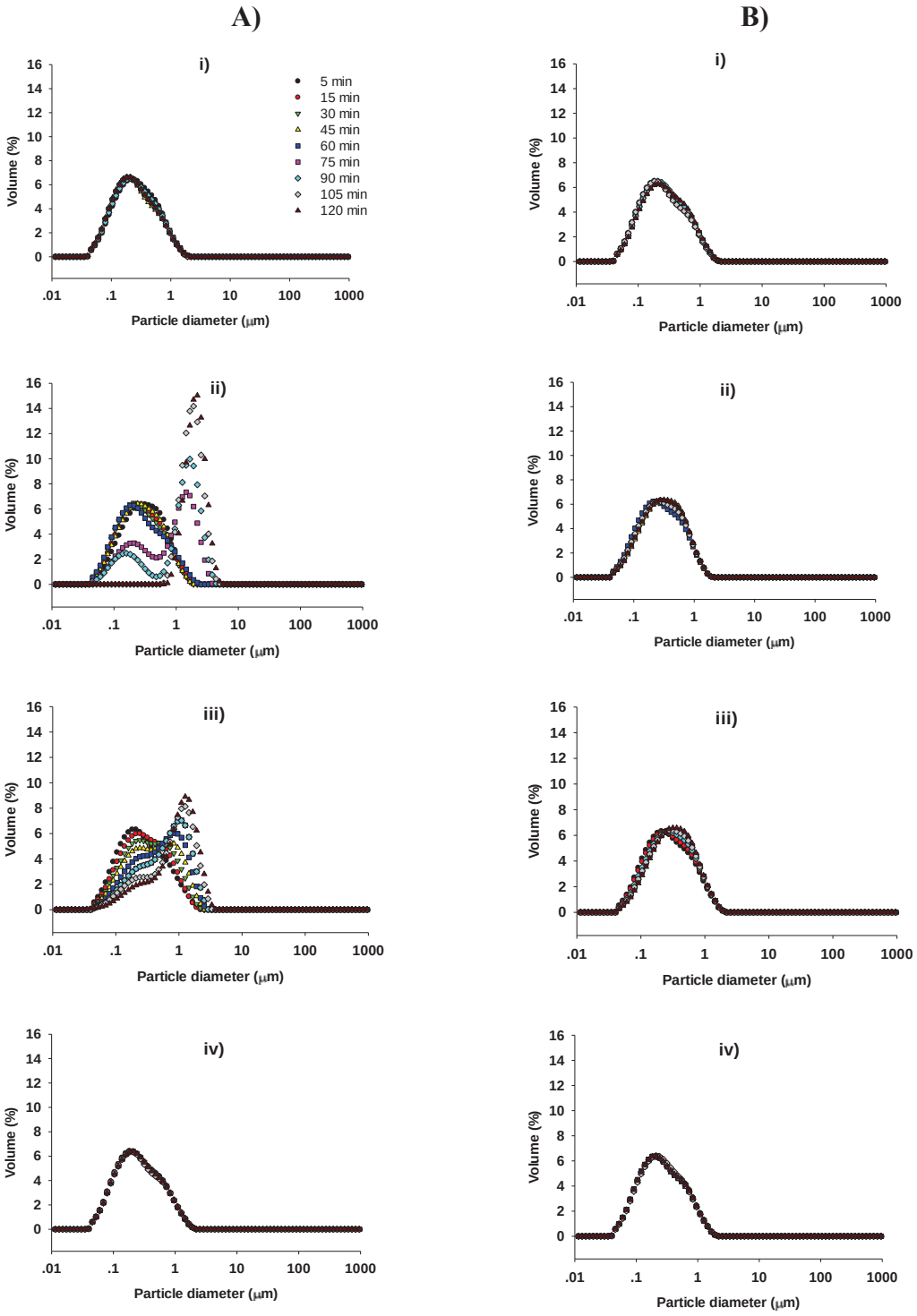


Figure 5.6 **Changes in droplet size distribution of emulsions during digestion with SGF with pepsin and pregastric lipase (A) or with pregastric lipase (B) at pH 2 (i), pH 3.5 (ii), pH 4.5 (iii) and pH 5.5 (iv) at 37°C, 10 rpm, for 120 min. Analysed with Walstra solution A.**

5.4.3 Confocal microscopy

Comparison of confocal images (Figure 5.7-10) confirmed that flocculation of the fat globules occurred promptly in the early phase of *in vitro* digestion at pH 3.5–5.5, but that this flocculation was slowly broken down during the subsequent period of digestion. Examination of emulsions that had been treated with Walstra solution A (Figure 5.11-14) showed that increases in droplet size from coalescence were greatest at pH 3.5 (d_{43} changes from 0.408 to 1.651 μm), less pronounced at pH 4.5 (d_{43} changes from 0.419 to 0.953 μm) and not apparent at pH 5.5. Neither flocculation nor coalescence (Figure 5.7) was apparent at pH 2.0.

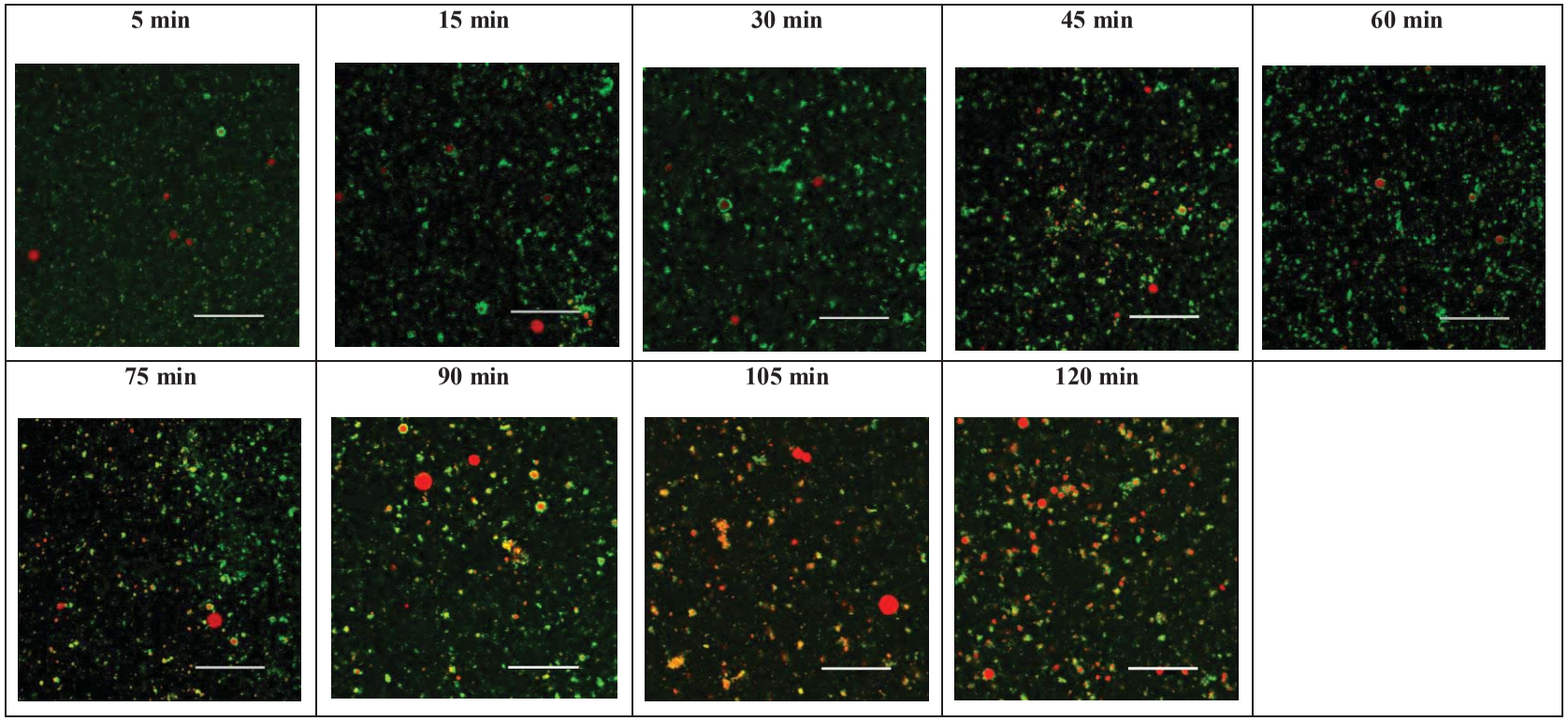


Figure 5.7 **Changes in the microstructure of liquid infant milk emulsions after digestion with SGF with pepsin and fungal lipase at pH 2 as a function of incubation time. Analysed without Walstra solution A. Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

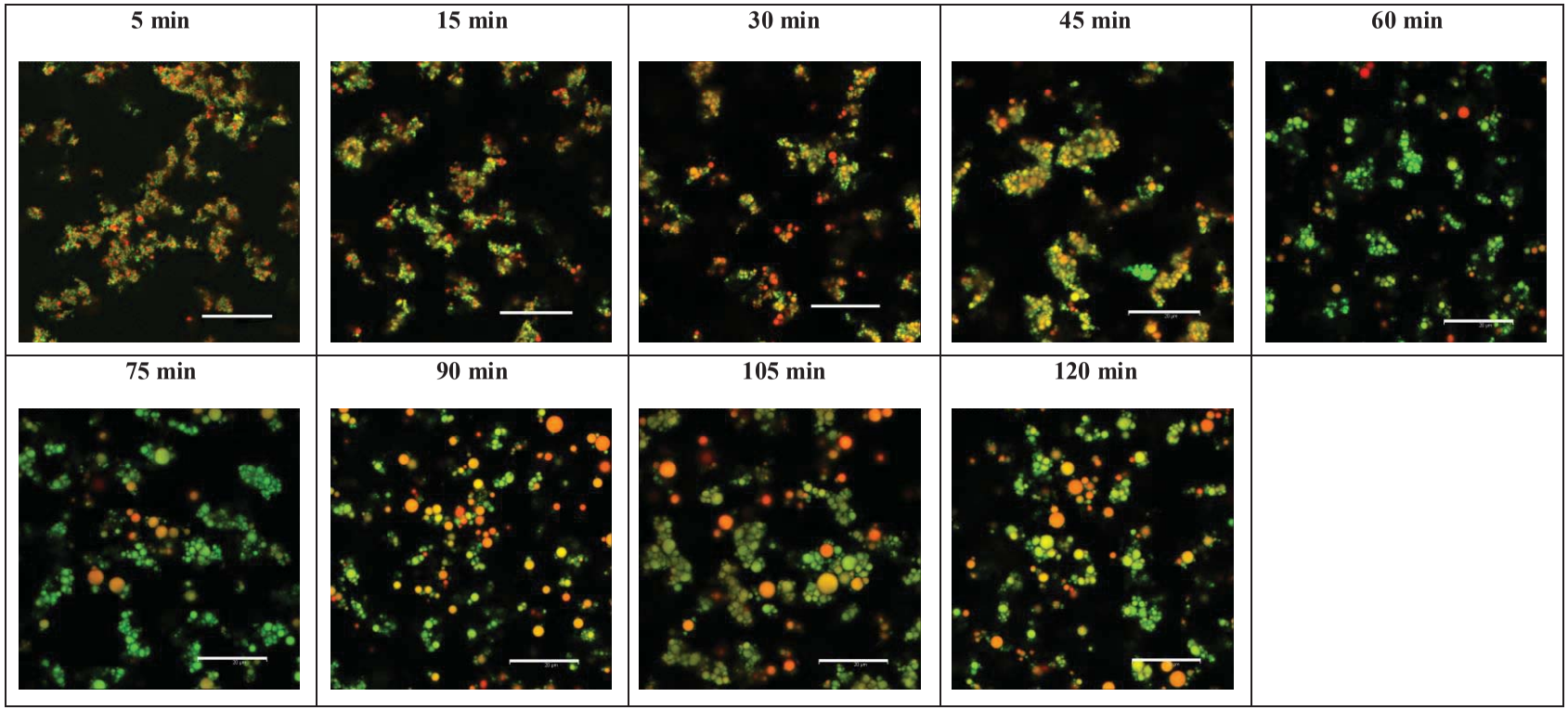


Figure 5.8 **Changes in the microstructure of liquid infant milk emulsions after digestion with SGF with pepsin and fungal lipase at pH 3.5 as a function of incubation time. Analysed without Walstra solution A. Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm**

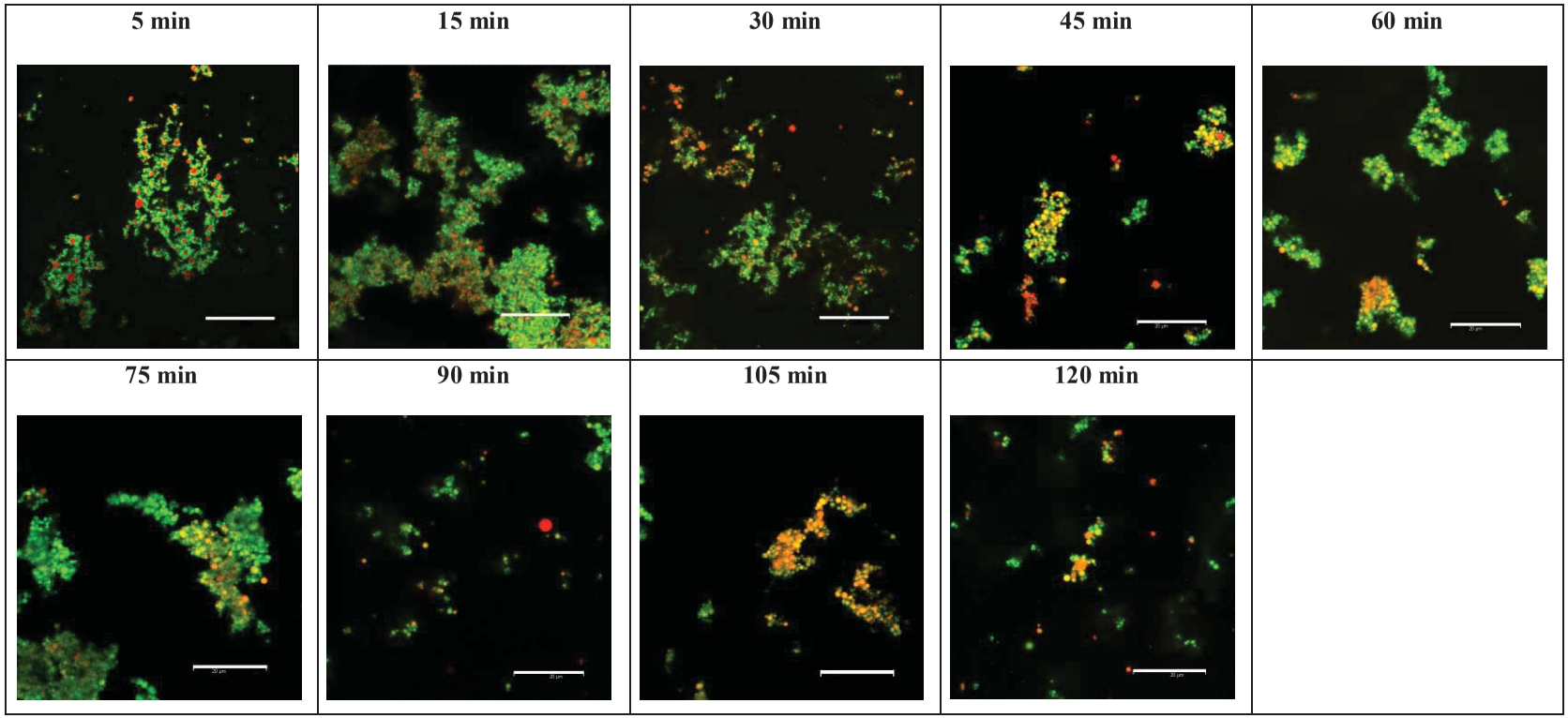


Figure 5.9 **Changes in the microstructure of liquid infant milk emulsions after digestion with SGF with pepsin and fungal lipase at pH 4.5 as a function of incubation time. Analysed without Walstra solution A. Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

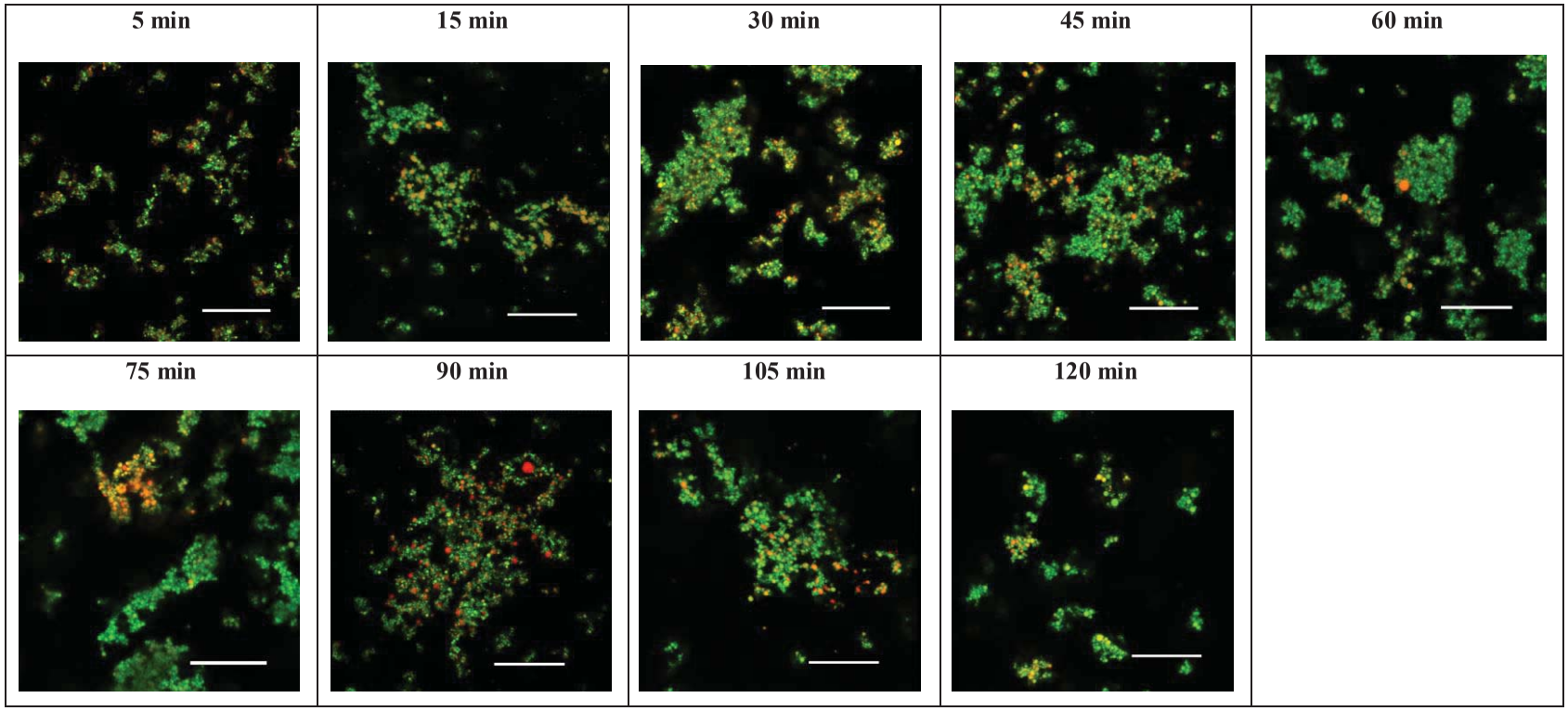


Figure 5.10 **Changes in the microstructure of liquid infant milk emulsions after digestion with SGF with pepsin and fungal lipase at pH 5.5 as a function of incubation time. Analysed without Walstra solution A. Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

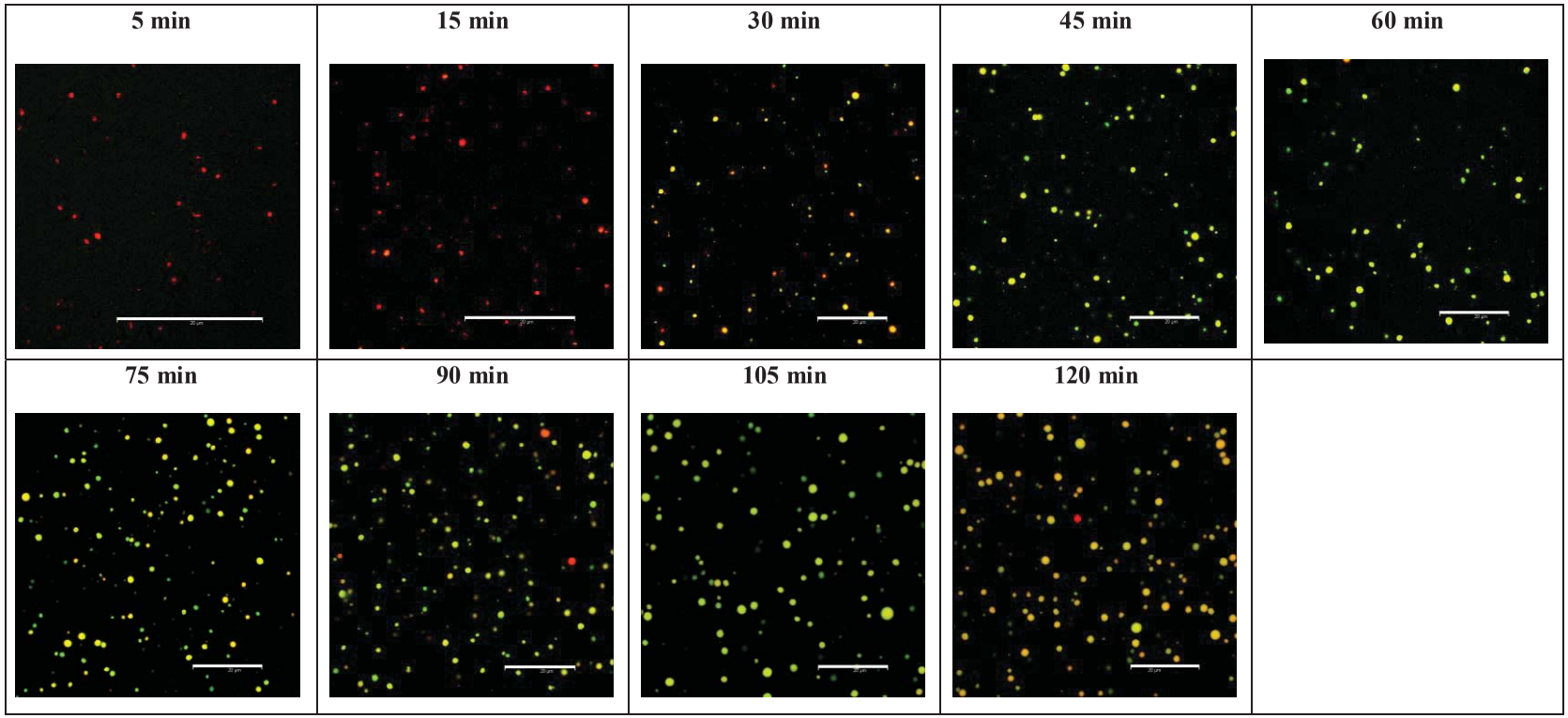


Figure 5.11 **Changes in the microstructure of liquid infant milk emulsions after digestion with SGF with pepsin and fungal lipase at pH 3.5 as a function of incubation time. Analysed with Walstra solution A. Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

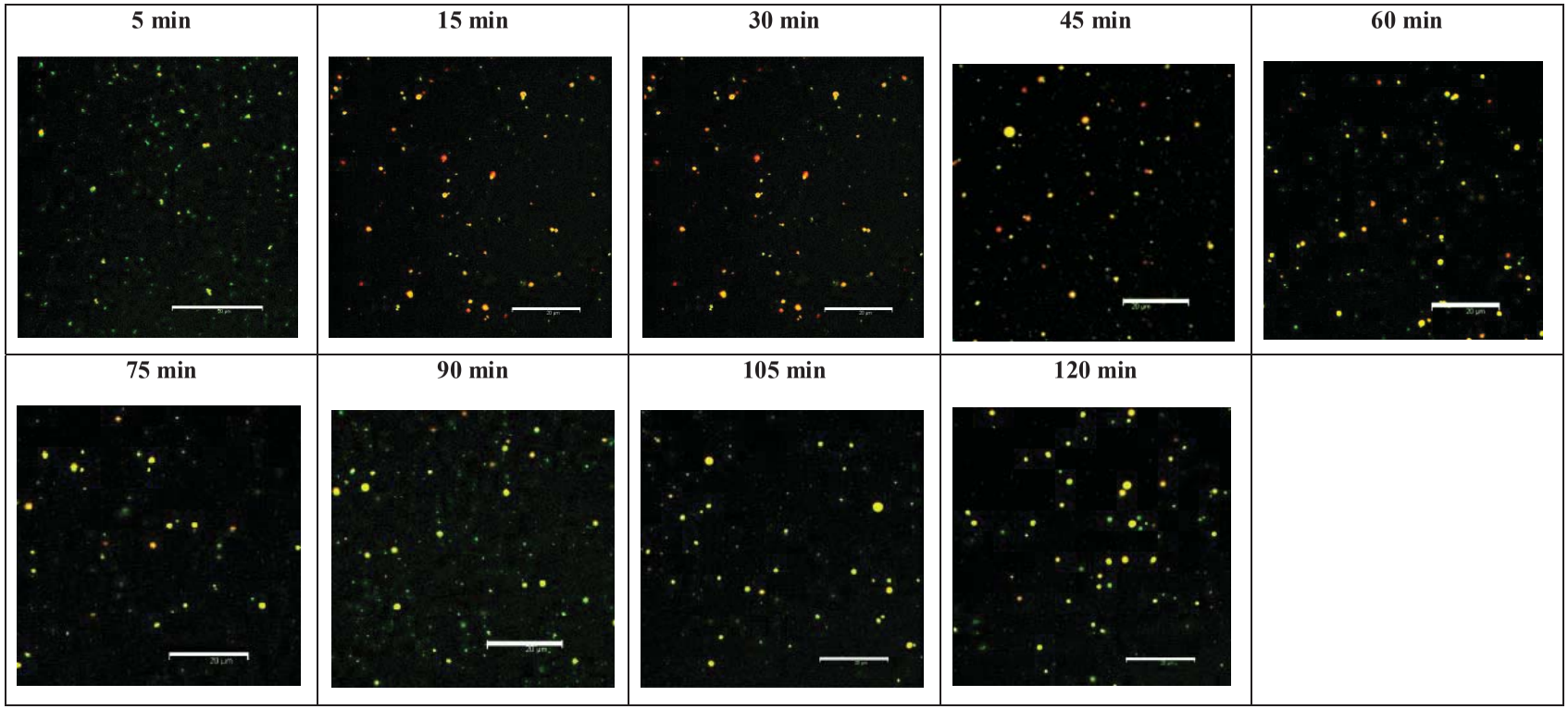


Figure 5.12 **Changes in the microstructure of liquid infant milk emulsions after digestion with SGF with pepsin and fungal lipase at pH 4.5 as a function of incubation time. Analysed with Walstra solution A. Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

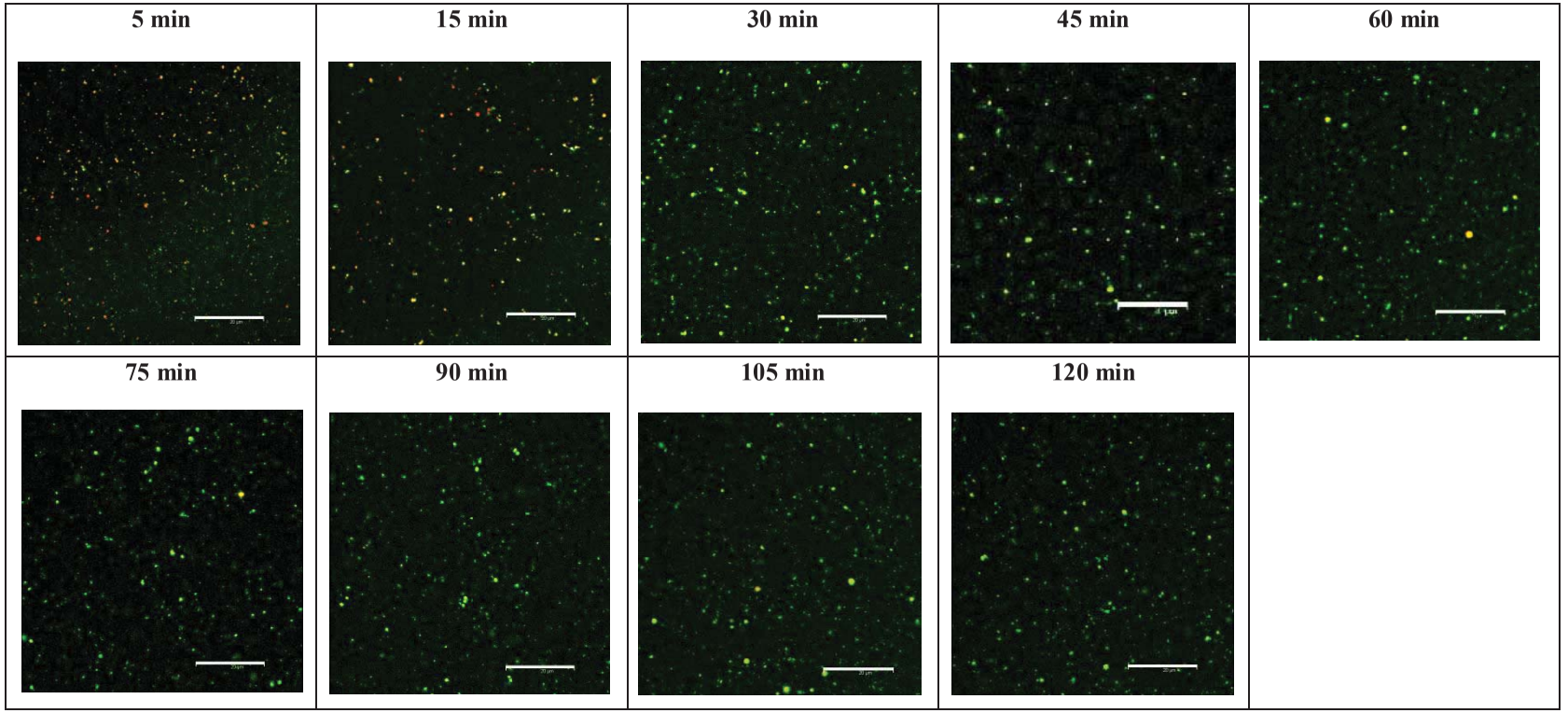


Figure 5.13 **Changes in the microstructure of liquid infant milk emulsions after digestion with SGF with pepsin and fungal lipase at pH 5.5 as a function of incubation time. Analysed with Walstra solution A. Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

5.4.4 Surface charge

The mean ζ-potential value of the formula milk used in this work was –38.4 ±1.5 mV at neutral pH. The changes in ζ-potential for samples undergoing digestion at pH 2 are shown in Figure 5.14A. At this pH all samples carried a high positive charge indicating that pH << p*I* for the protein component of the emulsion. Dynamic ζ-potential measurements remained unchanged for the *in vitro* systems containing pepsin but no lipase, whilst the systems containing fungal or pregastric lipase but no pepsin showed a drop in ζ-potential in the beginning of incubation, with no subsequent change in the remainder of the experiment. When both enzymes (pepsin and lipase) were present, an initial drop in ζ-potential was observed over the first 15 min, with little significant variation thereafter. The ζ-potentials of all samples during *in vitro* digestion at pH 3.5 (Figure 5.14B) were initially positive corresponding to pH < pI. When both pepsin and lipase were present there was a steady decline in positive values over 120 min, as was the case for samples that contained pepsin alone. With lipase alone (both fungal and pregastric) at pH 3.5 the ζ-potential did not change from its initial value.

The ζ-potentials of samples from *in vitro* digestion at pH 4.5 (Figure 5.14C) with both pepsin and lipase and from digestion with pepsin alone showed an initial ζ-potential close to 0 (being close to the p*I* of the milk protein), thereafter displaying steadily increasing negative values over 120 min. The ζ-potentials of samples from *in vitro* digestion with lipase alone did not change significantly, with slightly negative values that remained close to zero. The ζ-potentials of samples *in vitro*

digestion at pH 5.5 (Figure 5.14D) with pepsin alone and with lipase alone initially showed strongly negative values with no significant change over 120 min.

Comparisons of the temporal patterns of ζ-potential responses under the various pH conditions together indicated that ζ-potential declined, when both pepsin and lipase were present, over the same pH range in which flocculation and coalescence were most rapid i.e. at pH 3.5 and 4.5. It is also noteworthy that similar changes occurred over a similar pH range when pepsin alone was present but not when lipase alone was present. In the presence of pepsin only, although the proteins at emulsion interface may be hydrolysed, the protein–stabilised interface is still strong enough to resist rupture of the interfacial film, thus preventing droplet coalescence from occurring.

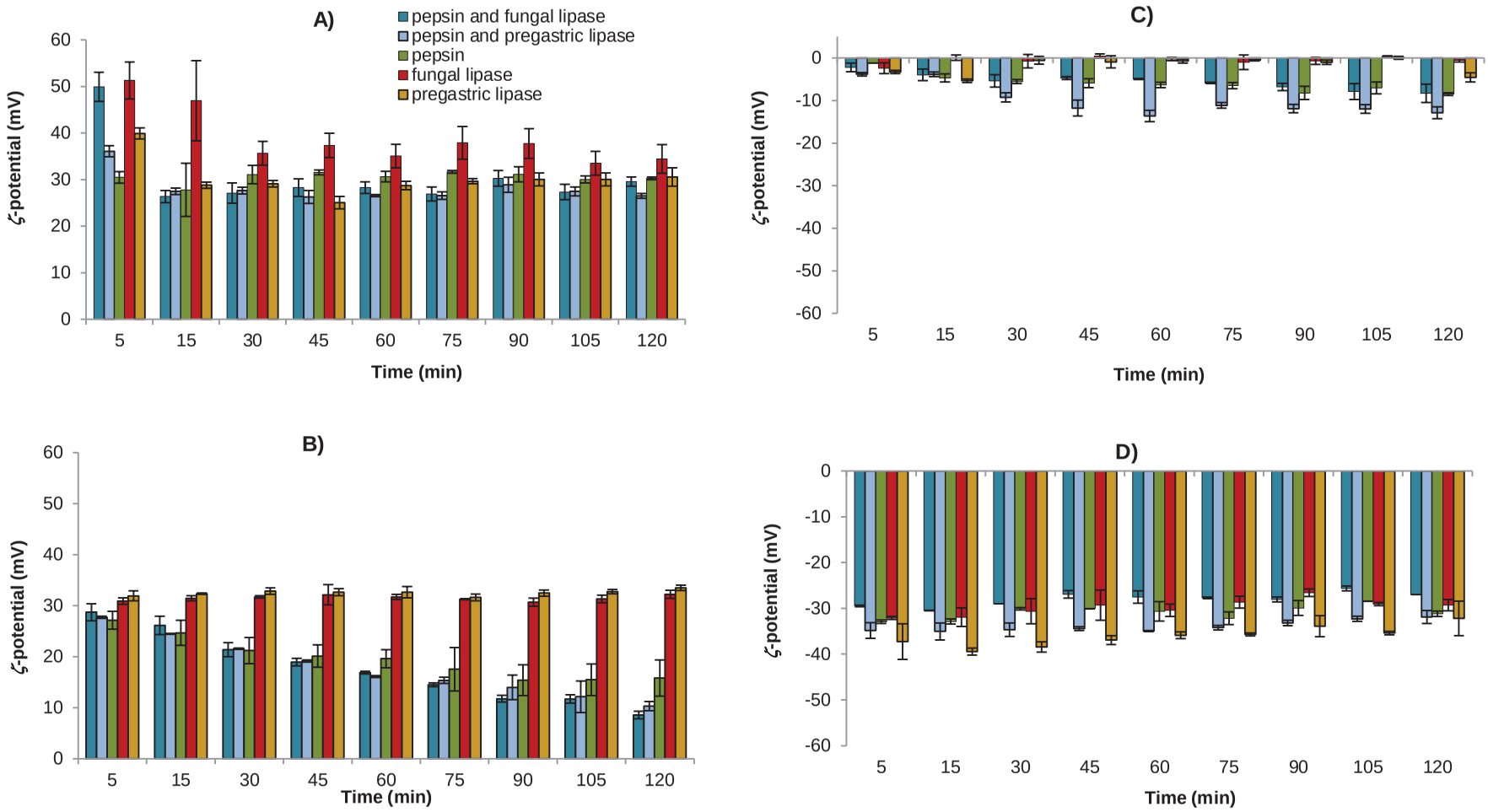


Figure 5.14 **Changes of ζ-potential of liquid infant milk emulsions during digestion with SGF and enzymes at pH 2 (A), 3.5 (B), 4.5 (C) and 5.5 (D), at 37°C, 10 rpm, for 120 min. The error bars represent the standard deviation of five repeat measurements.**

5.4.5 Comparing with other commercial infant formulae

The particle analysis of two commercial liquid formulae before digestion showed the typical small droplet size of homogenised milk. The droplet diameter (d_{43}) of S–26® (UHT liquid) and Enfamil® (retort liquid) was 0.338 and 0.371, respectively. In contrast, the droplet size of powder formula S–26® (powder) and Karicare® (powder) was bigger as typical of a dried product. The droplet diameter (d_{43}) was 1.156 and 0.693, respectively.

The particle size of the emulsion was observed during digestion at pH 3.5 analysed by light scattering (Figure 5.15). The results show that the size of the emulsions increased with the digestion time, from 5 to 120 min with the exception of UHT treated emulsion. S–26® and Karicare® powders howed a much greater change of droplet size during digestion. Interestingly, the size distribution of Karicare® powder changed abruptly after 30 min. However, at the end of digestion the droplet size was lower than 10 μm, as found in S–26® (liquid) and S–26® (powder).

After digestion at pH 3.5 with fungal lipase and pepsin, liquid infant formulae (both UHT and retorted milk) showed lower ζ-potential than powder infant formulae (Figure 5.16). Enfamil® (retort) liquid formula showed the lowest ζ-potential which implies that the electrostatic repulsion force between the oil droplets was the weakest compared to other infant formulae.

In Figure 5.17–5.19, the confocal microscopy results showed the flocculation and coalescence of commercial infant formulae after digestion at 3.5 with pepsin and

lipase. The S–26®, Karicare® powder formulae and Enfamil® liquid (retorted) formula showed more flocculation and aggregation than S–26® liquid (UHT) formula exhibiting a variety in size.

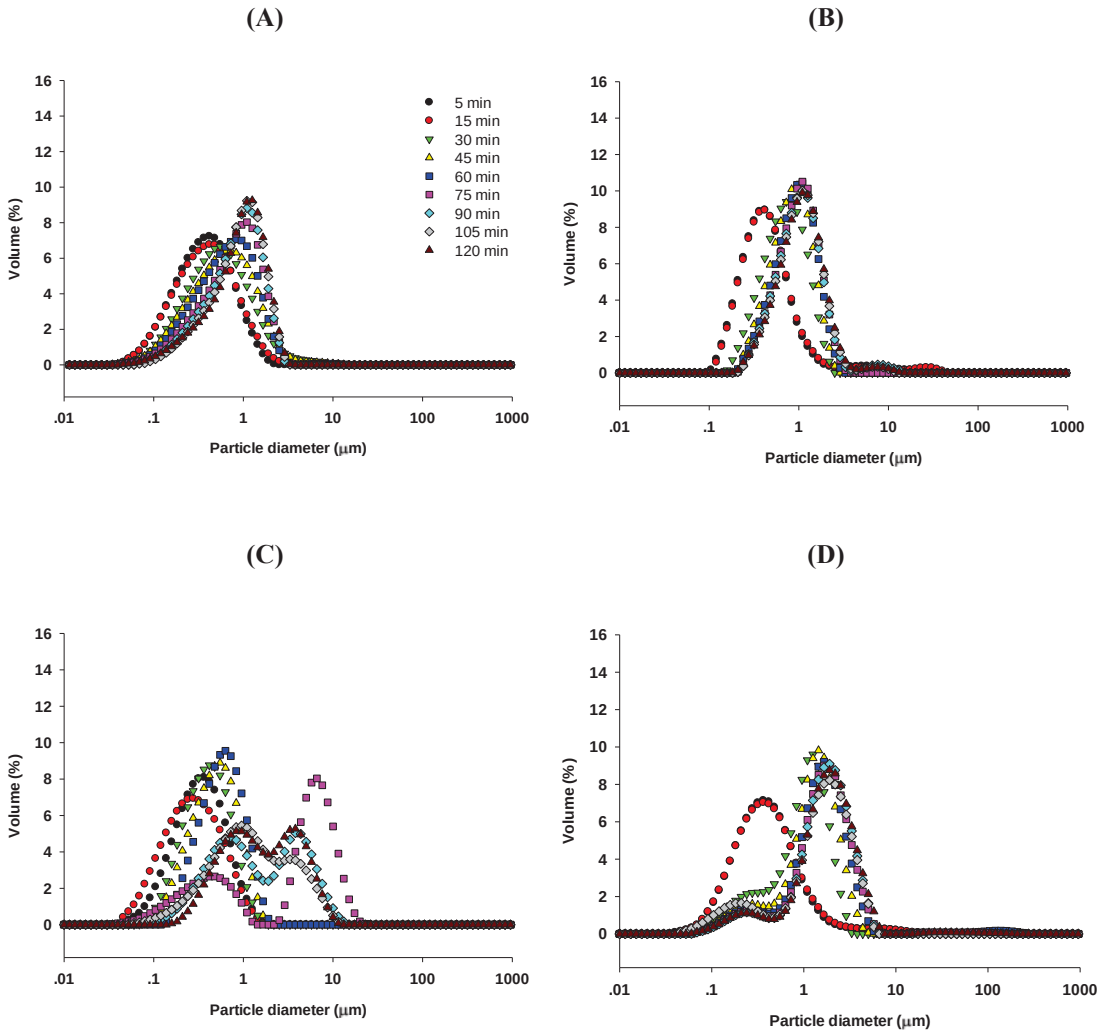


Figure 5.15 **Changes in droplet size distribution of S–26® (UHT liquid) (A), Enfamil® (retort liquid) (B), S–26® (powder) (C), and Karicare® (powder) (D) emulsions during digestion with SGF with pepsin and fungal lipase, at pH 3.5, at 37°C, 10 rpm, for 120 min. Analysed with Walstra solution A.**

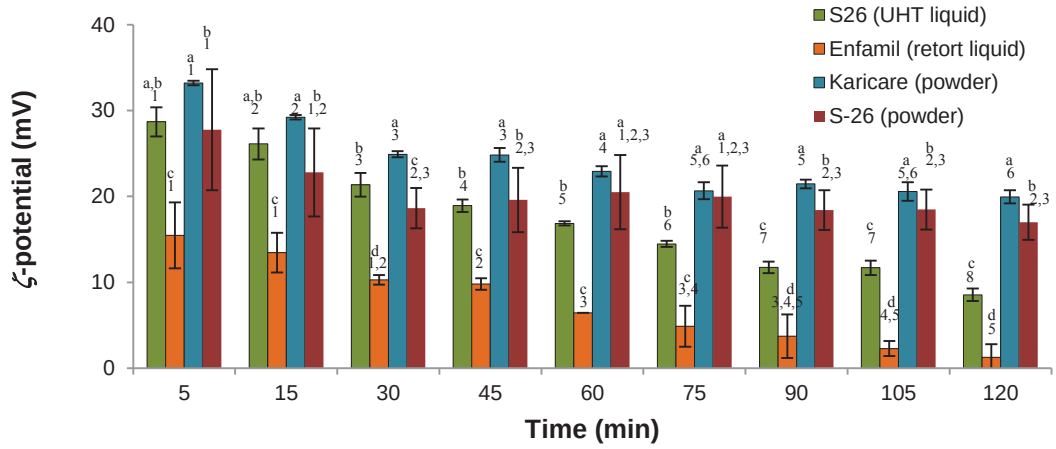


Figure 5.16 **Changes of ζ-potential of different commercial infant formulae during digestion with SGF with pepsin and fungal lipase, at pH 3.5, at 37°C, 10 rpm, for 120 min. The error bars represent the standard deviation of five repeat measurements.**

Note: Different letters between samples at a given time indicate significant difference using one way ANOVA test (P=0.05). Different numbers between times at a given sample indicate significant difference using one way ANOVA test (P=0.05).

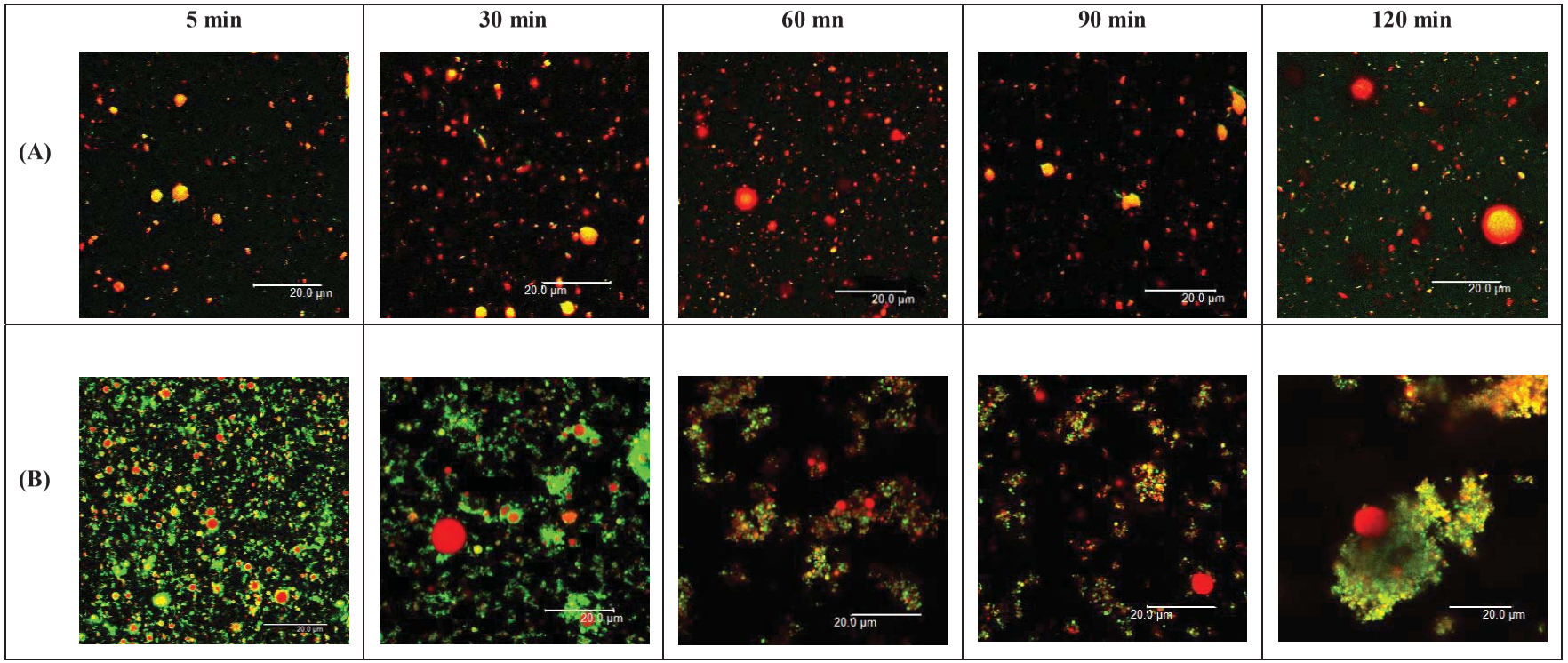


Figure 5.17 **Confocal micrographs S–26® (powder) during digestion with SGF with pepsin and fungal lipase, at pH 3.5, for 120 min. Analysed with Walstra solution A (A) and without Walstra solution A (B). Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar represents 20 µm.**

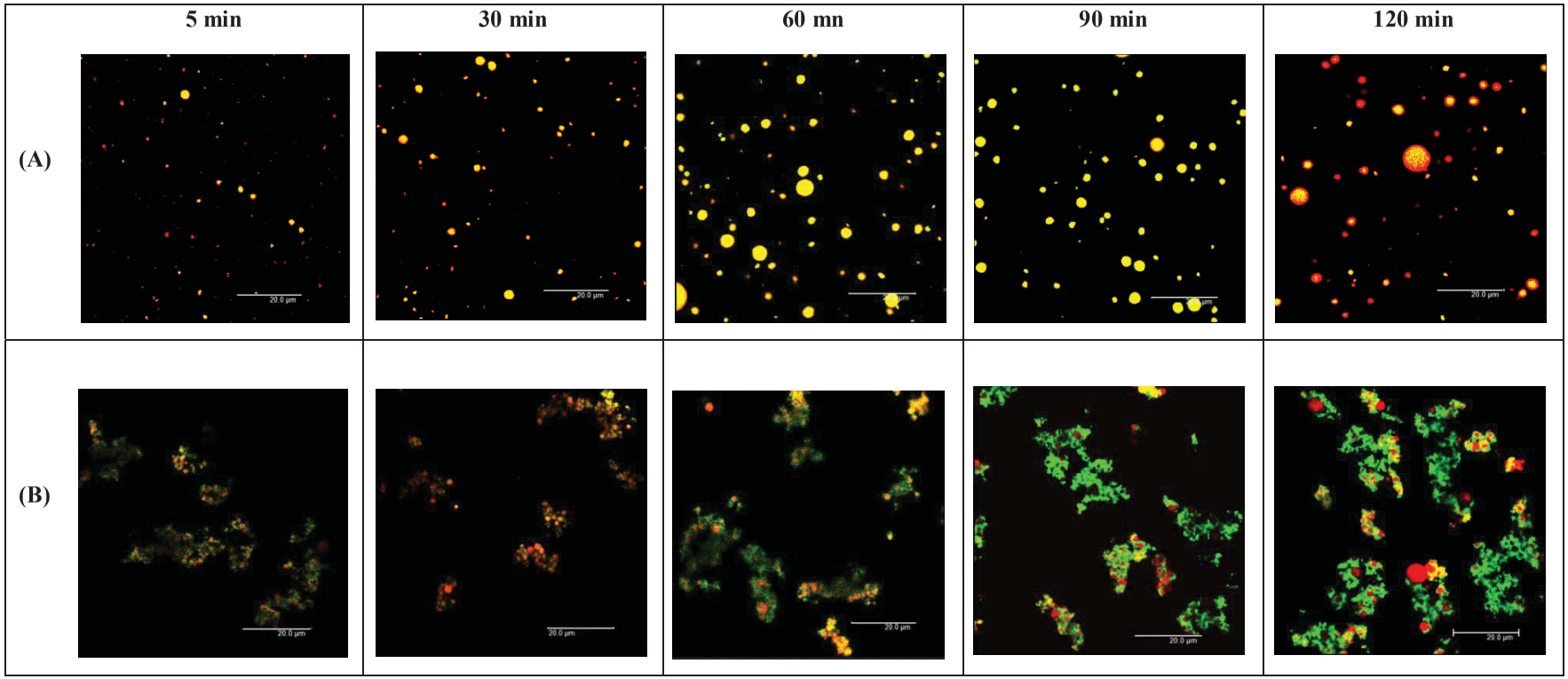


Figure 5.18 **Confocal micrographs Karicare® (powder) after digestion with SGF with pepsin and fungal lipase, at pH 3.5, for 120 min. Analysed with Walstra solution A (A) and without Walstra solution A (B). Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar represents 20 µm.**

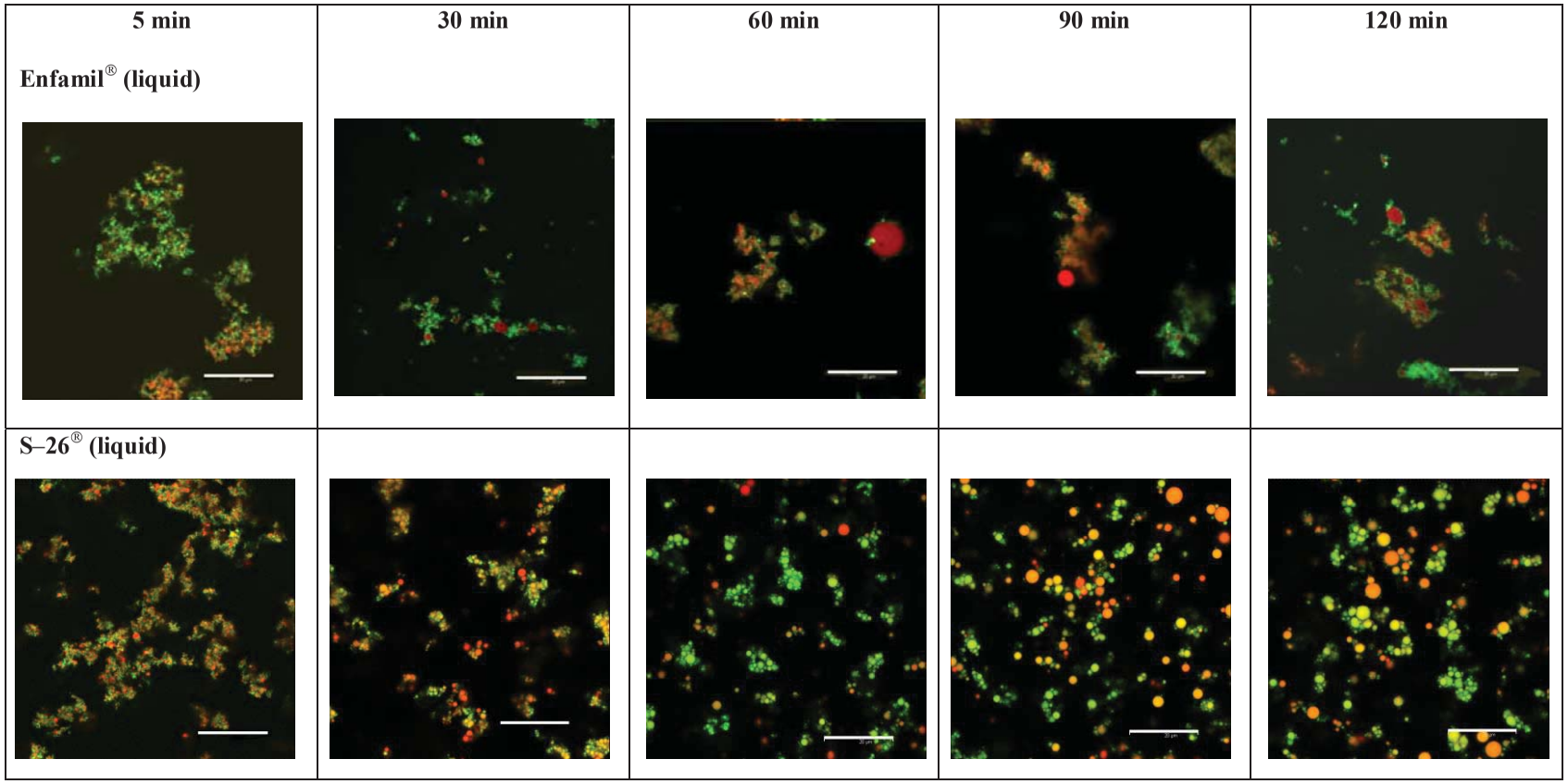


Figure 5.19 Confocal micrographs of Enfamil® (retort liquid) and S–26® (UHT liquid) after digestion with SGF with pepsin and fungal lipase, at pH 3.5, for 120 min. Analysed without Walstra solution A. Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar represents 20 µm.

5.5 Discussion

The results from this study indicate the importance of constructing *in vitro* systems of enzyme digestion that reproduce conditions *in vivo*. The demonstration that oil droplet coalescence (a likely structural consequence of lipolysis) is influenced by the shear rate developed by the stirrer, highlight the need for careful design of the mechanics of *in vitro* reactors. The demonstration that coalescence is maximised when both proteolytic and lipolytic enzymes are present indicates that hydrolytic enzymes may work in synergy to efficiently disrupt the physical structures found in food materials. Accordingly, it is considered appropriate that *in vitro* systems should recapitulate the entire suite of enzymes to which a substrate would be exposed *in vivo* and not limit them to those which are perceived to be of relevance to the principal macro-nutrient whose digestion is being investigated.

5.5.1 Shear during *in vitro* digestion

The susceptibility of droplets to coalesce or aggregate in shear flow is known as orthokinetic stability (Dickinson, 1997). The slower rate of increase in oil droplet size at lower stirring speeds i.e. at lower shear, suggests that the process of coalescence is to some extent dependent upon either the collision frequency (shear based on Smoluchowski's theory) or collision efficiency (hydrodynamic and colloidal interaction between droplets) (McClements, 1999). In this respect it is noteworthy that, if the physical conditions that are applied do not reflect the low rates of shear found in the human stomach, then the process of coalescence may be artificially physically augmented and the other factors that govern the normal

physiological digestion of fats may be less apparent. This is particularly true given that the fat concentration in these biological emulsion systems is relatively dilute, and thus droplet–droplet contact is likely to be dependent on either association through the promotion of colloidal interactions, or as a consequence of encounter frequency as driven through shear conditions.

5.5.2 Effects of proteolysis and lipolysis on aggregation and coalescence

Human and porcine pepsins exhibit a complex sequence of conformational changes that influence their activity and may facilitate their transit from the sites of secretion into the lumen without damaging epithelial proteins (Campos and Sancho, 2003). They are reported to be inactive in environments with pH above 4 but are activated in environments with pH values below this, the pK_a value of this activated enzyme being 3.5. This pK_a is close to the ambient pH at which maximum coalescence was observed and fits with maximal changes in ζ-potential over pH range 3.5–4.5 there being less change in ζ-potential at pH 2. This is indicative that hydrolysis of both serum and interfacial proteins occurs preferentially under conditions of low pH.

Coalescence requires close approximation of oil droplets followed by failure of the interfacial structure separating them (McClements and Li, 2010). Therefore, whilst approximation and coalescence of droplets may be induced by force alone, in a low shear environment such as the lumen of the stomach, it is more likely that coalescence is aided by aggregation. Aggregation of formula emulsions is likely to be maximal when lumen pH approaches the p*I* of the proteinaceous components of

the interfacial layer, thereby minimising surface charge of the emulsion. Confocal microscopy and particle size measurements showed that flocculation was also observed at ambient pHs more distant from the p*I* of protein, presumably as a result of surface proteolysis with consequent reduction in surface charge and/or the facilitation of hydrophobic interactions.

The loss of interfacial mechanical stability leading to coalescence has been hypothesised to be a consequence of one of two possible mechanisms: surface proteolysis resulting in a reduction in the viscoelastic integrity of the protein stabilised interface; or, alternatively adsorption of fatty acids at the oil/water interface through a mechanism of orogenic displacement as a consequence of lipolysis.

The size spectra and the confocal micrographs, showing that droplet size does not change in the absence of pepsin, underscore the particular role of pepsin in generating droplet flocculation and subsequent interfacial proteolysis in formula feeds. Indeed they demonstrate that the range of shear forces that were applied, which included stirring rates up to 100 rpm were all insufficient to induce film rupture between neighbouring droplets unless the intervening material had initially undergone some degree of proteolysis. Accordingly, findings indicate that proteolysis is not directly contributory to changes in the coalescence stability of the emulsions.

However, it is interesting to note that both the ζ-potential measurements (Figure 5.14), and the particle size data indicate that, in the absence of pepsin (Figure 5.5B), the adsorbed protein layer is not hydrolysed at the oil/water interface, apparently

inhibiting lipase adsorption and hence lipolysis. This behaviour is also observed at pH 5.5 (Figure 5.5Aiv) when pepsin is included, and can be attributed to the fact that the protease has little activity at this elevated pH. Given that lipolysis does not take place with lipase alone, significant hydrolysis of the proteins in the interfacial layer appears to be a mechanistic requirement, presumably by way of detaching charged and polar domains. Such a process would render the interface more hydrophobic, causing droplet aggregation (notably at pHs some distance from the p*I*) as well as providing more favourable binding sites for lipase adsorption.

Once the lipase is able to adsorb to the droplet interface, hydrolysis of the triglyceride into diglycerides and free fatty acids can commence. The fatty acids generated during hydrolysis compete at the interface with the adsorbed protein. The structural integrity of the interfacial layer may be compromised through the orogenic displacement of protein by fatty acids (van Aken et al., 2011) This will consequently reduce the ability of the viscoelastic protein layer to stabilise the interface, since the adsorbed layer of fatty acids will be expected to provide poor mechanical stability in comparison (van Aken et al., 2011). In dilute emulsions where droplets are non-associating, poor surface stability does not necessary lead to coalescence, providing sufficient repulsive forces between droplets are maintained. However, droplet association through flocculation (either through charge or hydrophobic interactions) can cause coalescence even in dilute emulsions by bringing poorly stabilised droplets into close proximity, to the point that 'spontaneous' film rupture between neighbouring droplets can take place (Dukhin et al., 2003, Dukhin et al., 2001, van Aken, 2003).

Finally, it is interesting to note that, when *in vitro* digestion was conducted at pH 2, the emulsion was flocculated for only the first 30 min of digestion. These floc structures were progressively eroded as digestion proceeded, allowing the initial droplet distribution to be regained. Therefore, there was little change in particle size distribution by the end of the period of *in vitro* digestion. At this low pH the adsorbed protein layer was found to be highly positively charged, effectively preventing extensive flocculation perhaps pending more extensive proteolysis. Whilst the drop in ζ-potential measurements indicated that some proteolysis of the adsorbed protein layer did appear to have occurred, it is arguable whether any reduction in surface charge would be sufficiently substantial to prevent electrostatic repulsion.

In terms of the lipase, several systems were digested in duplicate with fungal and with pregastric lipase, since the optimal pH of fungal lipase (pH 7.5) (Manuel, 1999) differs from that of mammalian lipases (pH 5.6-6.5) (Lai, Mackenzie, O'Connor, & Turner, 1997),. Similar trends were observed with both enzymes. However, the calf pregastric lipase was less stable and reproducibility correspondingly was impaired so fungal lipase was used for the remaining of the study.

5.5.3 Different processing of commercial infant formulae

Karicare® powder infant formula appeared more digestible than S–26® powder infant formula due to a greater increased in particle size (The d_{43} at 120 min was 2.405 and 1.199, respectively). Both products contain soy lecithin. It is mindful to note that different soy lecithin differs in phospholipids profile and fatty acid

composition. The main difference observed between the two products is that the whey proteins in S–26® powder formula are mainly α-lactalbumin while that in Karicare® powder formula is a mixture of α-lactalbumin and β-lactoglobulin.

A previous study has shown that α-lactalbumin was hydrolysed by pepsin rapidly during *in vitro* digestion. However, in the presence of phospholipids, it was found that the interaction between α-lactalbumin and phosphatidylcholine (one of the major constituent in phospholipid) was associated with a decreased proteolysis. Thus, resulting in a slower rate of gastric digestion (Moreno et al., 2005).

It is also possible that heat process conditions have a negative effect on the digestibility of the product. Lacroix et al (2008) studied the effect of heat process on digestibility of milk proteins. It was reported that the digestive kinetics for UHT milk is faster than pasteurised milk. This is due to heat induced aggregation of whey protein and casein which formed a softer casein coagulum and enhanced the susceptibility to proteolytic enzymes to break down the proteins.

As infant formula is a complex system, more work needs to be done to have a better understanding of its behaviour under *in vitro* gastric digestion and to confirm the hypothesis.

5.6 Conclusions

Together the results of this study indicate that the digestion of formula milks requires the concerted action of proteolytic and lipolytic enzymes. In spite of the smaller oil droplet size and correspondingly greater oil/water surface area available for

enzymatic attachment in formula feeds, the results suggest that differences in the proteinaceous layer at the oil/water interface from those in biological milks (Guo et al., 1996) may render their digestion less efficient (Armand et al., 1996). Variations in formulation and processing conditions may additionally influence the structure and composition of the interfacial proteinaceous layer of manufactured milks. This may in turn influence susceptibility to both proteolysis and lipolysis, with further consequence for digestibility.

Whilst the concerted action of proteolytic and lipolytic enzymes may serve to secure an adequate digestibility, it is important to note that such synchrony would best be achieved if both enzymes had similar pK_a's and their synthesis and secretion to commence equally promptly at birth, which not the case in human infants.

In devising formula feeds with an oil/water interface that is more amenable to digestion by the neonate, it is important to note that *in vitro* evaluations should employ physiological shear rates if inappropriate augmentation of coalescence and disruption of the proteinaceous interface are to be avoided. Again, given the sensitivity of digestion to the ambient pH, due allowance must be made for temporal variation in the postprandial gastric pH with the secretory rates of HCl and the buffering capacity of the formula feed. It is also important to note that understanding of the effect of ambient pH on the kinetics of human gastric lipase is incomplete, as is knowledge of the extent to which the actions fungal lipases are analogous.

Chapter 6 The effect of lactoferrin on physical changes in lecithin stabilised emulsions during *in vitro* gastric lipolysis[2]

6.1 Abstract

The effect of the interaction of and alignment of protein with the oil/water interface in stable emulsions on *in vitro* simulated gastric lipolysis by *Rhizopus oryzae* lipase was explored using Intralipid®, a commercial soy oil emulsion stabilised by lecithin, before and after electrostatic binding with lactoferrin.

Lipolysis of 20% untreated Intralipid®, as evidenced by increase in droplet size i.e. d_{43} and by confocal microscopy, took place at pH levels between 3.5 and 5.5 and that lipolysis was evident with lipase alone and with mixtures of pepsin and lipase at pH 3.5. Conversely, no coalescence was evident on digestion with lipase alone at pH levels below 5.5 of Intralipid® treated with lactoferrin. However, coalescence of droplets in treated Intralipid® did take place at pH levels above 2 when both pepsin and lipase were present. The ongoing decrease in droplet charge in lactoferrin treated emulsions at pH 3.5 and 4.5 in digesta containing lipase or both pepsin and lipase indicated ongoing lipolysis was taking place.

This research concluded that the interaction of lactoferrin with the oil/water interface of soy droplets inhibited the action of lipase pending digestion by pepsin.

[2]Part of the contents presented in this chapter has been published as a paper in Food Hydrocolloids: Lueamsaisuk, C., Lentle, R. G., MacGibbon, A. K. H., Matia-Merino, L., & Golding, M. (2014a). 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? DOI: 10.1016/j.foodhyd.2014.08.010

6.2 Introduction

Given that lipolytic digestion depends upon the location of the lipolytic enzyme on the oil/water interface either alone as in the case of gastric lipases or with the aid of other elements such as colipase in the action of pancreatic lipase it is likely that oligomeric fractions and other dietary proteins with significant hydrophobic regions that partition across the oil/water interface may sterically hinder their action. Such complexities may be particularly important in neonatal nutrition where milk is the principal diet and fat the principal energostatic metabolite. This chapter may expect to see particular adaptation to maximise lipolytic efficiency and minimise competitive proteinous steric exclusion of lipases.

An understanding of such adaptation is particularly important in devising breast milk substitutes. However, variation in the composition of breast milk between individuals and with time (Malbon, 2006, Khan et al., 2013) along with difficulties in repeated sampling of the gastric contents of neonates compromise repeatability. Thus it is necessary to simulate the physical and chemical environment *in vitro*.

In the previous chapter, changes in the coalescence stability of formula emulsions were examined *in vitro*. The findings confirmed that the coalescence of emulsion droplets that characterises the onset of enzymatic lipolysis was most likely to occur at pH between 3.5 and 4.5, in the presence of both gastric lipase and pepsin. Further when incubation was carried out with either lipase or pepsin alone coalescence was either slowed or did not occur. It was also shown that propensity to coalescence

increased with rate of shear indicating that it was necessary to limit shear rate to those which would normally be encountered in the gastric lumen.

These findings fitted with a hypothesis that droplet interface caused by the adsorption on the droplet interface of free fatty acids lead to coalescence but that this process was inhibited by a layer of adsorbed protein that acted as a barrier for lipase adsorption and required synchronous action of gastric protease to disperse.

To validate this hypothesis and further explore how this apparent enzymatic co-dependency facilitates the digestion of protein–stabilised emulsions, this chapter shows how a pH dependent layer-on-layer technique was utilised to coat a phospholipid–stabilised (negatively charged) emulsion system with a protein coat (positively charged lactoferrin-stabilised) by electrostatic binding, and examine how this change in interfacial composition influenced the stability of the emulsion under *in vitro* gastric conditions.

6.3 Materials and methods

6.3.1 Materials

6.3.1.1 Intralipid®

Intralipid®, a lecithin–stabilised emulsion of soya oil was purchased from Fresenius Kabi Australia Pty Limited, NSW Australia. The solution contained 20% triglyceride, 1.2% phospholipid and 2.2% glycerol. The details of Intralipid® are

described in Chapter 3. A 3.6% (v/v) solution of Intralipid® was prepared by dilution with Milli-Q water (3.6 mL of 20% Intralipid® added to 16.4 mL Milli-Q water).

6.3.1.2 Lactoferrin

Bovine lactoferrin was a gift from Fonterra Research and Development Centre, Palmerston North, New Zealand. A 1% (w/v) of lactoferrin powder in 3.6% (v/v) Intralipid® was prepared by first dissolving 0.2 g of lactoferrin powder in 16.4 mL Milli-Q water, adjusting pH to 6.8 and stirring for one hour to allow complete dissolution. 16.4 mL of this solution were then added 3.6 mL of 20% Intralipid® in the mixture stirred at room temperature for a further 2 hours before use.

6.3.1.3 Enzymes

Porcine pepsin (porcine, 800–2500 U/mg) and fungal lipase extracted from *Rhizopus oryzae* (80 U/mg) were used (details mentioned in Chapter 3).

6.3.1.4 Walstra solution A

The Walstra solution A used in the determination of emulsion particle size in subsamples of digestate, comprised 0.375% (w/v) of EDTA and 0.125% (w/v) of Tween 20 from BDH Chemicals (BDH Ltd., Poole, England) made up in RO water at pH 10.

6.3.2 Methods

6.3.2.1 *In vitro* digestion of emulsion samples

Either 20 mL of the 3.6% Intralipid® solution alone or the similarly diluted Intralipid® with 0.2 g of lactoferrin solution per 20 mL were each sheared at a speed of 100 rpm for two hours in a 400 mL beaker at 37°C prior to the commencement of *in vitro* digestion.

Simulated gastric fluid (Intralipid® solution: SGF ratio = 20:50 v/v) was then added. The pH of the mixture was maintained at either 2, 3.5, 4.5 or 5.5 using 1 M HCl or 1M $NaHCO_3$. The mixture was incubated at 37°C and stirred at 10 rpm in a 400 mL beaker for a total of 2 h. One mL aliquots of digestate were taken every 15 min for subsequent determination (details mentioned in Chapter 3).

6.3.2.2 Determination of emulsion-SGF mixtures

The effects of the protein–stabilised emulsion, various pH and enzymes on droplet stability and size were explored by comparing the droplet size distributions. Characterisation of droplet structure was examined by confocal microscopy. The charge on the fat droplets was quantified by determining the electrical potential termed the ζ-potential as described in Chapter 3.

6.4 Results

6.4.1 Droplet size distribution

The mean droplet size (d_{43}) of diluted Intralipid® at pH 7 prior to commencement of digestion was 0.32 μm whilst that of lactoferrin treated Intralipid® was 0.4 μm (Figure 6.1). There were no changes in droplet size determined either with or without Walstra solution on incubation at pH 3.5 for 120 min with no enzymes added (Figure 6.2).

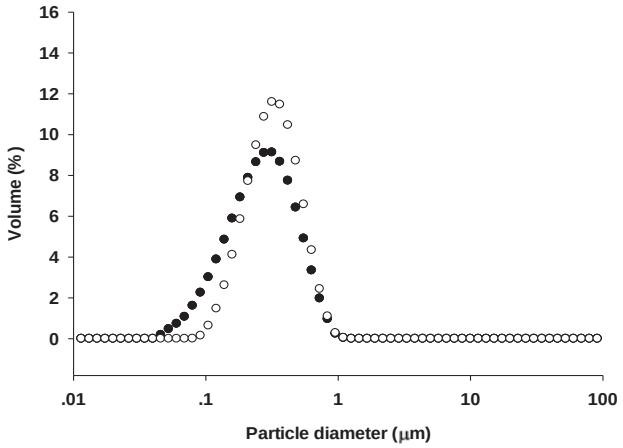


Figure 6.1 **Droplet size distributions of freshly made 3.6% (v/v) Intralipid® emulsion (●) and 3.6% (v/v) Intralipid® emulsion treated with lactoferrin (○). Analysed with Walstra solution A.**

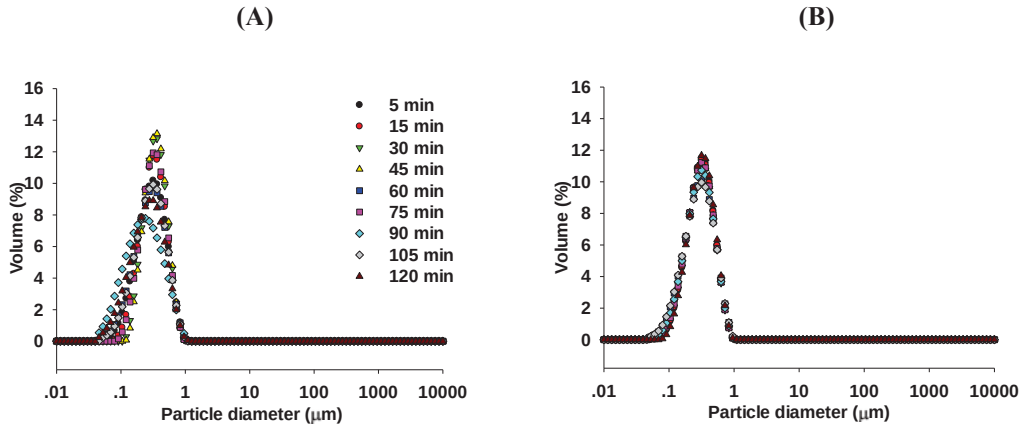


Figure 6.2 **Changes in droplet size distribution of Intralipid® 3.6% (v/v) during incubation with SGF without enzymes at pH 3.5 at 37°C, 10 rpm, for 120 min. Analysed without Walstra solution A (A) or with Walstra solution A (B).**

Droplet diameter of aliquots of digesta containing pepsin and lipase, determined with Walstra solution, tended to increase over time at pH levels above 2 and was maximal at pH 3.5 (Figure 6.3A). Droplet size as determined by (d_{43}) after treatment of aliquots of digestate with Walstra solution A did not change with pepsin alone at pH 3.5 but increased over two hours on incubation with lipase alone and with lipase plus pepsin the increase being greater in the latter (Figure 6.2B). Mean droplet size did not change with any of the enzyme combinations at pH levels above or below this value.

Droplet diameter of Intralipid® emulsions treated with lactoferrin incubated with both pepsin and lipase tended to increase over time at all pH levels above 2.0, being maximal at pH 5.5 (Figure 6.4A) as was droplet size (d_{43}). Droplet size did not increase on incubation of lactoferrin treated Intralipid® emulsions with pepsin alone but there was some low level increase in this parameter on incubation with lipase alone at pH 5.5 (Figure 6.4B).

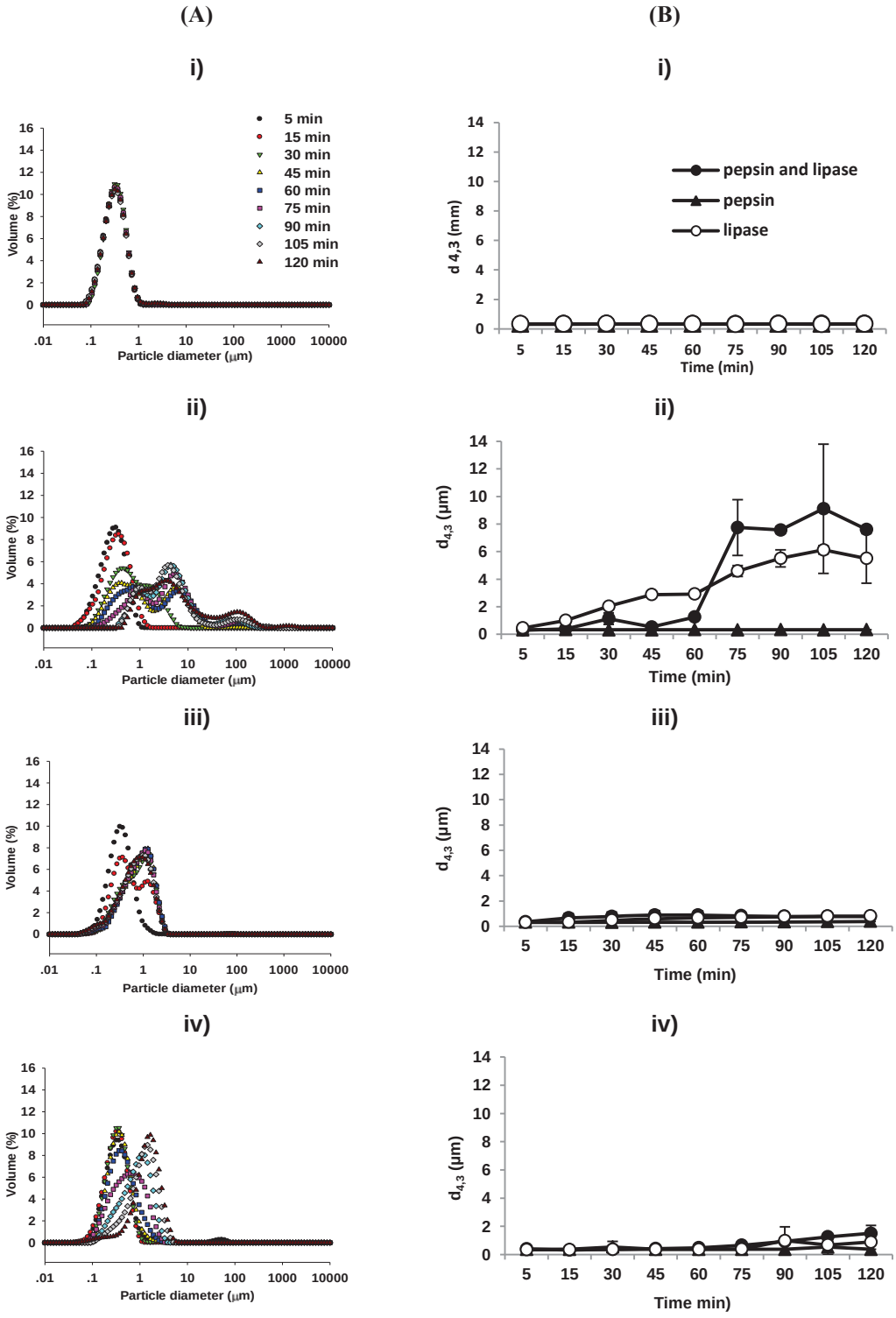


Figure 6.3 **Changes in droplet size distribution (A) and diameter d_{43} (B) of 3.6% (v/v) Intralipid® emulsion during digestion with SGF with pepsin and fungal lipase at pH 2 (i), pH 3.5 (ii), pH 4.5 (iii), pH 5.5 (iv) at 37°C, 10 rpm, for 120 min. Analysed with Walstra solution A. The error bars represent the standard deviation of three repeat measurements.**

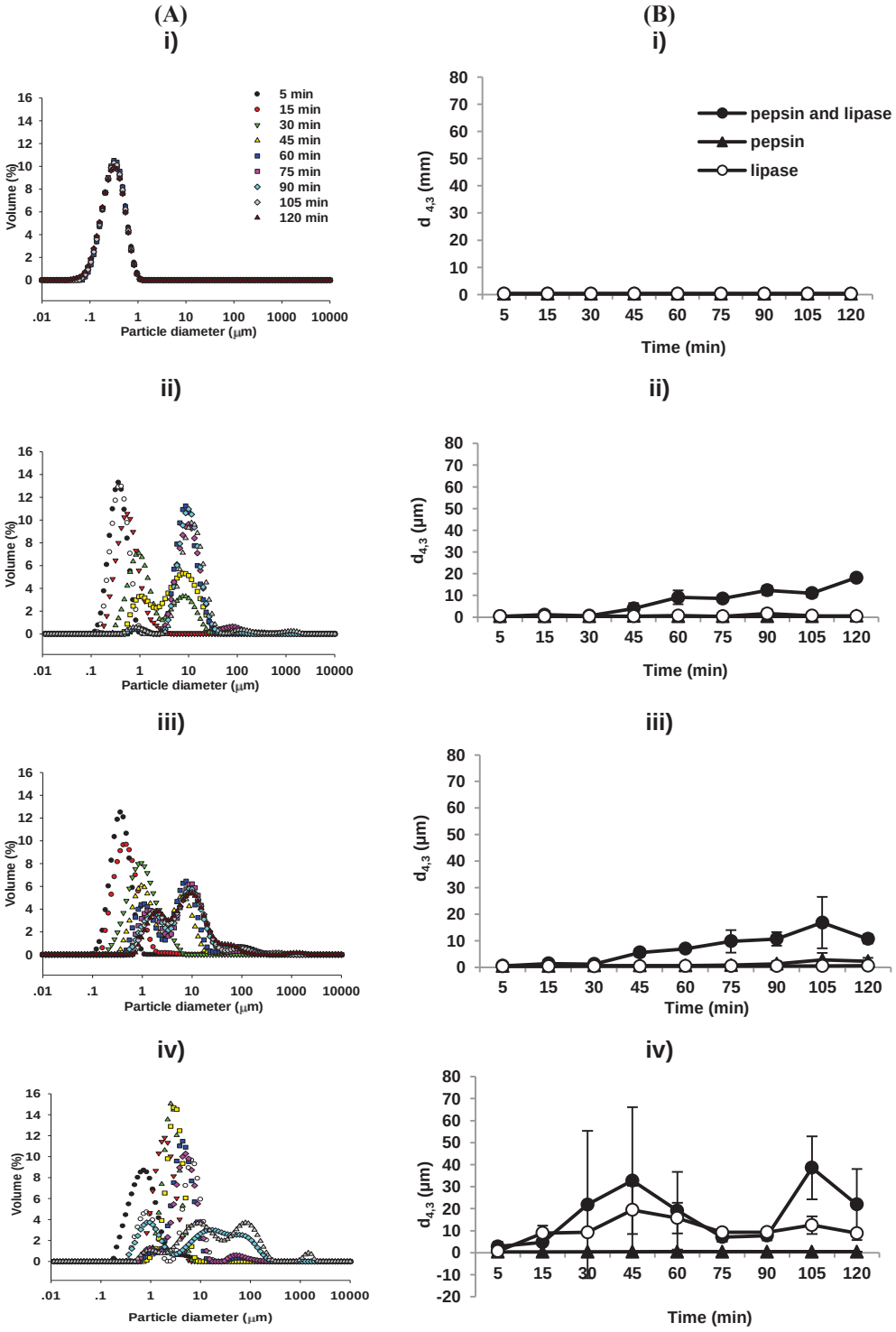


Figure 6.4 **Changes in droplet size distribution (A) and diameter d_{43} (B) of 3.6% (v/v) Intralipid® treated with 1% (w/v) lactoferrin during digestion with SGF with pepsin and fungal lipase, at pH 2 (i), pH 3.5 (ii), pH 4.5 (iii), and pH 5.5 (iv), at 37°C, 10 rpm, for 120 min. Analysed with Walstra solution A. The error bars represent the standard deviation of three repeat measurements.**

6.4.2 Confocal microscopy

Confocal microscopic assessment of aliquots of digestate treated with Walstra solution A (Figure 6.5 and Figure 6.6) and without Walstra solution A (Figure 6.7) confirms the time dependent changes is droplet diameter found with the Mastersizer. Droplet diameter on incubation of Intralipid® solution with both pepsin and lipase was maximal at pH 3.5 (Figure 6.5) whilst droplet size of Intralipid®/lactoferrin emulsions incubated with the two enzymes increased at all pH levels above 2 and was maximal at 5.5.

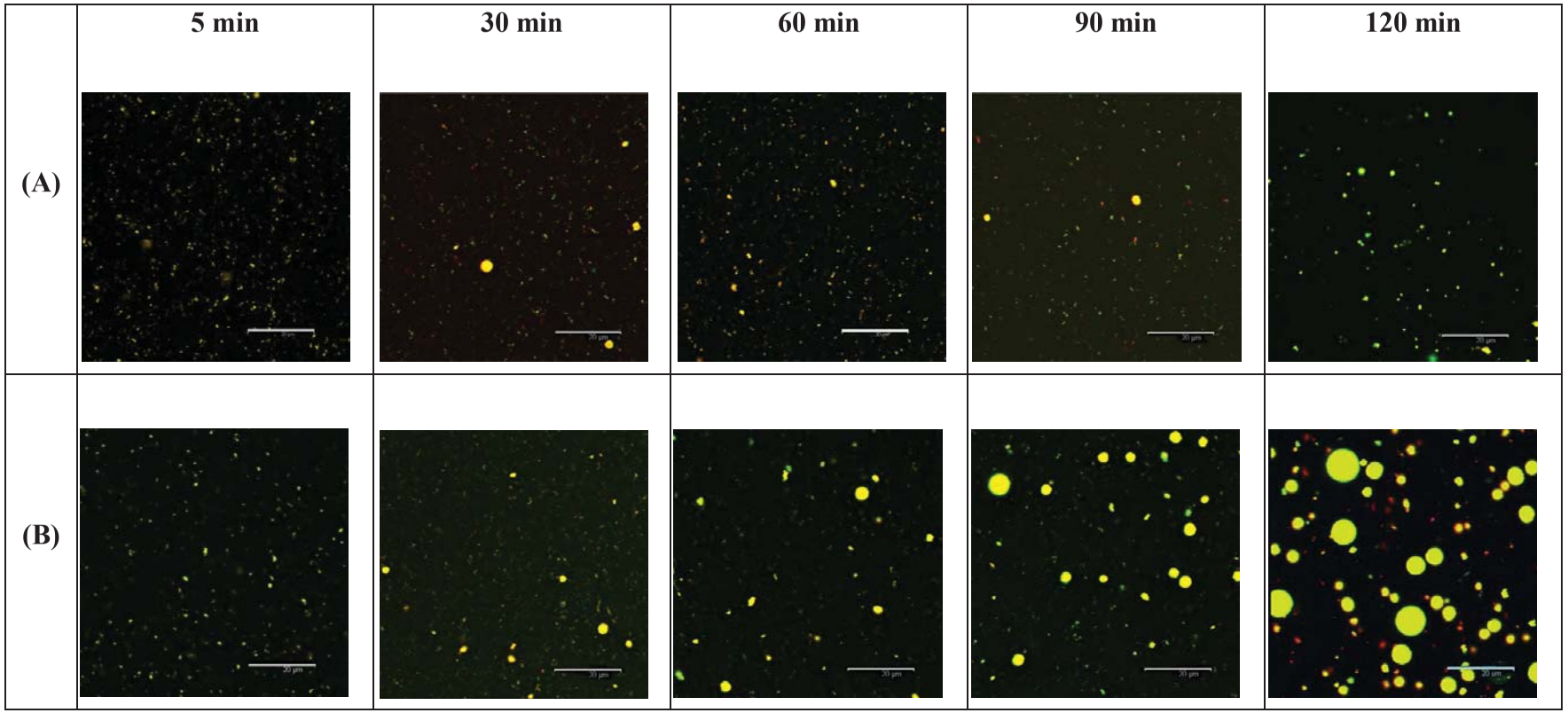


Figure 6.5 **Changes in the microstructure of 3.6% (v/v) Intralipid® during digestion with SGF with pepsin and fungal lipase, at pH 2 (A), pH 3.5 (B), pH 4.5 (C) and pH 5.5 (D), at 37°C, 10 rpm, for 120 min. Analysed with Walstra solution A. Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

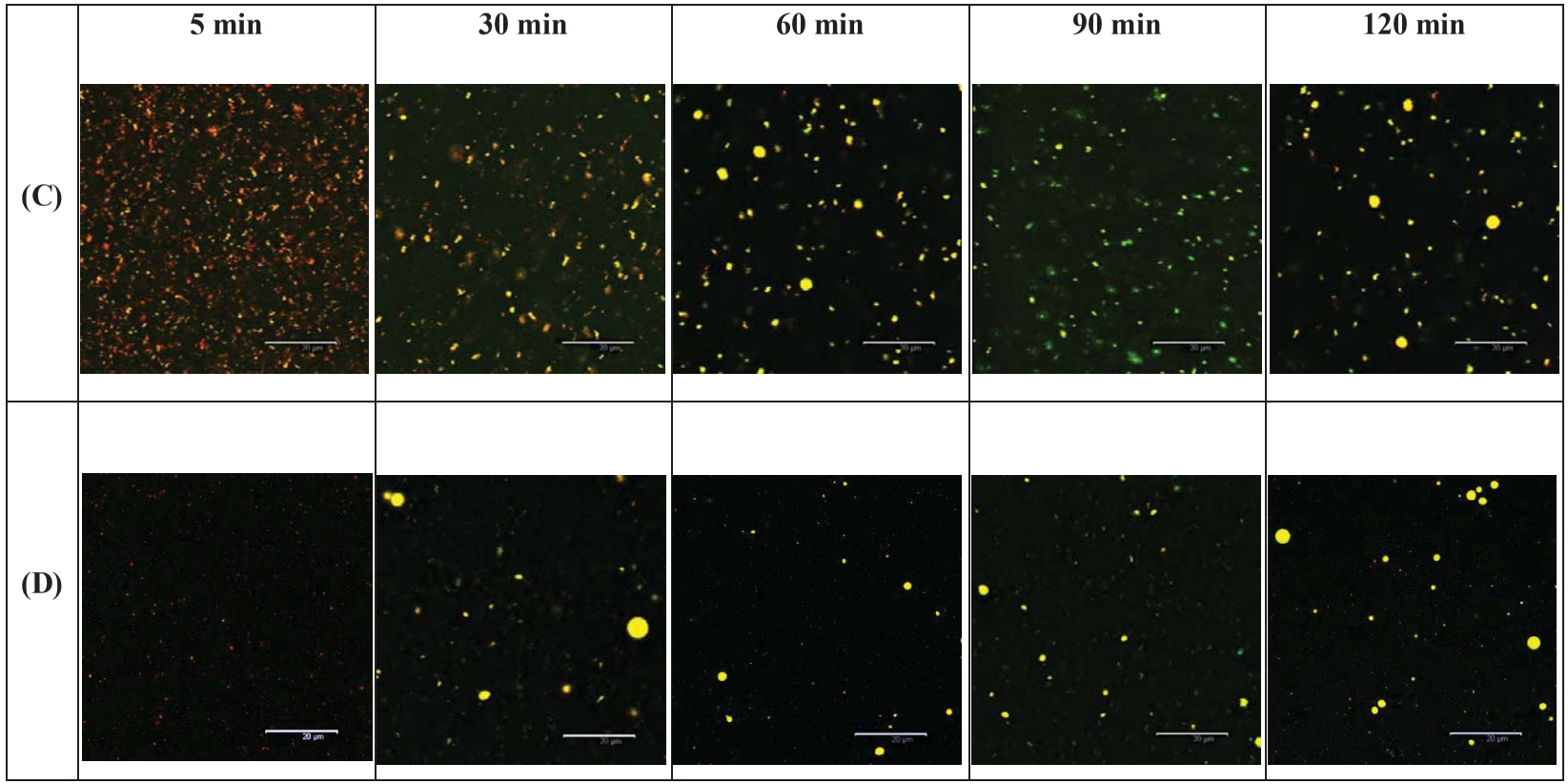


Figure 6.5 (Continued).

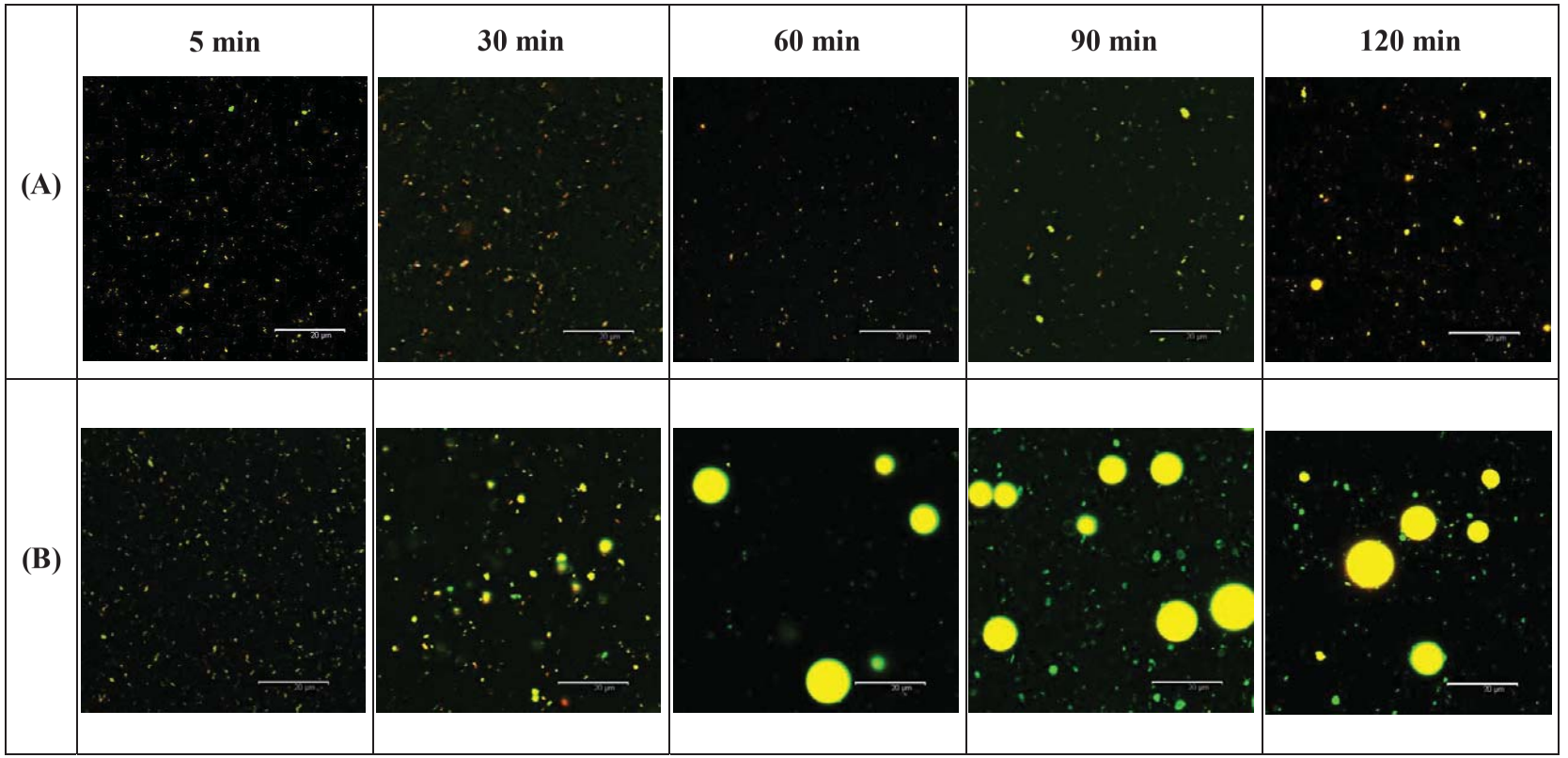


Figure 6.6 **Changes in the microstructure of 1% (w/v) lactoferrin treated 3.6% (v/v) Intralipid® during digestion with SGF with pepsin and fungal lipase, at pH 2 (A), pH 3.5 (B), pH 4.5 (C) and pH 5.5 (D), at 37°C, 10 rpm, for 120 min. Analysed with Walstra solution A. Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

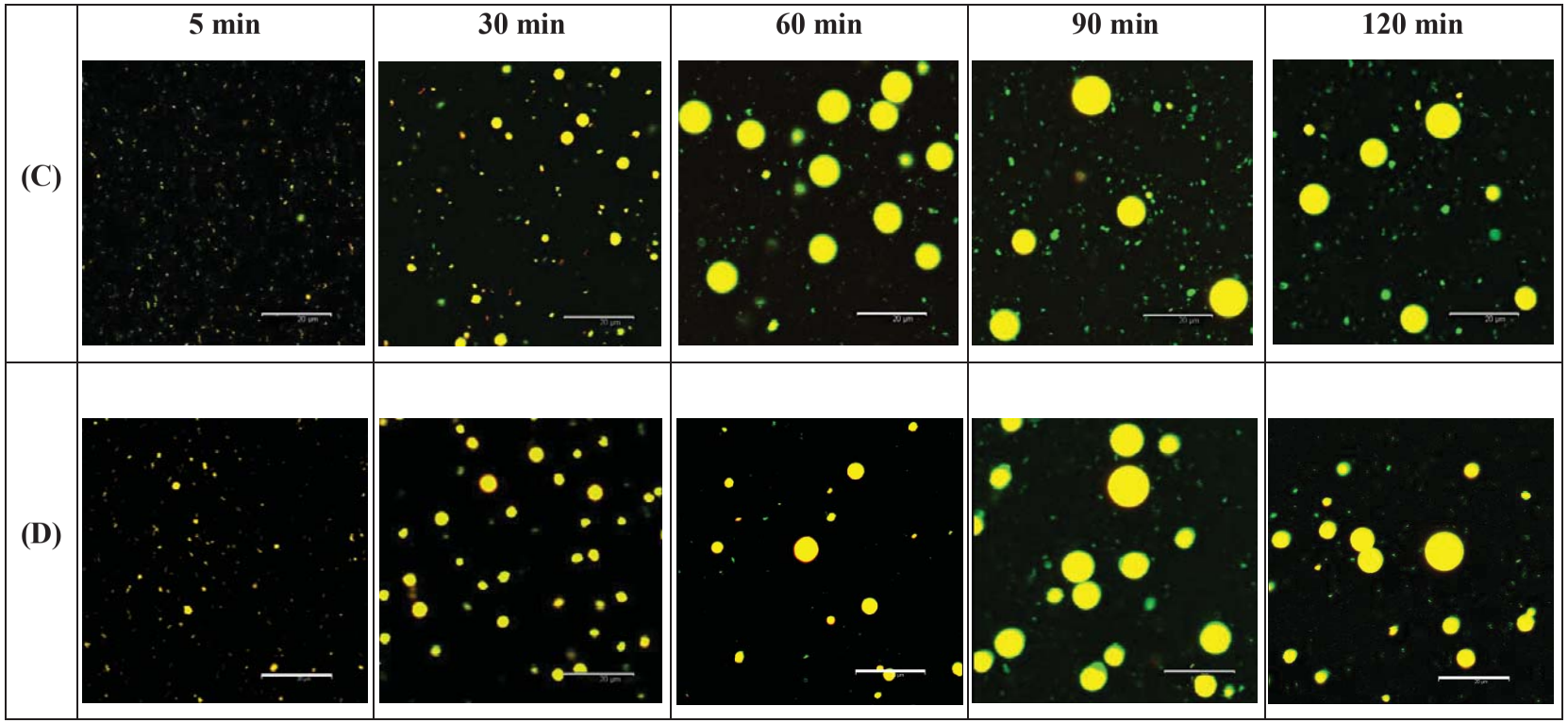


Figure 6.6 (Continued).

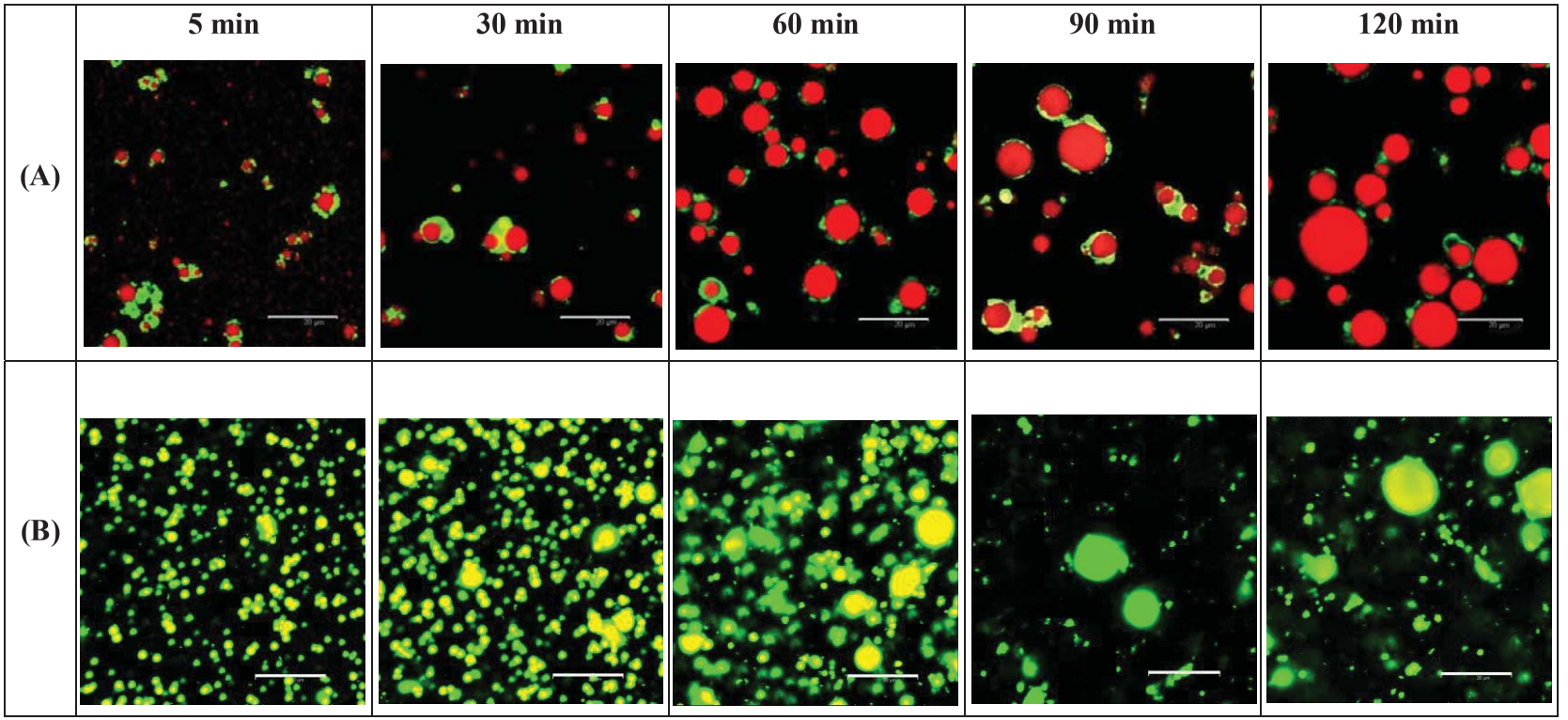


Figure 6.7 **Changes in the microstructure of 1% (w/v) lactoferrin treated 3.6% (v/v) Intralipid® during digestion with SGF with pepsin and fungal lipase, at pH 3.5 (A) and pH 5.5 (B), at 37°C, 10 rpm, for 120 min. Analysed without Walstra solution A. Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

6.4.3 Surface charge

The ζ-potential of the 3.6% (v/v) Intralipid® emulsion was –44.06 mV at neutral pH whereas lactoferrin treated Intralipid® emulsion had a high positive charge +16.6 mV at the same pH (pH 7).

The ζ-potentials of the aliquots 3.6% (v/v) Intralipid® emulsion sampled in the absence of enzymes or during digestion with fungal lipase and or pepsin (Figure 6.8A) were consistently positive at a pH of 2 and consistently negative at pH levels above 2 and changed little during the course of digestion at any pH.

The ζ-potentials of the aliquots 3.6% (v/v) Intralipid® emulsion treated with lactoferrin sampled in the absence of enzyme or during digestion with fungal lipase and or pepsin (Figure 6.8B) were consistently positive at all pH levels but became less positive over time at pH at pH 4.5 and 5.5 when lipase was present either alone or in combination with pepsin.

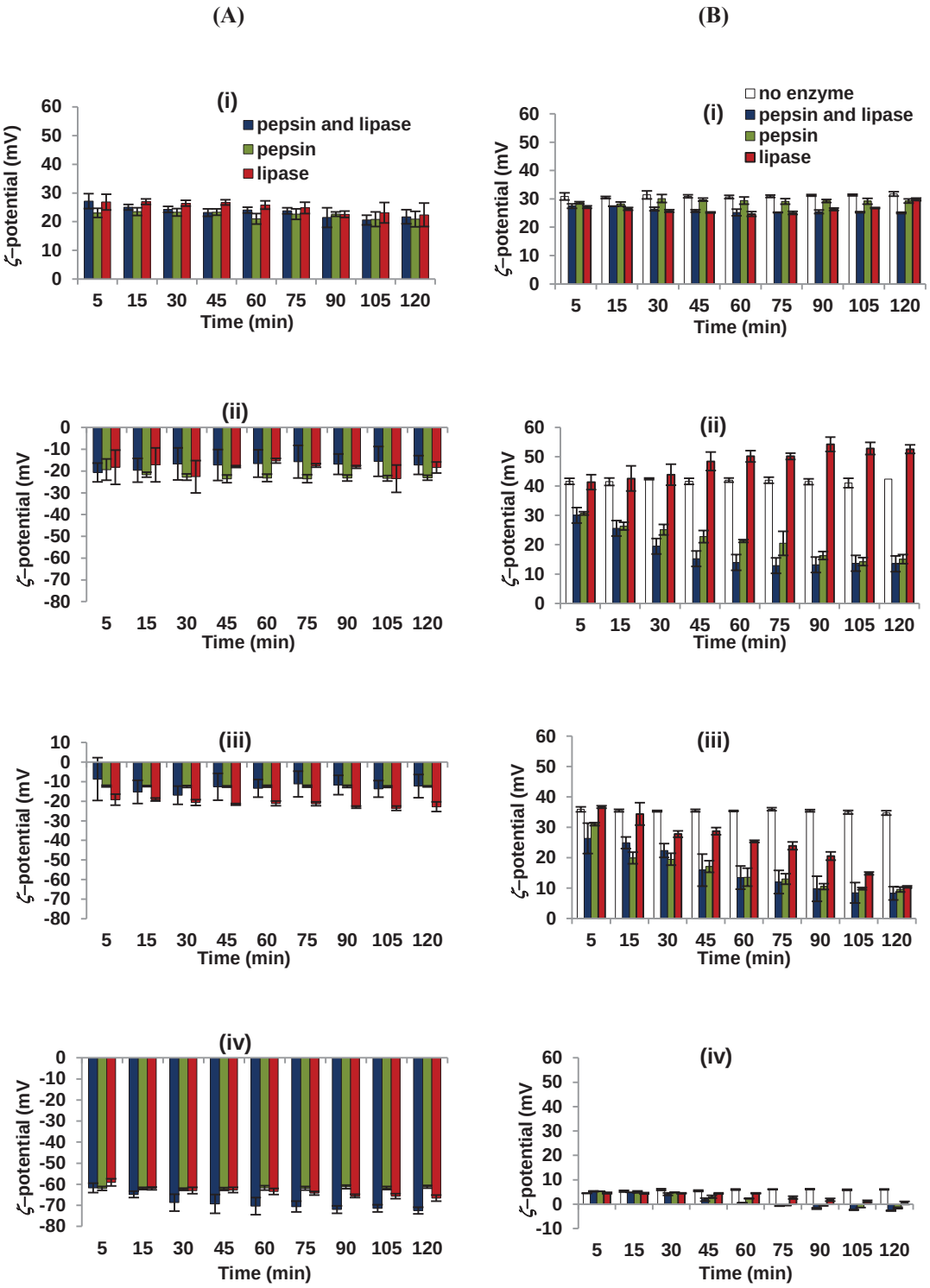


Figure 6.8 **Changes of ζ-potential of 3.6% (v/v) Intralipid® emulsion (A) and 3.6% v/v Intralipid® emulsion treated with 1% (w/v) lactoferrin (B) during digestion with SGF at pH 2 (i), 3.5 (ii), 4.5 (iii) and 5.5 (iv), at 37°C, 10 rpm, for 120 min. The error bars represent the standard deviation of five repeat measurements.**

6.5 Discussion

The coalescence of droplets in untreated Intralipid® during simulated gastric digestion at pH 3.5-5.5 (Figure 6.3 and Figure 6.4) may be attributed to a destabilising change in the physical characteristics of their oil/water interfaces resulting from lipolysis. It is known that the free fatty acids generated by ongoing lipolysis are surface active (Thomas et al., 2012), and able to adsorb to the oil/water interface (Wabel, 1998). This is likely to displace the original phospholipid membrane and promote coalescence. Changes in the droplet of a previously stable emulsion thus may provide an indirect indication that lipolysis is occurring. It is evident that the original phospholipid oil/water interface of the untreated Intralipid® emulsion did not inhibit the absorption and action of fungal (*Rhizopus oryzae)* lipase. It is likely that the phospholipid membrane might not inhibit the action of human gastric lipase. Similar levels of coalescence were observed when digestion was carried out with either fungal lipase alone, or in combination with pepsin. This observation is to be expected as there is no protein present at the interface so that pepsin would not have any influence on the stability of the Intralipid® emulsion, since. This conclusion was further validated by the finding that the size of emulsion droplets did not change during digestion with pepsin alone.

The absence of coalescence in digestates containing either or both of the two enzymes at pH 2 could result from effects on either the ionisation characteristics or surface charges of the reaction components. The catalytic site of the lipase is buried under a lid or cap domain in both, human gastric lipase (Roussel et al., 1999) and in *Rhizopus oryzae* lipase (Beer et al., 1996). The reactive site is separate from the

binding site and its function is likely to be independent of interfacial structures and activation (Verger, 1980, Derewenda et al., 1992). Electrostatic effects on the conformation of the active sites of fungal lipase may inhibit its activity whilst effects on the change of the enzyme or the droplets may inhibit lipase location on the oil/water interface. The optimum pH of *Rhizopus oryzae* lipase is reported to be 7.5 (p*I* 7.6) and it is reported to be stable in the range 4.6-7.5 (Hiol et al., 2000). Hence it appears that the first possibility is likely. Again it is conceivable that lipolysis may be occurring but that of droplet to droplet contact is inhibited from retention of their positive surface charge. Similarly the greater propensity to coalescence at pH 3.5 may result from minimisation of surface charge (relative to pH 4.5 and 5.5), reducing the repulsive forces between droplets, increasing the likelihood of contact and coalescence.

The addition of the protein lactoferrin to the Intralipid® emulsion was intended to create a protein coating on the surface of the droplets as a consequence of electrovalent bridging to the phospholipid membrane. The p*I* of lactoferrin is 8.7, hence the protein will carry a net positive charge at pH levels below this. At pH 3.5 and above, the ζ-potential values indicate showed that the untreated Intralipid® emulsion carries a negative charge (Figure 6.8A). These values became positive following treatment with lactoferrin (Figure 6.8B) confirming electrostatic crosslinking between the phospholipid and the lactoferrin had occurred. However, at pH 2 there was no change in surface potential on addition of lactoferrin. As both phospholipid and lactoferrin carried a positive charge but pH 2 no electrostatic crosslinking would be expected to occur, as was evidenced by the lack of change in

ζ-potential in lactoferrin treated Intralipid® at pH 2.0. Correspondingly the lack of any change in the droplet size when the lactoferrin treated Intralipid® was digested with either fungal lipase or with pepsin alone at pH 2, is consistent with the fact that interfacial composition is not modified by the addition of the lactoferrin so that the outcomes reflect those obtained with untreated Intralipid®.

The effects of treatments with the two enzymes at pH levels between 3.5 and 5.5 differed considerably from those obtained with the unmodified Intralipid®. There was no detectable change in droplet size on digestion with pepsin alone indicating that proteolysis of the lactoferrin coat, which is evidenced by the progressive downward shift in ζ-potential with respect to the preparation containing no enzyme (Figure 6.8B), did not compromise emulsion stability. The phospholipid membrane to which the protein was electrostatically bound was unaffected by the binding with lactoferrin or by the subsequent digestion process and continued to stabilise the droplets after the lacttoferrin had been digested.

The apparent maintenance of droplet stability on digestion with lipase alone is likely due to lactoferrin forming a physical barrier that prevents the hydrophobic surfaces of *Rhizopus oryzae* lipase (Beer et al., 1996) from engaging with that of the droplets. Alternatively it could be postulated that lipase was able to form a hydrophobic linkage with the droplet but that the fatty acids produced on lipolysis subsequently bound to lactoferrin rather than partitioning into and destabilising the phospholipid coat of the droplet. However, given that the pH conditions during digestion are appreciably below pK_a of most fatty acids, and thus that the fatty acids produced are not likely to be ionised, this is less likely to be the case. The rapid coalescence of the

emulsion on *in vitro* digestion with both lipase and pepsin at pH 3.5 and 4.5 indicates that the location of lipase on the oil/water interface and subsequent lipolysis, is facilitated by proteolysis of the layer of adsorbed lactoferrin by pepsin.

The increase in droplet size observed when the emulsion was digested with lipase alone at pH 5.5 appears inconsistent with observations made at pH 3.5 and 4.5. However, the charge neutral state of the Intralipid®/lactoferrin emulsion at this pH (Figure 6.8B) may allow greater flocculation, increasing the likelihood of coalescence of droplets by bringing them into closer contact. This hypothesis is supported by the results of confocal microscopy (Figure 6.7) showing the formation of clusters of droplets at pH 3.5 and 5.5 in the absence of Walstra solution A.

6.6 Conclusions

The findings of this study are consistent with those of previous chapter that investigated physical changes of formula emulsions during *in vitro* gastric digestion, in indicating that gastric lipolysis may be inhibited by a layer of protein adsorbed at the oil/water interface. Pepsin may thus have a dual role in directly facilitating proteolysis, and indirectly facilitating lipolysis through improving accessibility of the lipase to the interface.

The results illustrate the complex physical interplay between the macronutrient components of the diet both before and during the process of digestion and the need for synergism of the various enzymes which digest them. The latter observations underscore the importance of including the full range of enzymes in the *in vitro* studies regardless of the substrate e.g. pepsin in studies of lipid digestion. Again, in

highlighting the differing physical outcomes at different ambient pHs, the studies show that the significant changes in gastric pH values during the postprandial period (Bovo et al., 1995) may influence the relative activities of the suite of gastric enzymes and hence the products of their synergy.

Chapter 7 Structure dynamics of human breast milk during simulated gastric digestion

7.1 Abstract

Human breast milk is reported to be more readily digested than infant formulae (Splinter and Schreiner, 1999). In the particular case of lipid digestion, there is greater dependency on digestion progressing in the stomach rather than in the small intestine due to the immaturity of pancreas. This is opposed to the situation in the adult, where lipid digestion occurs predominantly in the small intestine.

It can be argued that the characteristics and physicochemical properties of breast milk have developed to optimise digestion in the infant since the digestive physiology of the infant differs from that in the adult. Therefore the levels of secretions of gastric lipase observed in the infant stomach and the ambient pH level is higher in the infants than in adults. Conversely, the structures and composition, generated during the manufacture of infant formula, may differ from those of human milk and render the milk digestable.

To explore how the conditions in the stomach of infants impact on the structure and stability of natural human milk, the behaviour of breast milk explored under simulated infant gastric conditions was examined over a range of ambient pH conditions (pH 3.5, 4.5 and 5.5). Accordingly, structural changes to human milks were observed as a consequence of exposure to pepsin and fungal lipase (separately and in combination) at 10 rpm stirring speed for a range of times up to 2 h. The

effects of digestion on the emulsion structure and stability were examined by determining the droplet size, microstructure and charge.

Droplets were found to be resistant to coalescence across all applied conditions, in marked contrast to observations made for protein-stabilised emulsions reported in earlier chapters. Structural changes arising from flocculation (attributed to protein precipitation) were observed at pH 4.5, otherwise gastric conditions had little apparent impact on emulsion structure. Changes in droplet surface potential indicated that surface proteins of the MFGM were hydrolysed, however, in spite of this, these droplets were seen to be remarkably resistant to coalescence. Whilst this lack of coalescence could indicate that lipolysis was not taking place, it is more likely that lipolysis does, but that the biological emulsion system was not susceptible to coalescence. This is possibly either due to lack of aggregation of fat droplets during digestion (thereby minimising coalescence arising from droplet–droplet contact), or as a consequence of the stabilisation of droplets by fatty acids derived from lipolysis (due to the distinct differences in triglyceride composition between human and formula milks). These observations are consistent with the argument that excessive coalescence under gastric conditions would serve to reduce digestive efficiency and would be biologically less favourable.

7.2 Introduction

Milk is the first and only food ingested by human infants for a period of time (0–6 months) (Damodaran et al., 2008). The efficient digestion of milk is important for the infant to assimilate the nutrients that are necessary for optimal development and growth (Serpero et al., 2012). Breast milk is reported to be better digested by the

infant compared to formula milks. Breast feeding has long been promoted as its nutrient profile is perfectly to balance the requirements of human infants with respect to fatty acids, amino acids, vitamins and minerals (Haumont, 2010, Groh-Wargo and Sapsford, 2009, Berenhauser et al., 2012, Arslanoglu et al., 2009). Whilst the composition of human breast milk varies widely between countries, among women and between feedings, it is still recommended as the optimal nutrition source, as it produces more favourable results in the infant than lipid emulsions or infant formulae (Groh-Wargo and Sapsford, 2009, Lapillonne et al., 2013, Fink, 2013).

The fat globule size in human breast milks range from 0.2 μm to more than 15 μm with a mean size of 4 μm (Michalski et al., 2005a). The globules comprise a large triacylglycerol core that is coated by a biological membrane being composed of a complex structure of polar lipids, sphingolipids, cholesterol, proteins, phospholipids and glycolipids (Argov et al., 2008).

As already discussed, the digestive processes of the infant are less well developed due to insufficient pancreatic developmental (Hamosh, 1996). Because of this, the action of gastric lipase plays a more significant role in lipid digestion (Hamosh, 1996, Roman et al., 2007). One consideration as to why lipids in maternal milk are more readily digested than in formula milks is that the human breast milk emulsion provides a more favourable interface for gastric lipase adsorption. Based on observations made in chapters 5 and 6, it is hypothesised that this may be due to the surface being composed mainly of phospholipids, as opposed to the proteinaceous surface layer observed in the milk droplets in formula milks, which appears to act as a barrier to lipase adsorption.

However, it may also be considered that fat digestion and absorption of human milk is improved by the action of endogenous lipoprotein lipase and bile salt–stimulated lipase content which are secreted in the milks (Haddad et al., 2012, Ramírez et al., 2001, Lapillonne et al., 2013).

To test these hypotheses, changes in lipid structure and surface charge in the fresh and heated human breast milk during *in vitro* gastric digestion (37°C, pH 3–5 with contain pepsin and/or fungal lipase) with low shear (Lueamsaisuk et al., 2014b) were determined.

7.3 Materials and methods

7.3.1 Materials

7.3.1.1 Maternal milk

Maternal milk was obtained from healthy general volunteers in Palmerston North, New Zealand. Surplus milk was collected from mothers who were breast feeding babies between 1 and 3 months of age. Mothers thus were asked to donate the milk. Milk expressed through the use of a breast pump after the baby had been fed was used. Milk samples were transferred to sterilised containers and stored at 4°C. The information from donors on their general health and medical history, the age, health, and nutritional status of their baby was collected. This study was approved by the Massey University Human Ethics Committee.

7.3.1.2 Enzymes

Porcine pepsin (porcine, 800–2500 U/mg) and fungal lipase extracted from *Rhizopus oryzae* (80 U/mg) were used (details mentioned in Chapter 3) as described below.

7.3.1.3 Walstra solution A

Walstra solution A was used in determination of emulsion droplet size to break up any protein-mediated flocs. The solutions consisted of 0.375% (w/v) EDTA and along with 0.125% (v/v) Tween 20 (BDH Ltd., Poole, England) made up in RO water at pH 10 (as described in Chapter 3).

7.3.2 Methods

7.3.2.1 Simulated gastric fluid

As presented in Chapter 3, simulated gastric fluid (SGF) was prepared by dissolving 200 mg of sodium chloride and 1.3 ml of 1 M HCl in 80 ml of Milli-Q water. The pH of the solution was adjusted to 3.5, 4.5 and 5.5 and the total volume made up to 100 ml. An amount of 450 mg of dry powdered porcine pepsin (porcine, 800–2500 U/mg) and 20 mg of dry powdered *Rhizopus oryzae* lipase equivalent to 1600 IU (80 U/mg) were then added to this solution and dispersed by constant stirring at 37°C, at 10 rpm for 10 min.

7.3.2.2 *In vitro* digestion of milk samples

Twenty mL of breast milk was mixed with 50 mL of SGF (milk: SGF ratio = 1:2.5 v/v) at 37°C. The pH of the mixture was maintained at either pH 3.5, 4.5 or 5.5 by

the appropriate ongoing drop−wise addition of either 1 M HCl or 1 M $NaHCO_3$. The mixture was incubated at 37°C for 2 h at 10 rpm. A 1 ml sample of digestate was removed from the beaker every 15 min (details mentioned in Chapter 3). The effect of heating human breast milk samples at 65°C for 15 min was also explored to study its enzyme activity i.e. bile salt digested lipase, etc.

7.3.2.3 Determination of emulsion-SGF mixtures

The droplet size distributions were analysed by light scattering technique using Mastersizer. The droplet structure was examined by Confocal microscopy. The charge on the fat globules was quantified by determining the electrical potential termed the ζ-potential using Zetasizer as described in Chapter 3.

7.4 Results

7.4.1 Particle size analysis

The analysis showed that the fat droplets in the human breast milk samples had a bimodal distribution, with a first modal peak around 1 μm and a second peak modal at around 5 μm (Figure 7.1).

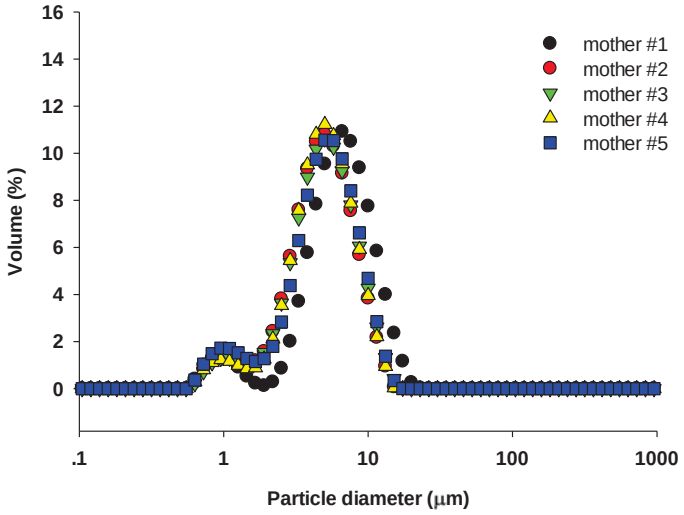


Figure 7.1 **Droplet size distribution of human milk from 5 mothers. Analysed with Walstra solution A.**

The droplet size of human milk measured at three pH levels (pH 3.5, 4.5 and 5.5) without enzymes added and with and without Walstra solution A are shown in Figure 7.2. The size distribution in human milk was found to display an increase over time in the absence of enzymes and in the absence of Walstra solution A, with the most pronounced changes observed at pH 4.5 (Figure 7.2ii)).

However, on addition of Walstra solution A, droplet size distributions were found to match that of the native milk emulsion, irrespective of pH.

Droplet size measurements of maternal milks incubated in the presence of both pepsin and lipase did not show any significant changes in particle size distribution when treated with Walstra irrespective of pH. However, droplet size increases were observed in the absence of Walstra treatment, again being most apparent at pH 4.5.

The droplet size distribution of the emulsions digested at pH 3.5, 4.5 and 5.5 with pepsin alone (Figure 7.4A) or with fungal lipase alone (Figure 7.4B) showed no

discernible change in particle size distribution over the course of simulated digestion, irrespective of pH conditions. Comparison of droplet size distributions of heated milks against non-heated samples under gastric conditions comprising both pepsin and lipase and at pH 3.5 is shown in Figure 7.5. Particle sizes after treatment with Walstra dissociating solution did not show any discernible changes over the course of the incubation period, irrespective of whether the samples had been heated or not.

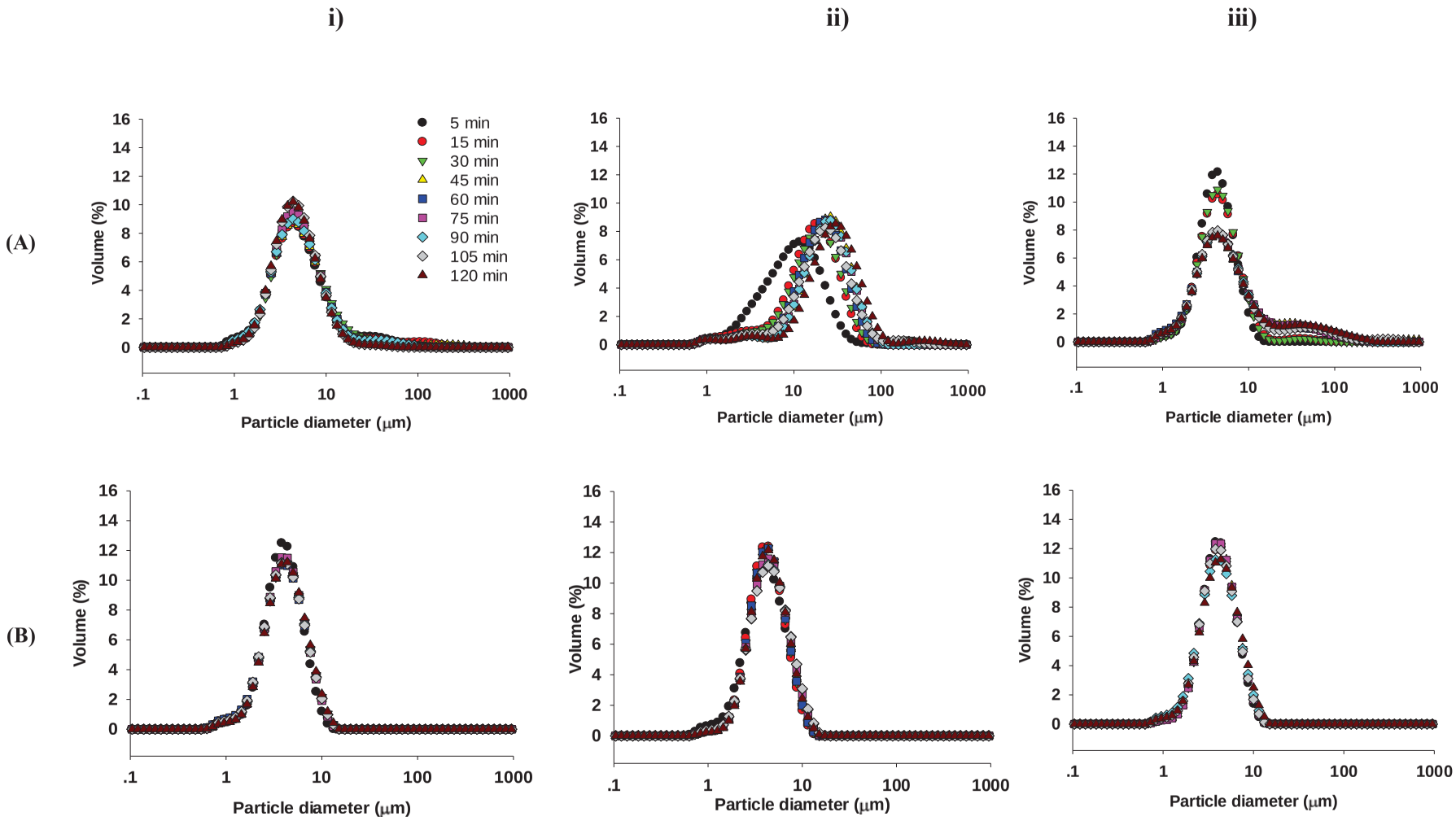


Figure 7.2 **Changes in droplet size distribution of human breast milk emulsions during incubation with no enzyme, at pH 3.5 (i) (n=2), pH 4.5 (ii) (n=2) and pH 5.5 (iii) (n=2), at 37°C, 10 rpm, for 120 min. Analysed without Walstra solution A (A) and with Walstra solution A (B).**

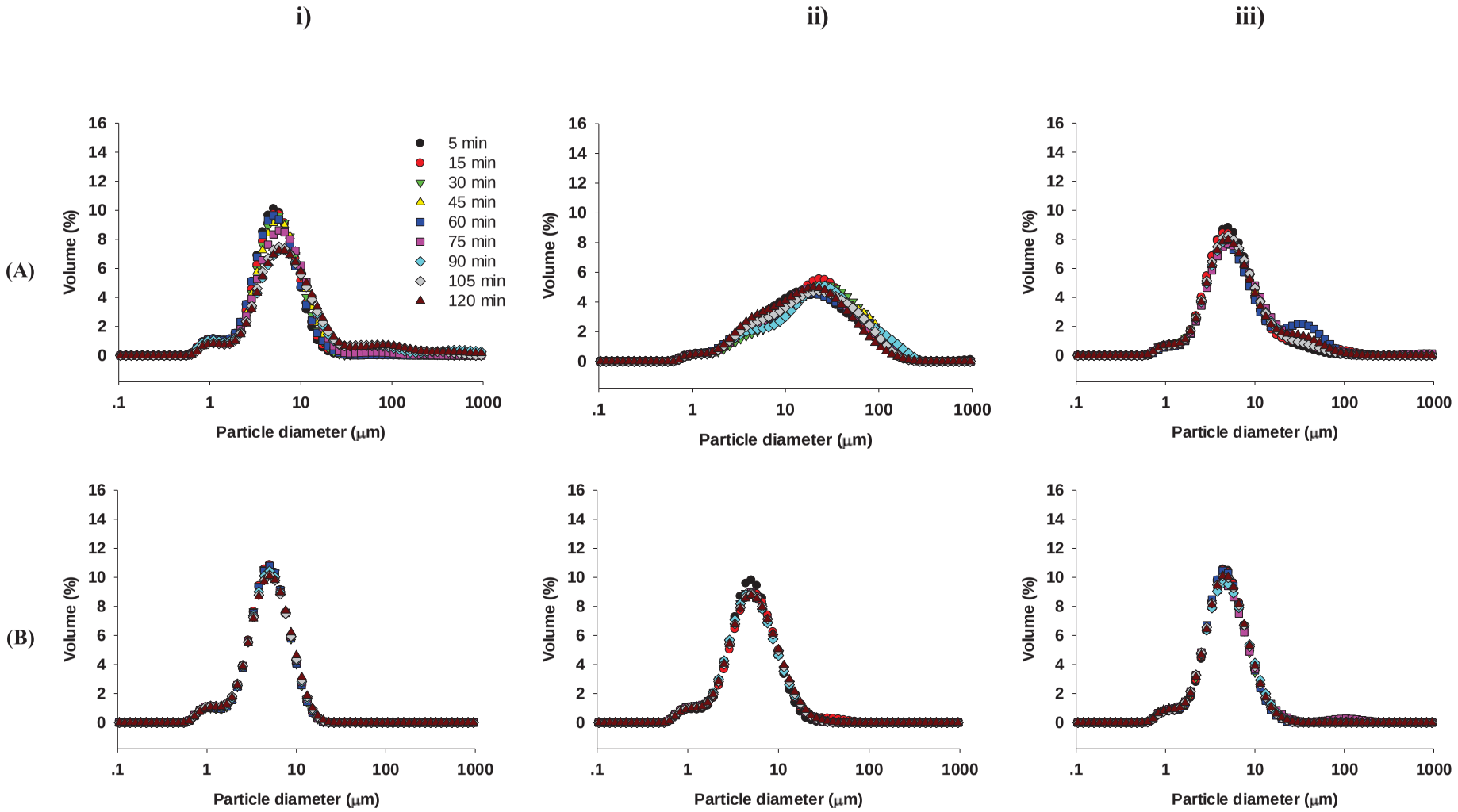


Figure 7.3 **Changes in droplet size distribution of human breast milk emulsions during digestion with SGF with pepsin and fungal lipase at pH 3.5 (i) (n=5), pH 4.5 (ii) (n=2) and pH 5.5 (iii) (n=2), at 37°C, 10 rpm, for 120 min. Analysed without Walstra solution A (A) and with Walstra solution A (B).**

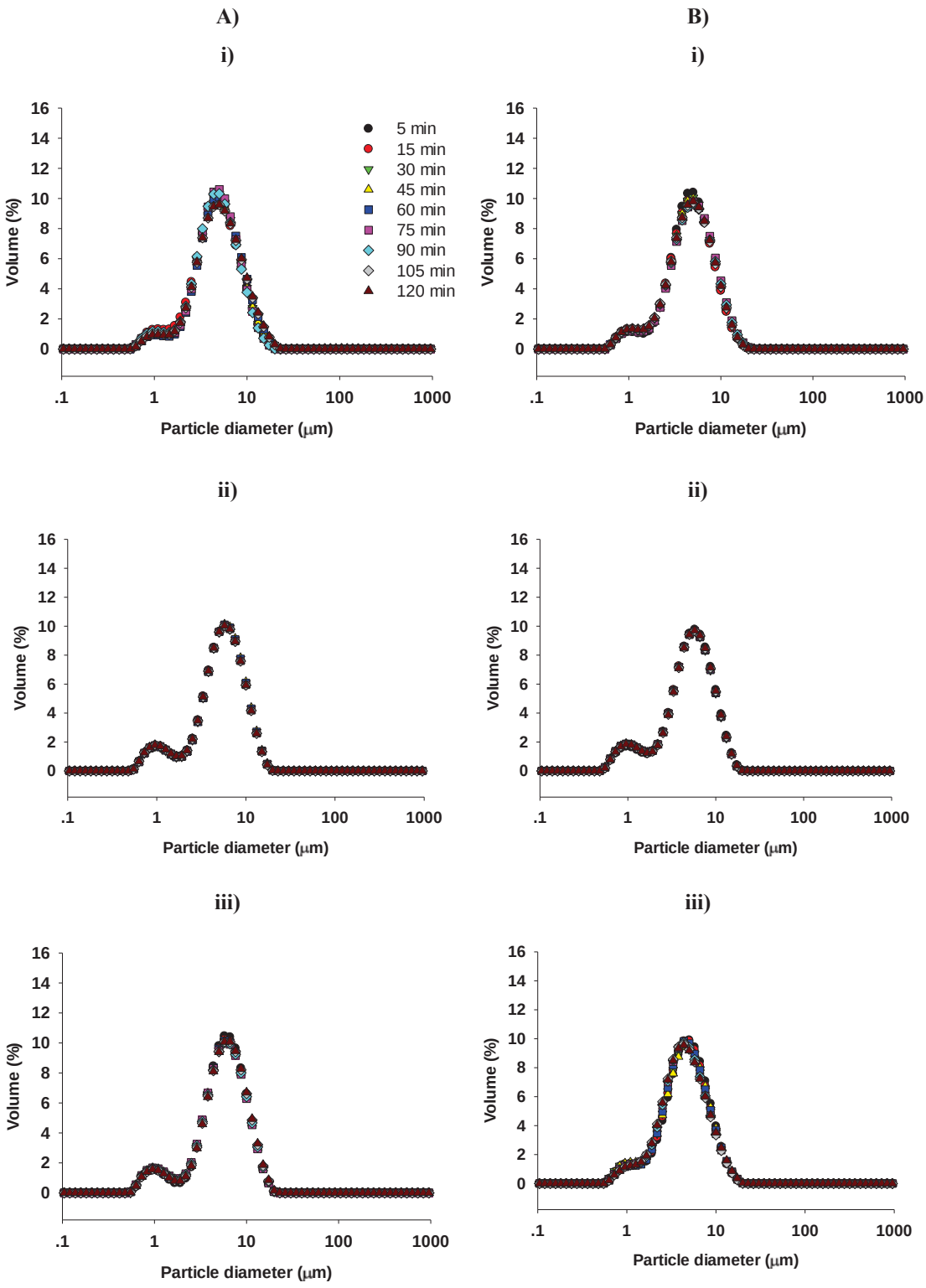


Figure 7.4 **Changes in droplet size distribution of human breast milk emulsions during digestion with SGF with pepsin (A) or with fungal lipase (B) at pH 3.5 (i) (n=2), pH 4.5 (ii) (n=2), and pH 5.5 (iii) (n=2) at 37°C, 10 rpm for 120 min. Analysed with Walstra solution.**

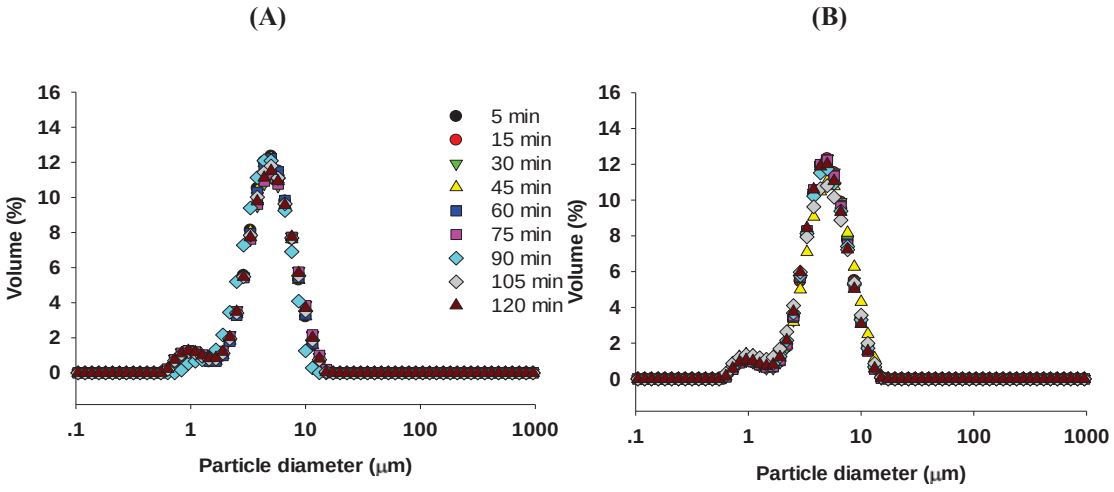


Figure 7.5 **Droplet size distribution of human breast milk emulsions during digestion with SGF with pepsin and lipase at pH 3.5 at 37°C, 10 rpm, for 120 min, in the present of unheated (A) or heated (65°C, 15 min) (B) milk before digestion. Analysed with Walstra solution A.**

7.4.2 Confocal microscopy

The microstructure of fresh breast milk samples from five mothers prior to digestion indicated the presence of different amounts of proteinous membrane surrounding the fat droplets (Figure 7.6). The micrograph results should be changed to reflect that samples were taken at different stages of laction. At all stages of lactation i.e. 11 weeks (Figure 7.6A), 10 weeks (Figure 7.6B), 7 weeks (Figure 7.6C), 6 weeks (Figure 7.6D) and 4 weeks (Figure 7.6E) postpartum. There was no discernible variation in the droplet size before and after feeding within the individual mother (Figure 7.7).

Samples incubated in simulated gastric fluid comprising pepsin and lipase are shown in Figures 7.8 to 7.10 for pH 3.5–5.5. In the samples treated with Walstra solution A,

a number of lipid droplets decreased. This was attributed to dilution with this solution. The fat droplets in the confocal micrographs showed no signs of aggregation, after treated with the addition of the Walstra solution A. The results of both the light scattering technique and micrographs confirm that after incubation the fat globule structure did not discernibly change.

At pH 3.5 after digested with pepsin alone or lipase alone, the micrographs showed some clusters of protein and fat globules when the emulsions were treated with SGF with pepsin alone (Figure 7.11) or lipase alone (Figure 7.11) without diluting it with Walstra solution A (Figure 7.11−12A) as opposed to when diluted in Walstra solution A (Figure 7.11−12B), where only individual droplets were observed.

Comparison between heated and unheated breast milk samples (Figure 7.13) showed that the droplet size appeared visually larger in the micrograph of the unheated than the heated after digestion, however, the size distribution results showed there was no marked difference between these samples.

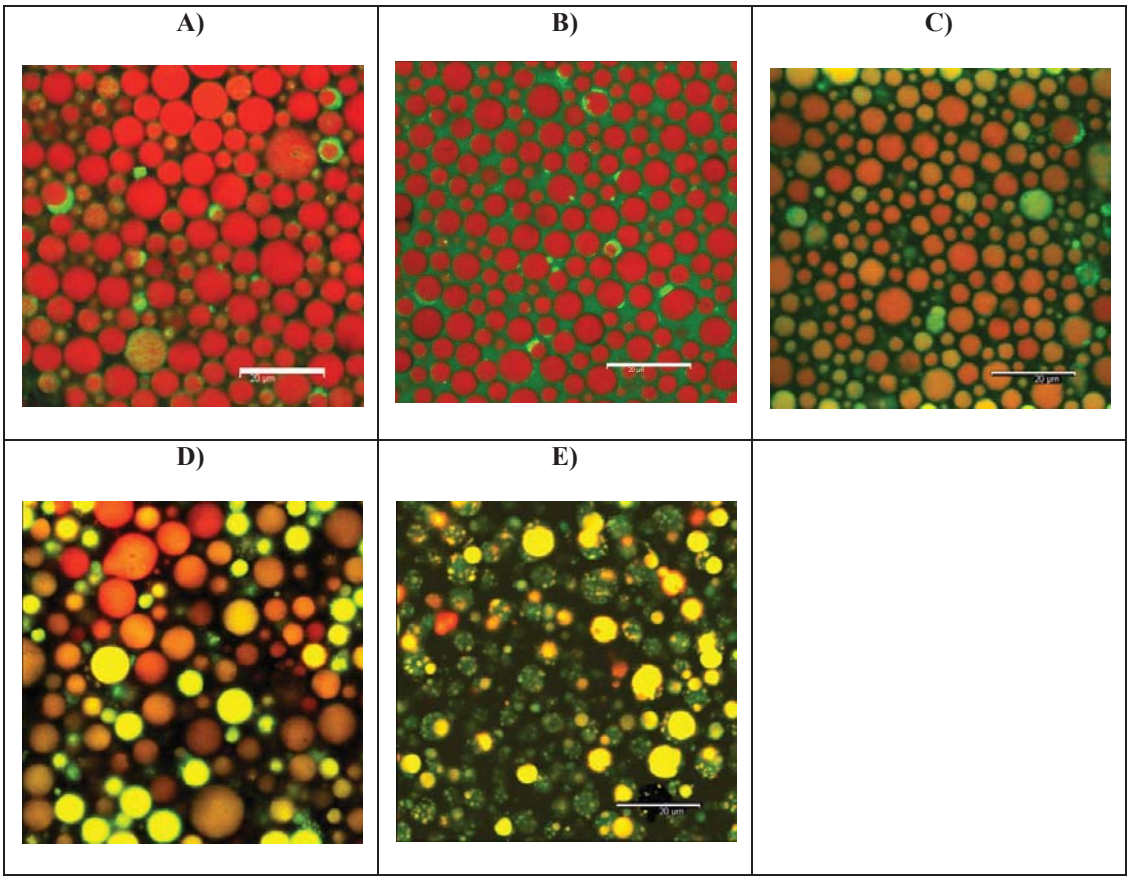


Figure 7.6 **The microstructure of fresh breast milks from 5 mothers (A, B, C, D and E). Analysed without Walstra solution A. Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

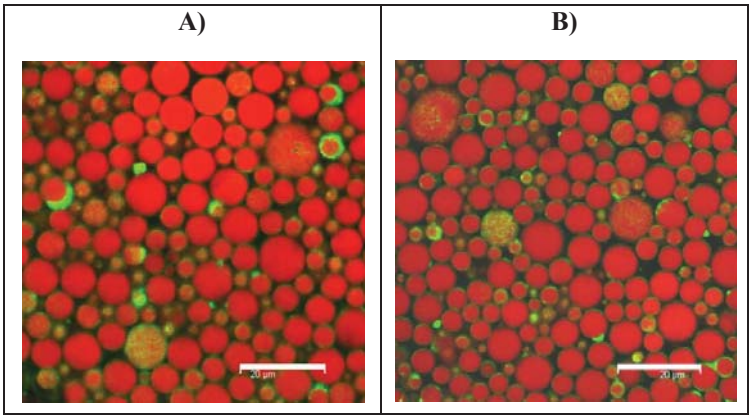


Figure 7.7 **Comparison of the microstructure of fresh breast milk between before feeding (A) and after feeding (B). Analysed without Walstra solution A. Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

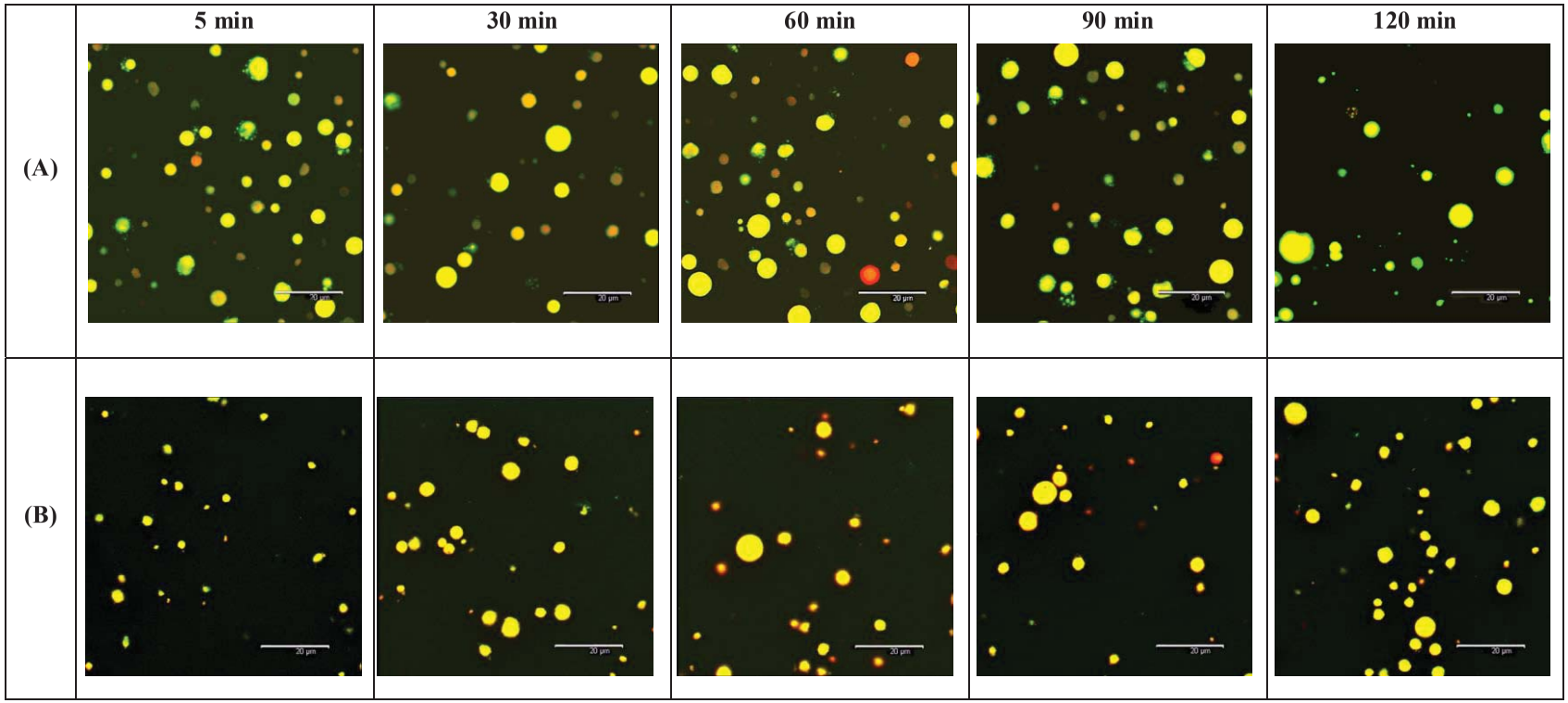


Figure 7.8 **Changes in the microstructure of human breast milk during digestion with SGF with pepsin and fungal lipase at pH 3.5, at 37°C, 10 rpm, for 120 min. Analysed without Walstra solution A (A) or with Walstra solution A (B). Samples were stained with Nile blue (for fat) and Fast green (for protein). Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

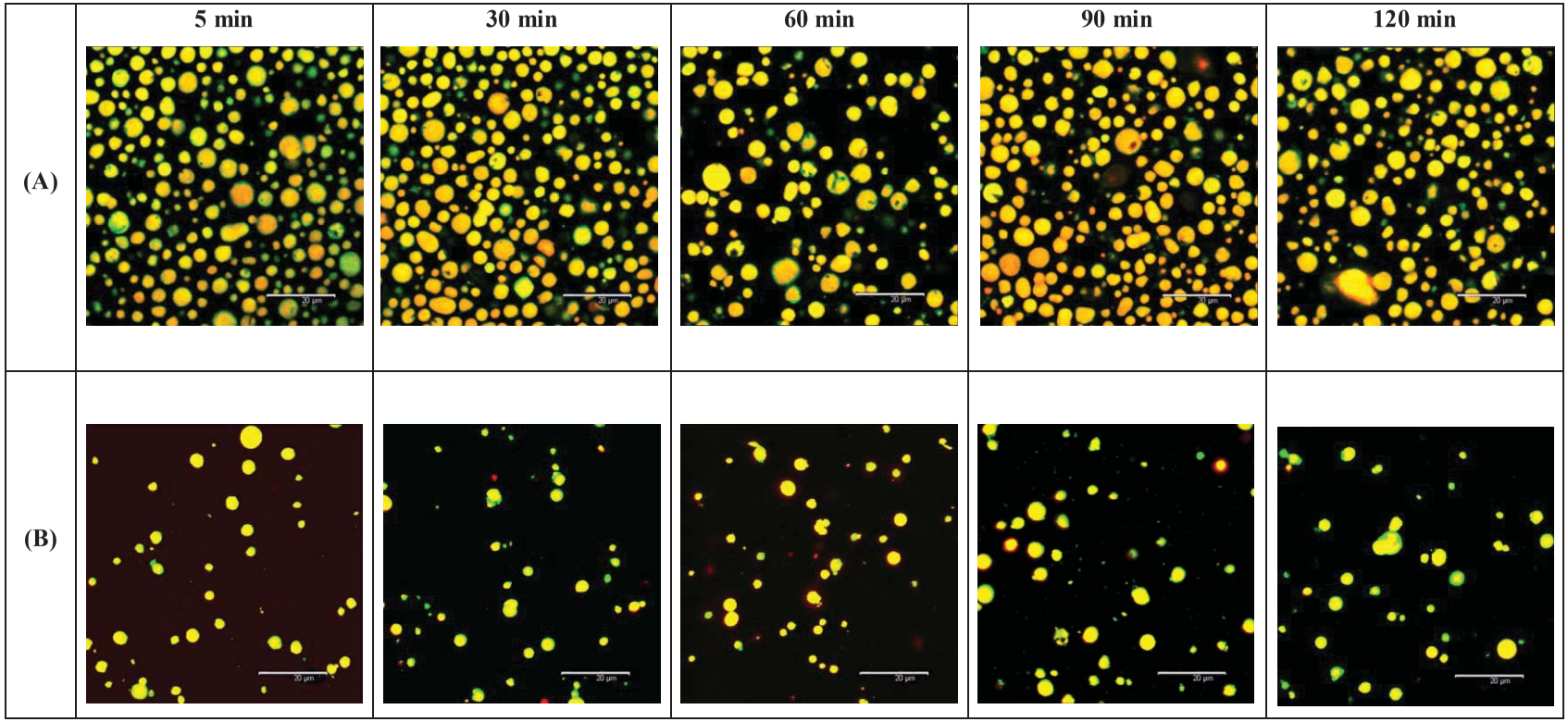


Figure 7.9 **Changes in the microstructure of human breast milk during digestion with SGF with pepsin and fungal lipase at pH 4.5, at 37°C, 10 rpm, for 120 min. Analysed without Walstra solution A (A) or with Walstra solution A (B). Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

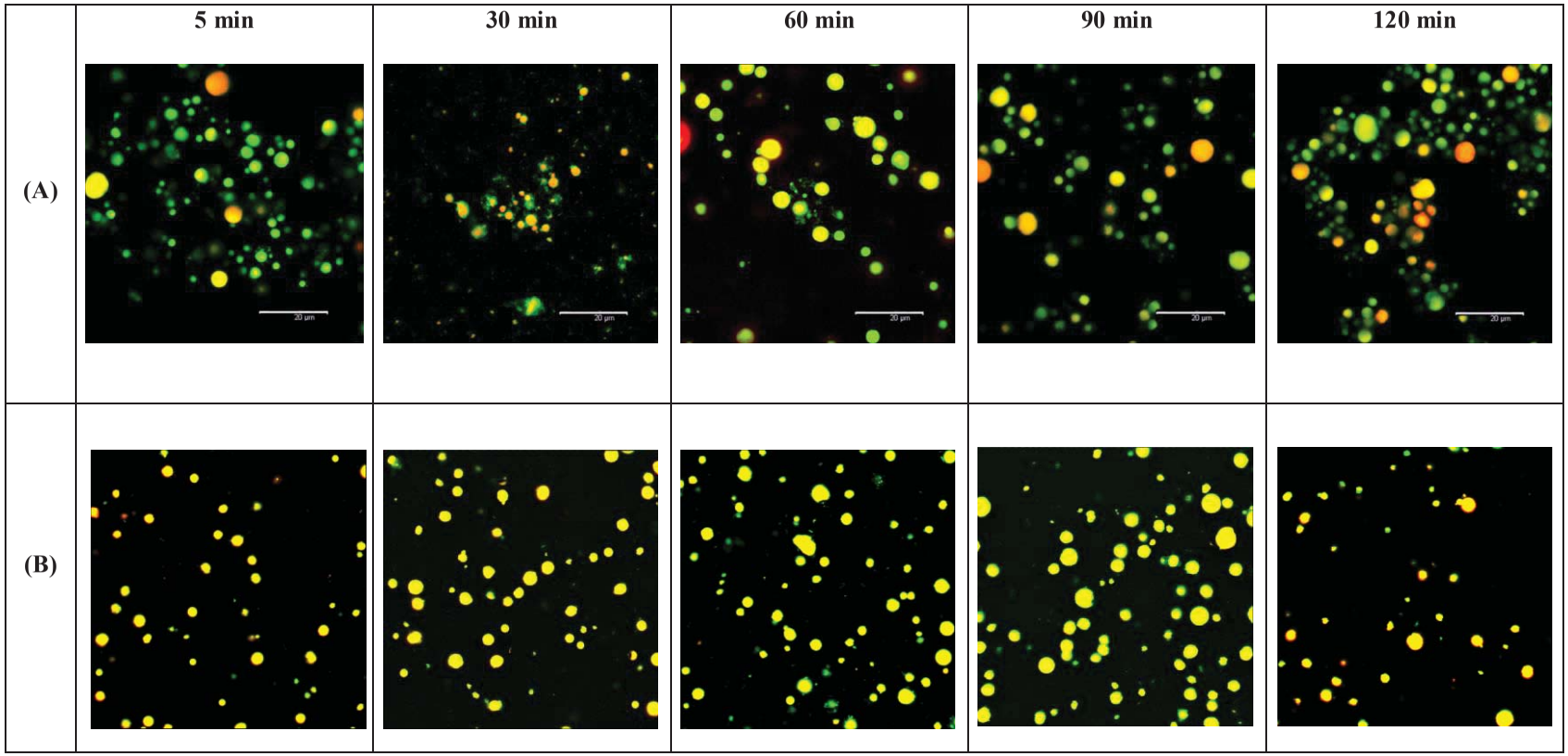


Figure 7.10 **Changes in the microstructure of human breast milk during digestion with SGF with pepsin and fungal lipase at pH 5.5, at 37°C, 10 rpm, for 120 min. Analysed without Walstra solution A (A) or with Walstra solution A (B). Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

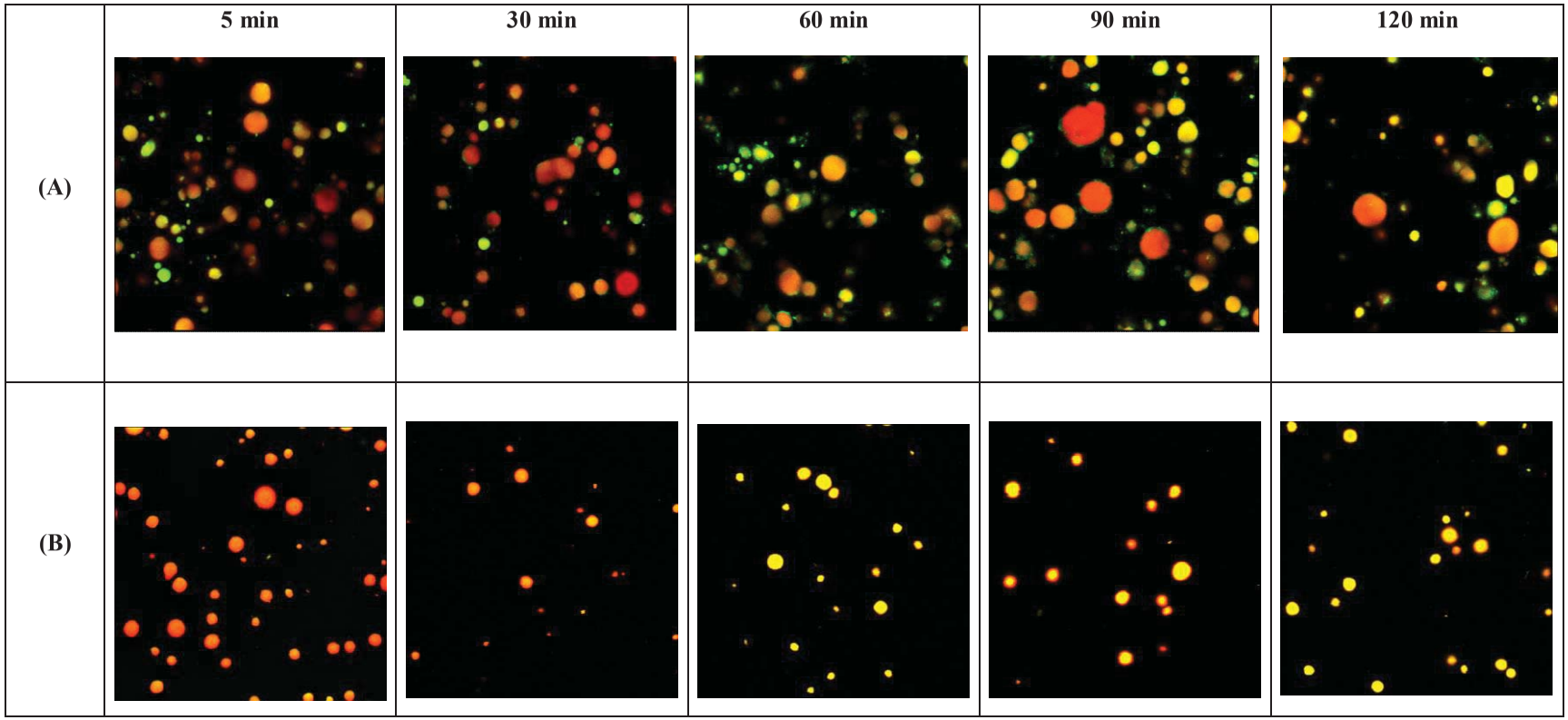


Figure 7.11 **Changes in the microstructure of human breast milk during digestion with SGF with pepsin only at pH 3.5, at 37°C, 10 rpm, for 120 min. Analysed without Walstra solution A (A) or with Walstra solution A (B). Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

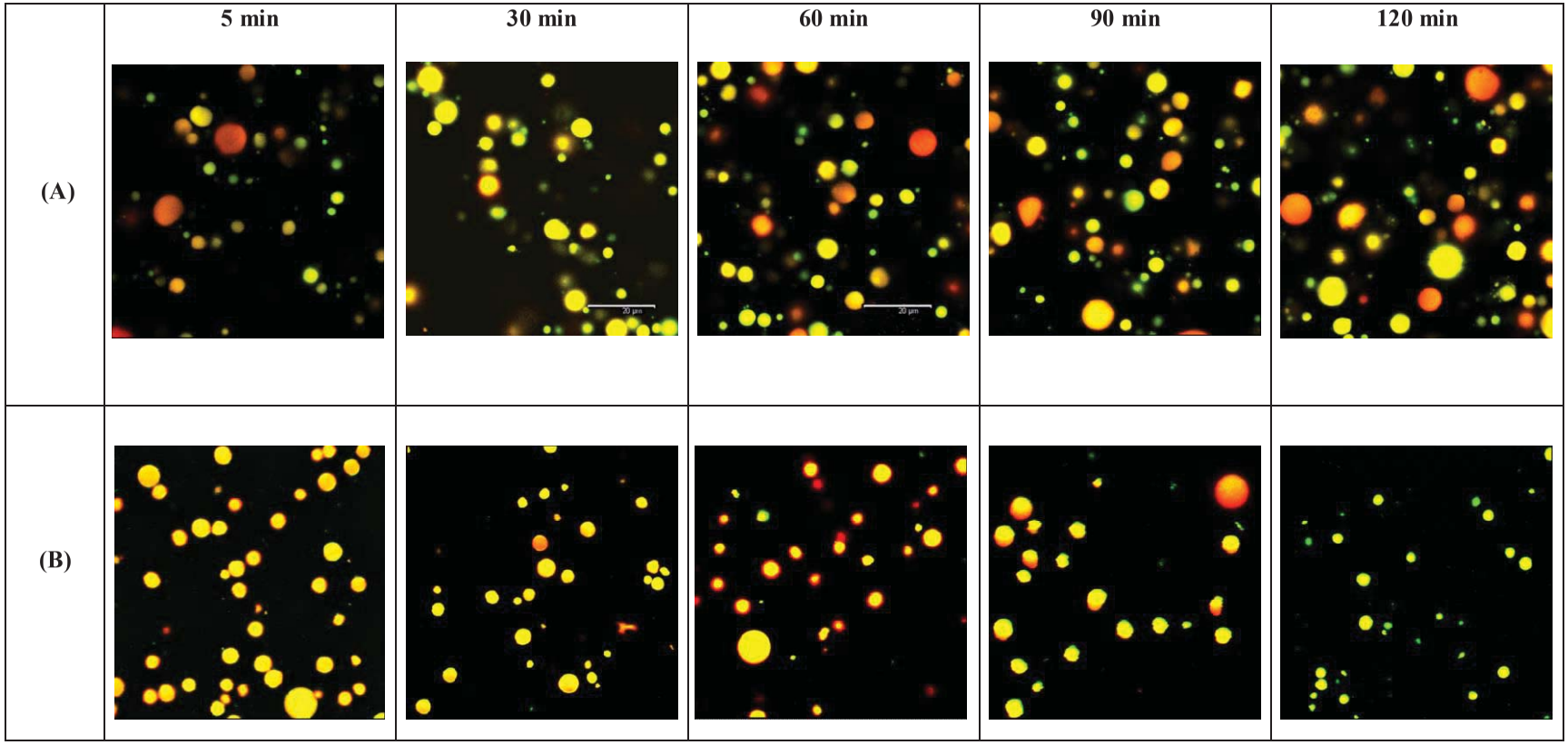


Figure 7.12 **Changes in the microstructure of human breast milk during digestion with SGF with fungal lipase only at pH 3.5, at 37°C, 10 rpm, for 120 min. Analysed without Walstra solution A (A) or with Walstra solution A (B). Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

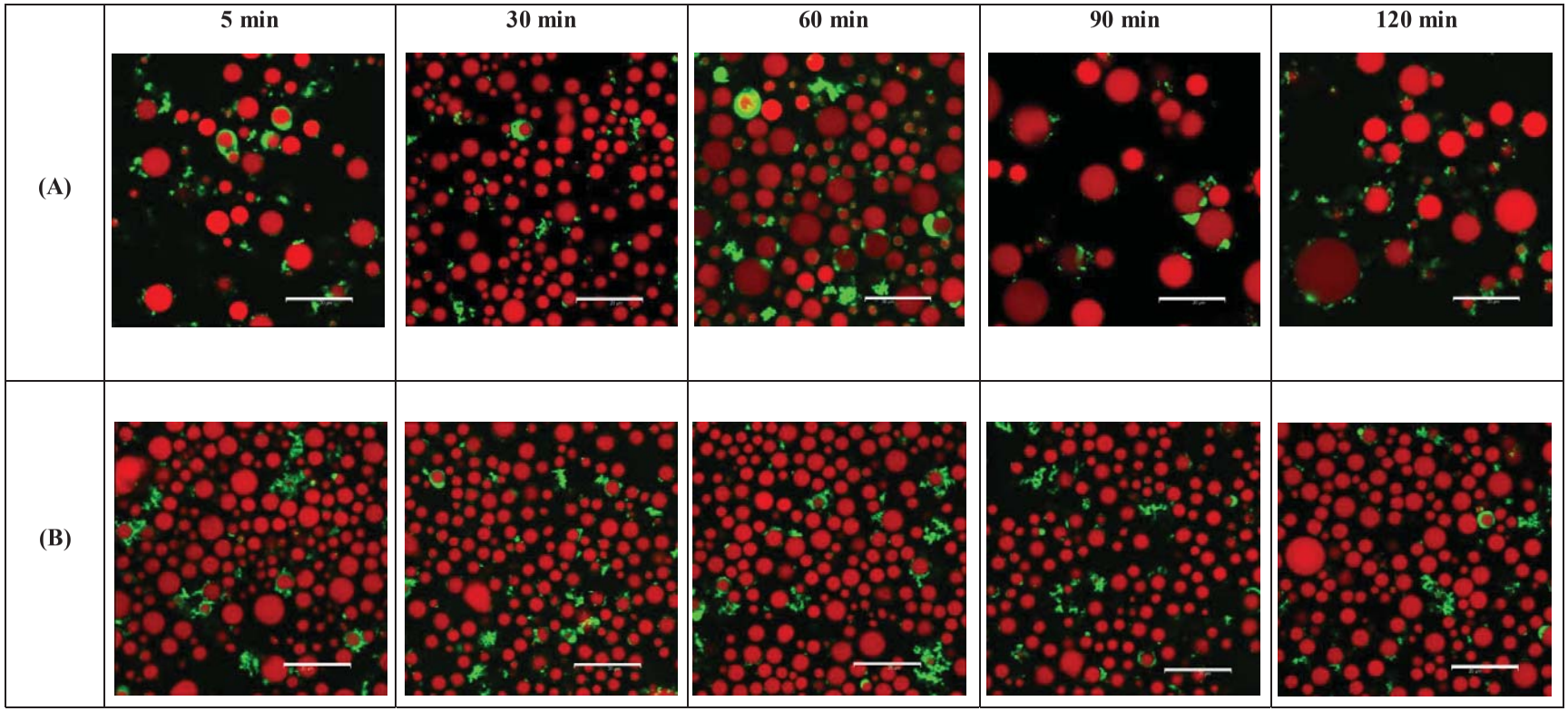


Figure 7.13 **Changes in the microstructure of unheated human breast milk (A) and heated human breast milk at 65°C, 15 min (B) before digestion with pepsin and fungal lipase at pH 3.5, at 37°C, 10 rpm, for 120 min. Analysed without Walstra solution A. Samples were stained with Nile blue (for fat) and Fast green (for protein). Scale bar corresponds to 20 µm.**

7.4.3 Droplet charge

The breast milk analysed with Milli-Q water at neutral pH (pH 7) before digestion had an average ζ-potential value of about –20 mV (Figure 7.14). After adjustment to pH 3.5 from pH 7, the charge changed abruptly from negative to positive surface potential (+25 mV). At pH 4.5 and 5.5 the emulsions had negative charges –8.5 mV and –30 mV, respectively.

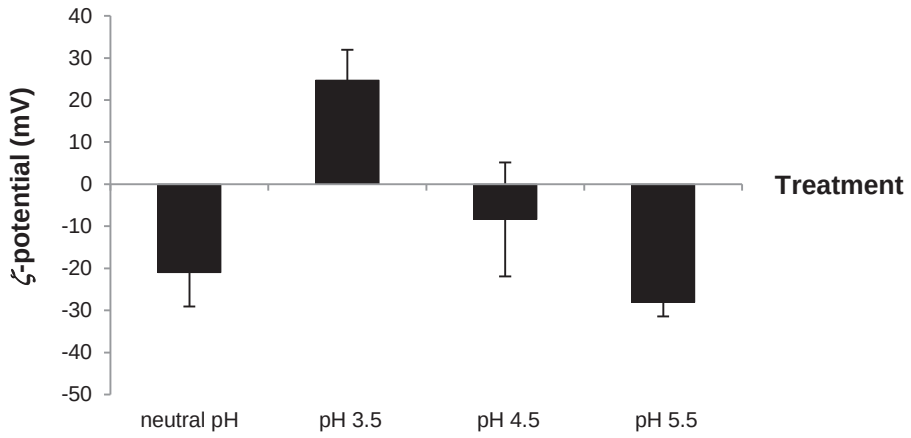


Figure 7.14 **Changes of the ζ-potential of the fresh human breast milk at different pH (without enzyme). The error bars represent the standard deviation of five repeat measurements. *Neutral pH (n=5), pH 3.5 (n=5), pH 4.5 (n=2) and pH 5.5 (n=2).**

When milk samples were incubated at pH 3.5, 4.5 and 5.5 without enzymes (Figure 7.15) there was minimal change over time. However, when milk samples were digested with a combination of pepsin and lipase, gradual decreases in ζ-potential values were observed at pH 3.5 and 4.5, but no noticeable changes were detected at pH 5.5. When milk samples were digested with pepsin alone the relative change in ζ-

potential was more pronounced at pH 3.5 than at pH 4.5, with little change at pH 5.5. When digested only with lipase, the ζ-potential of the emulsion did not change much at pH 3.5 and 5.5 (Figure 7.17A). In contrast at pH 4.5, the ζ-potential fluctuated (Figure 7.17B). In summary, a combination of pepsin and lipase decreased the zeta potential compared to that of milk treated with pepsin alone or lipase alone, within pH 3.5- 4.5 range. At pH 5.5 (Figure 7.17C), the ζ-potential of all treatments did not change during digestion.

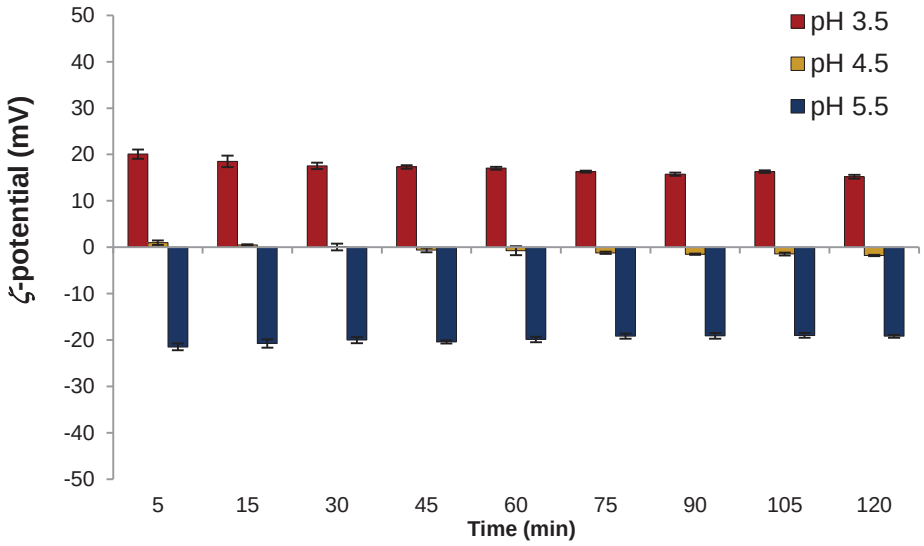


Figure 7.15 **Changes of the ζ-potential of human breast milk during incubated without enzyme at different pH (n=2) at 37°C, 10 rpm, for 120 min. The error bars represent the standard deviation of five repeat measurements.* pH 3.5 (n=2), pH 4.5 (n=2) and pH 5.5 (n=2).**

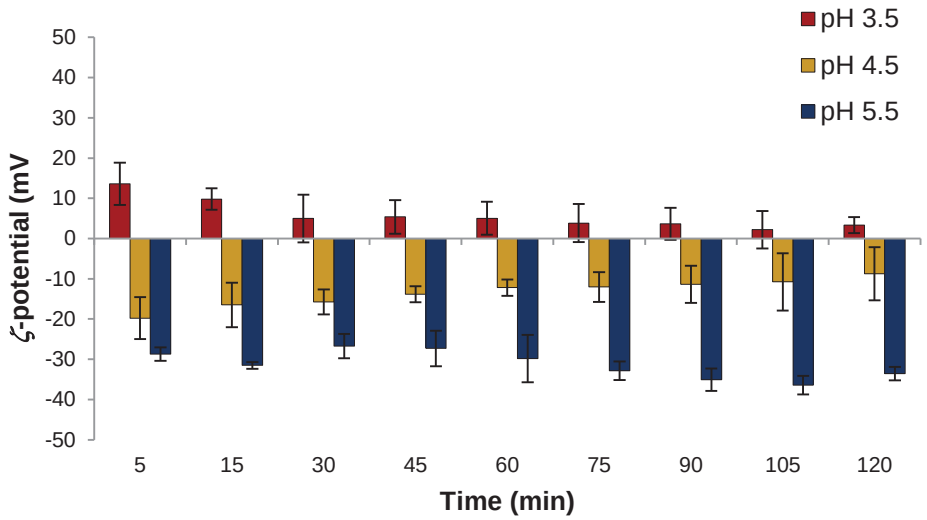


Figure 7.16 **Changes of the ζ-potential of human breast milk during digestion with SGF with pepsin and lipase, at different pH, at 37°C, 10 rpm for 120 min. The error bars represent the standard deviation of five repeat measurements.**

*** pH 3.5 (n=5), pH 4.5 (n=2) and pH 5.5 (n=2).**

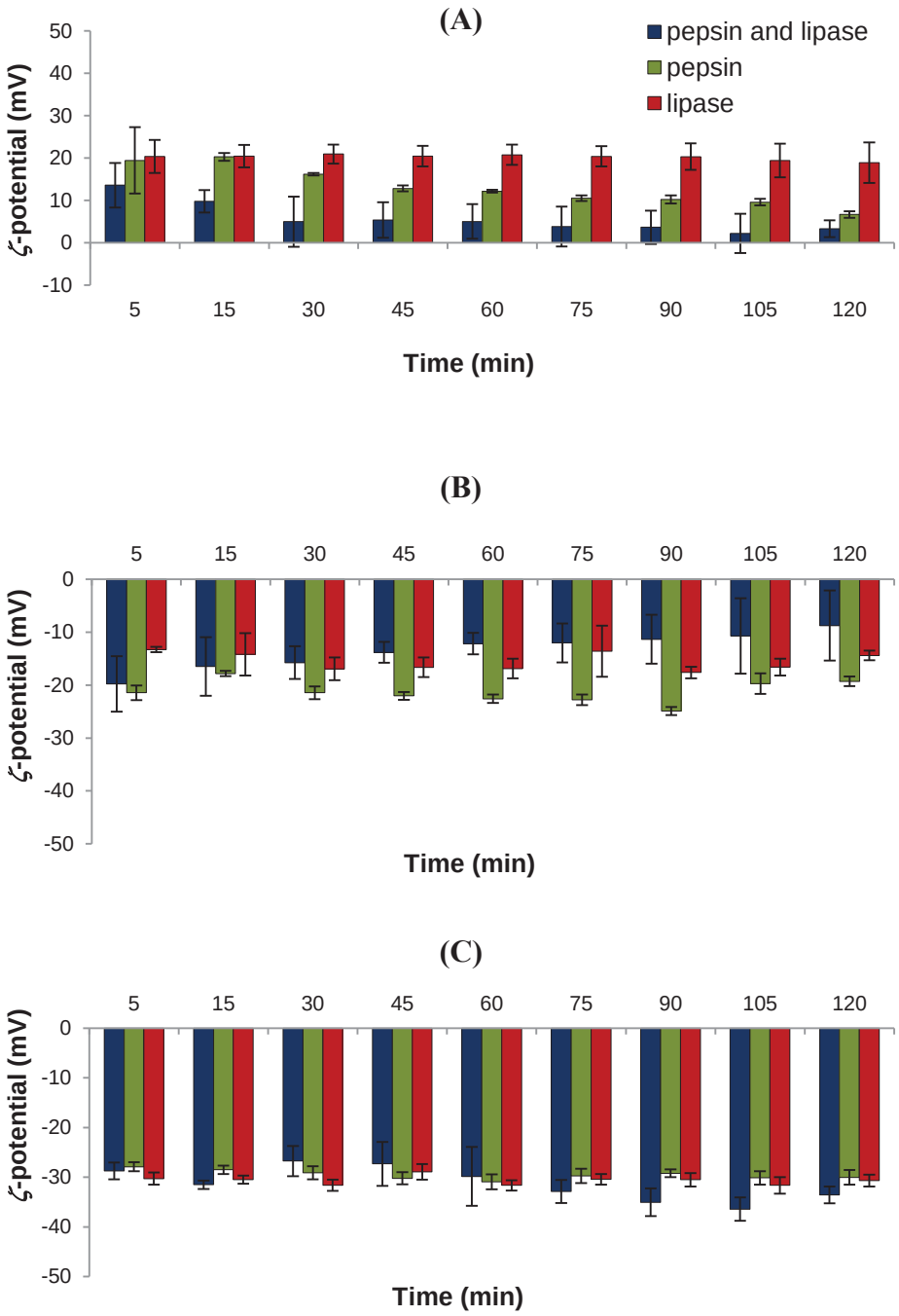


Figure 7.17 **Changes of the ζ-potential of human breast milk during digestion with SGF with enzymes at pH 3.5 (A), pH 4.5 (B) and pH 5.5 (C) at 37°C, 10 rpm, for 120 min. The error bars represent the standard deviation of five repeat measurements. * pH 3.5 (n=2), pH 4.5 (n=2) and pH 5.5 (n=2).**

7.5 Discussion

7.5.1 Particle size and microstructure

Fresh human milk samples collected in this study indicated that a mean droplet size diameter (d_{43}) of 5 μm was close to that obtained by Michalski et al. (2005) (4.4 μm) and by Rüegg & Blanc (1981) (5.2 μm). Previous research has reported that the size of fat globules in human milk ranges from 0.3 to 15 μm with a mean diameter between 3.5 and 5 μm (Lopez and Ménard, 2011, Michalski et al., 2005b). The present work demonstrated that the size distribution of fat droplets in fresh breast milk from five mothers before digestion with the simulated gastric fluid did not vary greatly. Confocal microscopy did, however, show some interesting variation in the extent to which protein was present at the interface (Figure 7.6). Whilst maternal milks are known to have a glycoprotein layer (Lopez and Ménard, 2011) bound to the oil-water interface, it cannot be determined based on the evidence provided whether the adsorbed protein material is glycoprotein in origin, or alternatively is a consequence of serum protein adsorption occurring due to expression and subsequent handling of the milk prior to analysis.

The main observation arising from the incubation study is the distinct absence of droplet coalescence during gastric digestion of human milk irrespective of the pH conditions, or the combination of enzymes applied. In fact the emulsion system appeared remarkably stable under simulated gastric conditions, with the main structural change appearing to be a consequence of protein precipitation at pH conditions approaching the isoelectric point of casein.

The absence of coalescence when the maternal milk is incubated with both enzymes present is at variance with observations made for both formula milks and for the model (Intralipid®) emulsion system. Whilst the native phospholipids and proteins located on the surface of the milk fat might be considered as acting as a barrier for lipase adsorption, this would appear contradictory to the biological imperative for achieving effective nutrient digestion (particularly in considering the increased reliance on gastric digestion during infancy). Additionally, it is already well established that up to 50% of lipolysis of milk occurs during gastric digestion for infants (Atkinson and Lonnerdal, 1989). Based on this understanding it is postulated that lipase adsorption and lipolysis is occurring within the gastric model, but does not lead to droplet coalescence and emulsion destabilisation in the manner observed for formula milks.

It is possible to envisage a number of hypotheses that might account for this observation. Firstly, it could be argued that the absence of a fat droplet network (observed in confocal microscopy) would minimise the potential for droplet–droplet contact. Accordingly, at the low shear rates applied in the gastric model, film rupture as a consequence of droplet approach and film thinning would be less likely to occur. A second possibility is the different triglyceride composition of breast milk may result in a different population of fatty acids being produced compared to either infant formula or the Intralipid® emulsion. It is possible that the accumulation of specific fractions of saturated or high melting point fatty acids at the oil/water interface may enhance the mechanical stability of the interface, providing a mechanism against coalescence. Observations consistent with this hypothesis have been reported elsewhere, notably in a study carried out by Gallier et al. (2013),

which demonstrated that gastric digestion of non–homogenised bovine milk by rats did not appreciably show significant changes in droplet size distribution. They also indicated changes in the surface morphology of the droplets, which was attributed to the accumulation of lipolytic polar lipids. Whilst findings from this chapter clearly demonstrated the differences in dynamics of emulsion structuring between biological and manufactured milks, further investigation needs to be carried out to fully elucidate the mechanism responsible for maintaining droplet stability during gastric digestion of breast milk. A further observation from Figures 7.5 and 7.13 is that the additional endogenous lipase enzymes present in maternal milk, namely bile salt-stimulated lipase (BSSL) (Abrahamse et al., 2012, Lindquist and Hernell, 2010) and lipoprotein lipase (LPL) showed no changes in the droplet size distribution when compared to the heated milks, in which the BSSL and LPL were inactivated (Figure 7.5). Again, this may be indicative that droplet stability is not compromised by any changes at the oil-water interface arising from lipolysis for maternal milk.

7.5.2 Droplet charge

The electrostatic repulsions that are induced by the polar lipids, proteins and glycoproteins contained in the MFGM are contributed to the physical stability of fat globules by preventing their aggregation and coalescence in milk (Lopez, 2011).

Findings from Figure 7.14 showed an inversion of charge from negative at pH 5.5 to positive at pH 3.5. Accordingly, the reduction in surface potential at pH 3.5 and 4.5 could be attributed to either the hydrolysis of the MFGM glycoprotein layer or digestion of bound proteins from the serum phase. It is worth noting that there is a lack of change in surface potential during digestion when treated with lipase alone.

This is not necessarily an indication that lipase is unable to bind to the interface. It is speculated that for a predominantly phospholipid–stabilised MFGM, lipase adsorption is not hindered and thus lipolysis may still be proceeding in the absence of pepsin. An interesting consideration is whether the change in surface potential results in interactions with oppositely charged material (e.g. proteins) present in the serum phase. This may be due to the fact the droplet surface charge is in fact determined by the glycoprotein layer. Glycoproteins (i.e. mucin) are not readily digested by pepsin as protein binding within a nest of carbohydrate could prevent coalescence of the fat droplet. Therefore, glycoproteins act as the barrier to pepsin and also resistant to gastric digestion (Mather, 2011b, Mather, 2011a, Allen and Flemström, 2005, Le et al., 2012). Accordingly, in the absence of pepsin this remains bound to the interface even after adsorption of free fatty acids arising from lipolysis.

A particular observation at pH 4.5 is that whilst digestion with the combined pepsin–lipase results in a progressive decrease in surface charge, this is not observed for pepsin in the absence of lipase. This is perhaps unexpected as is not readily explainable. What is apparent from the surface charge measurements is that droplet stability, with respect to coalescence, is not influenced by the dynamic changes occurring at the interface during digestion.

7.6 Conclusions

This study provided an insight into understanding the physicochemical properties of human milk on *in vitro* lipid digestion under simulated infant physiological conditions (the combination and either of pepsin and lipase, pH at 3.5, 4.5, 5.5 and stirring speed of 10 rpm). Compared to formula milks and the model (Intralipid®)

emulsion, no significant coalescence of emulsion droplets was observed irrespective of pH or enzymatic combination. The hypothesis that the MFGM structure and composition acts as a barrier to lipolysis would seem counter to the requirement to achieving lipolysis during digestion in the stomach, particularly given the additional presence of BSSL and LPL within breast milk. Instead it is proposed that gastric lipolysis of human milk does occur, but does not necessarily lead to a destabilisation of the emulsion system in the same manner observed for formula milks. This may be a consequence of reduced droplet flocculation or the accumulation of milk–fat fatty acids at the interface imparting a stabilising layer. Further research is required to validate either of these hypotheses.

Chapter 8 Overview: conclusions and recommendations for future work

Fats are the principal energy source for the growing infant. Their digestion requires the breakdown of triacylglycerols that constitute the core of the maternal milk fat globule by lipolytic enzymes, both in the stomach and the small intestine. However, these digestive processes differ in the infant from those in adults (Borel et al., 1994; Hamosh, et al., 1981; Liao, et al., 1983). This study has provided valuable insight into the factors influencing the digestion of emulsions and the physicochemical changes occurring in the structure of an emulsion during its digestion by an infant, using an *in vitro* gastric model. This *in vitro* model has allowed comparisons to be made between human breast milk, existing formulae and model (Intralipid®) emulsions during incubation under simulated neonatal gastric conditions. This work has found firstly that the efficient digestion of fat in protein–stabilised emulsions required the concerted action of proteases and lipases. The hydrolysis of the interfacial protein layer, coupled with production and adsorption of fatty acids arising from lipolysis caused the fat droplets in the emulsions to coalesce, generating larger droplets of lower surface area. Secondly, that phospholipid–stabilised emulsions (including breast milk) appear to provide a more favourable interfacial domain for lipase binding, allowing lipolysis to occur without the dependency on proteolysis. Thirdly, that the shear and pH conditions had a significant effect on the relative stability of emulsion droplets during incubation.

The first objective of this thesis was to compare the physicochemical properties of breast milk, commercial infant formulae, and model protein-stabilised emulsions

prior to digestion. Subsequent objectives included the development of an appropriate *in vitro* model that simulated infant digestion and associated techniques to quantify droplet size during gastric digestion. The final objective was to use this system to compare maternal milk, infant formulae, phospholipid and lactoferrin-stabilised emulsions, so as to determine the influence of the biochemical and biophysical environment on their properties. An overview of these objectives is presented in Figure 8.1.

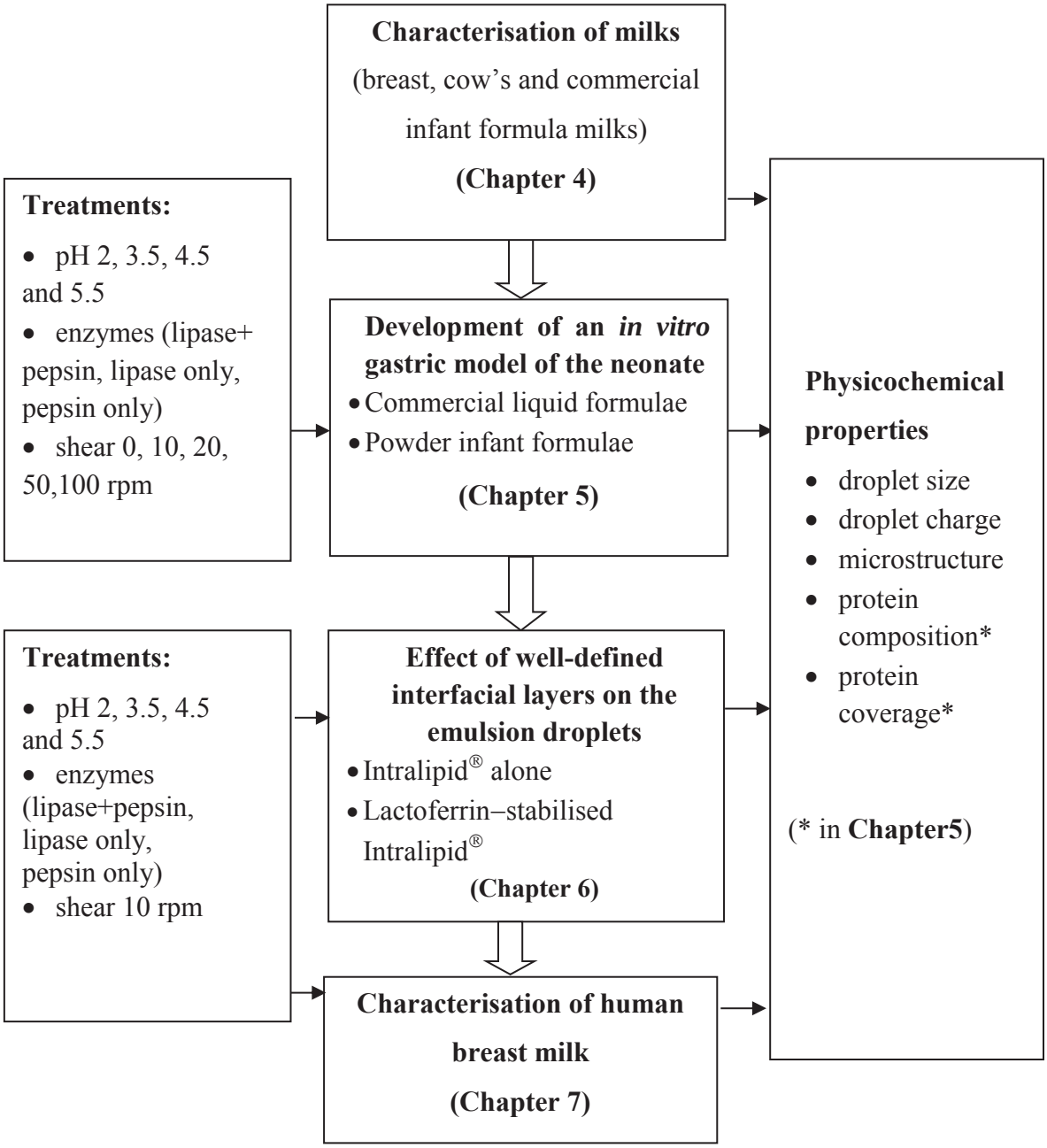


Figure 8.1 **General overview of the present thesis**

The characterisation of different milk emulsions in **Chapter 4** showed notable variation in composition of both protein and lipid (milk fat and vegetable oil) components, and pronounced differences in emulsion structure and surface composition. Both confocal microscopy and light scattering showed that liquid infant formulae had very fine emulsion droplet size distributions. Reconstituted powder formulae had marginally larger droplet size distributions, but droplet size in both liquid and reconstituted powder formulae were approximately an order of magnitude smaller than either native cow's milk or human breast milk.

The ζ-potential (surface charge) of human milk was significantly lower in relation to the other milks. This reflects the predominantly phospholipid composition of the oil-water interface. Confocal imaging showed extensive protein adsorption at the interface of the formula milks. The nature of these interfacial layers surrounding the lipid droplets was hypothesised as to be critical for efficient digestion of the lipids, influencing the stability of the lipid droplets through their susceptibility to aggregation and/or coalescence, which in turn would alter the surface area of lipid that is exposed to the action of enzymes in the stomach of the neonate.

The research in **Chapter 5** determined the influence of the conditions in the simulated gastric fluid (enzymes, pH and shear) of the *in vitro* model during digestion of formula milks. The proposed mode of action observed for formula milks digestion was then validated using a model emulsion system in which lactoferrin was electrostatically bound to the interface of a phospholipid stabilised emulsion, to differentiate the effect of the droplet size from that of the protein interface (**Chapter 6**).

Finally the *in vitro* model was then used to assess the effects of pH and shear on the digestion of the MFGs from human breast milk (**Chapter 7**). The results showed that during digestion, less relative changes to droplet size, droplet charge and microstructure were observed in the maternal milks in comparison to the infant formulae or Intralipid® emulsion. This would suggest that under the neonatal gastric digestion, the structure and composition of membrane coating fat droplets in maternal milks is a key factor in their infant gastric digestibility. Additionally, it was considered that variations in the range of fatty acids produced during lipolysis, as a consequence of differences in the triglyceride composition of the maternal, formula and Intralipid® emulsions, may also provide a contributing mechanism to structural dynamics during digestion.

Development of the in vitro model

The development of the in vitro model was intended to embody the neonatal gastric environment. Due to lower levels of stomach acid secretion, the pH conditions are elevated compared to adult physiology. This, coupled with the buffering effects associated with the consumption of milk, cause the neonatal gastric pH (pH 3.5-5.5) to be markedly higher than in the adult (pH 1.6-2.0). Therefore, in this study the gastric pH was fixed at 2, 3.5, 4.5 and 5.5 for 120 min of digestion time.

The enzymes, gastric lipase (derived from fungal sources or calf pregastric lipase) and pepsin (of porcine origin), were chosen to have similar characteristics to the human enzymes and similar range of pH. Whilst lipases are secreted by the gastric mucosa, it is important to note that additional milk lipases from breast milk (bile salt stimulated lipase and lipoprotein lipase) may participate in digestion of maternal

milk (but, importantly, will not contribute to the digestion of formula milk). Again, the shear rate in the infant's stomach is likely to be lower than that in the adult stomach.

Accordingly, a low stirring speed (10 rpm) was chosen so as to closely approximate the magnitude of shear generated during contractile activity in the infant stomach (Lueamsaisuk et al., 2014b). This study showed that shear affected the coalescence stability of oil droplets in the emulsion, which could provide an indirect indication of lipolysis in formula milks, as considered by the dynamic changes in d_{43} values (after dissociation in Walstra solution A) during digestion for 2 h.

During infant *in vitro* gastric digestion under standardised shear conditions, protein–stabilised emulsions (for both formula milks [**Chapter 5**] and lactoferrin–coated milks [**Chapter 6**]), were destabilised by the concerted action of both pepsin and lipase. The consequent droplet coalescence was attributed to the presence of free fatty acids arising from lipolysis accumulating at the oil-water interface. In spite of the smaller oil droplet size in formula feeds and correspondingly greater oil-water surface area available for enzymatic attachment, the presence of the proteinaceous layer at the oil-water interface may actually render their digestion less efficient.

In such protein–stabilised emulsions, gastric pepsin appears to govern lipolysis through improving accessibility of the lipase to the interface. Here, the initial hydrolysis of the proteins at the interface of the lipid droplets appears to be necessary before the lipid can be hydrolysed by the lipase (during the gastric stage of digestion).

It is important to note that the concerted action of pepsin and lipase in this manner could best be achieved if both enzymes had similar pK_a's and their synthesis and secretion was to commence equally promptly at birth, which is not the case in human infants (Lueamsaisuk et al., 2014b). The fact that lipase is most active at relatively higher pH levels than is pepsin may render the process less efficient. Hence, the pH in the stomach lumen needs to be low for maximal interfacial proteolysis by pepsin, whilst maximal lipolysis would require higher pH.

As observed in **Chapter 6**, no proteolysis is required for digestion of emulsion (Intralipid®) droplets stabilised with phospholipid membrane, as there is no protein barrier to lipase binding at the interface. Hence, coalescence of droplets from accumulations of fatty acid products is observed in the absence of pepsin.

As presented in **Chapter 7,** in human milk emulsions, structural dynamics similar to those for Intralipid® emulsion may be expected to occur, given that the stabilising interface of breast milk is comprised mainly of phospholipids (and could be reasonably assumed to be biologically favourable to lipase adsorption during digestion). However, human breast milk, showed the least structural changes as evidenced by a pronounced absence of flocculation and coalescence. This absence of coalescence, even in the presence of both gastric lipase and pepsin, is not necessarily an indication that lipolysis is not taking place. Rather it is considered that lipolysis and the formation of free fatty acids does not destabilise the maternal milk emulsion. This is possibly due to a reduction in droplet–droplet interactions based on the absence of any flocculation, or alternatively it could be due to stabilisation of droplets by the accumulating lipolytic products, such as high melting point free fatty

acids, at the interface. This may be likely to occur as the triglyceride composition of breast milk differs from that of both formula milks and the Intralipid® emulsion, which are based on vegetable oils and that do not contain significant quantities of these agents (Gallier et al., 2013).

While currently there are several continuous *in vitro* models that effectively mimic infant physiology this static *in vitro* model study is more rapid, less expensive, less labour intensive, and has no ethical restrictions allowing a large number of samples and conditions to be tested and compared. This reproducibility of this static *in vitro* model different factors to be tested individually in controlled conditions, i.e. the different initial pH, the effects of enzymes individually and in combination, and the different stirring speed. Also this *in vitro* model has an easy sampling which makes it suitable for physicochemical studies (droplet size, droplet charge and microstructure). The essential component of this *in vitro* static gastric digestion model is oil/water emulsion containing fat (which is hydrolysed by lipase) with or without protein coating (hydrolysed by pepsin) dispersed in aqueous phase or milk serum such as infant formulae. Moreover, there are some of the limitations of this *in vitro* model for example, 1) the build up of digested products that may affect the further lipid digestibility, 2) this *in vitro* model is for simulated gastric step only, may not be completely mimicking the process of gastrointestinal lipolysis wherethe gastric lipase initiates lipolysis which can be further progressed by the pancreatic lipase . Overall static models are important in setting the conditions of any future continuous models.

Finally, in devising formula feeds with an oil/water interface that is more amenable to digestion by the neonate, it is important to note that *in vitro* evaluation should

employ physiological shear rates to avoid inappropriate augmentation of coalescence and disruption of the proteinaceous interface. Again, given the sensitivity of digestion to the ambient pH, due allowance must be made for temporal variation in the postprandial gastric pH with the secretory rates of HCl and the buffering capacity of the formula feed. It is also important to note that the understanding of the effect of physiological pH on the kinetics of human gastric lipase is incomplete, as is the knowledge of the extent to which the actions fungal lipases are analogous to gastric lipase.

Future Work

This research has provided new insights as to how biological and manufactured emulsion structures change during *in vitro* gastric digestion that simulated the conditions in the infant stomach. The results have provided an indication as to the relative efficiency of gastric digestion of milks of different composition. However, it is acknowledged that whilst findings show pronounced differences in the manner in which breast and formula milk emulsions behave structurally on *in vitro* digestion, this does not necessarily account for all of the differences that have been reported for the lipid digestion and digestibility of maternal and formula milks (Michalski et al., 2005b, Armand et al., 1996, Garcia et al., 2014). To provide further understanding as to the effects of emulsion composition and structure on neonatal lipid digestibility, the following recommendations for future research are made:

- The evolution of changes in milk fat globules over the entire process of digestion (gastric and duodenal steps) needs further investigation for both native and homogenised milks, to better understand the biochemical and

biophysical mechanisms that influence digestion and bioavailability. Only the gastric step has been studied in this work. The development of an integrated *in vitro* model that accurately mimics the digestion conditions within the stomach and duodenum of infants would enable the study of relevant parameters such as shear, pH, enzymes (gastric lipase, pancreatic lipase, pepsin, colipase) and surface active agents (phospholipid, bile salts). The digestion of synthetic/formula feeds and maternal milk in the small intestine, in particular addressing how emulsion droplet size and the fatty acids that are release from the triacylglycerols in the gastric environment, impact on the development of micelles in the small intestinal environment, need to be explored. These are critical to the optimisation of formula feeds and to the understanding of the physical restraints that govern fat digestion in maternal milk. As there is a relative pancreatic insufficiency in the neonate the effect of supplementation with pancreatic lipase would be an interesting area of study.

- The relative secretion levels and enzymatic activities within the stomach and small intestine will have an effect on the physicochemical changes in the emulsions. These conditions change with time after ingestion and with the age and maturity of the infant, and so models need to be adaptable to be consistent with the changing gastrointestinal (GI) environment occurring due to development of the infant.
- Whilst the main objective of the study was to determine the structural changes taking place to milk emulsions during simulated gastric digestion, it is acknowledged that corresponding measurements of fatty acid release

would have been a valuable component of the study. This was attempted using gas chromatography–fatty acid methyl ester (GC–FAME), but was not successful due to difficulties in inhibiting enzyme activity without altering the structure of protein and fat. However, development of appropriate methodologies, particularly for dynamic analysis of fatty acid release during gastric lipolysis, is necessary in order to determine the correlation between the observed structural changes, and extent of lipolysis.

- Infant formulae are complex systems and historically the major focus has been on trying to match the composition to that of breast milk. However this work has showed that it is not just the composition that is important but that the structure of the emulsion is also important. This work has shown that interfacial composition of fat droplets is important. An understanding of the effect of ingredients on the interfacial composition and of the effects of processing (e.g. the impact of heat treatment) is necessary to improve digestive efficiency. The results of this study will provide a basis for infant formula manufacturers to investigate the optimisation of the emulsion structure of their infant formula.e. New improved formulae should result from the inclusion of these considerations in the design and testing of prospective infant formulations and components with respect to optimising the interfacial membrane composition, ingredients and processing so as to achieve maximal digestive efficiency.

- The interfacial structure of milk fat emulsions and the surface area that is available to enzymes are key to efficient fat digestion of human and formula milks. The glycerophospholipids that are present in the milk fat globule

membrane of human milk are considered good substrates for lipase adsorption. Hence, the presence of particular polar lipids at the interface may enhance or inhibit the activity of gastric lipase. Therefore the particular properties of polar lipids at the milk membrane needs to be elucidated. Moreover, apolipoproteins, which are naturally occurring proteins that bind and transport lipids in the circulation (Bolanos-Garcia and Miguel, 2003, Donma and Donma, 1989) could be considered as providing an appropriate interface of effective digestion.

- The development of novel *ex vivo* methodologies that mimic physical conditions in the lumen of the relevant gut component will be necessary to give physiological relevance to *in vitro* digestion studies.
- Finally, findings arising from these studies should ultimately be validated through the use of appropriate human studies to determine the effects of emulsion composition and structure, as observed through *in vitro* methodologies, in relation to digestive behaviour *in vivo*.

Appendix

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

18 November 2011

Chalida Lueamsaisuk
Institute of Food, Nutrition and Human Health
PN452

Dear Chalida

Re: Role of Emulsion Structure in Lipid Digestion of the Neonate

Thank you for your Low Risk Notification which was received on 1 November 2011.

Your project has been recorded on the Low Risk Database which is reported in the Annual Report of the Massey University Human Ethics Committees.

The low risk notification for this project is valid for a maximum of three years.

Please notify me if situations subsequently occur which cause you to reconsider your initial ethical analysis that it is safe to proceed without approval by one of the University's Human Ethics Committees.

Please note that travel undertaken by students must be approved by the supervisor and the relevant Pro Vice-Chancellor and be in accordance with the Policy and Procedures for Course-Related Student Travel Overseas. In addition, the supervisor must advise the University's Insurance Officer.

A reminder to include the following statement on all public documents:

> *"This project has been evaluated by peer review and judged to be low risk. Consequently, it has not been reviewed by one of the University's Human Ethics Committees. The researcher(s) named above are responsible for the ethical conduct of this research.*
>
> *If you have any concerns about the conduct of this research that you wish to raise with someone other than the researcher(s), please contact Professor John O'Neill, Director (Research Ethics), telephone 06 350 5249, e-mail humanethics@massey.ac.nz".*

Please note that if a sponsoring organisation, funding authority or a journal in which you wish to publish requires evidence of committee approval (with an approval number), you will have to provide a full application to one of the University's Human Ethics Committees. You should also note that such an approval can only be provided prior to the commencement of the research.

Yours sincerely

J. O'Neill

John G O'Neill (Professor)
Chair, Human Ethics Chairs' Committee and Director (Research Ethics)

cc Assoc Prof Matt Golding
Institute of Food, Nutrition and Human Health
PN452

Prof Richard Archer, HoI
Institute of Food, Nutrition and Human Health
PN452

Massey University Human Ethics Committee
Accredited by the Health Research Council

Research Ethics Office, Massey University, Private Bag 11222, Palmerston North 4442, New Zealand
T +64 6 350 5573 +64 6 350 5575 F +64 6 350 5622
E humanethics@massey.ac.nz animalethics@massey.ac.nz gtc@massey.ac.nz
www.massey.ac.nz

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

28 May 2013

Chalida Lueamsaisuk
IFNHH
PN452

Dear Chalida

Re: HEC: Southern A Application – 13/08
An examination of the structural and chemical changes in human milk during digestion

Thank you for your letter dated 28 May 2013.

On behalf of the Massey University Human Ethics Committee: Southern A I am pleased to advise you that the ethics of your application are now approved. Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely

Dr Brian Finch, Chair
Massey University Human Ethics Committee: Southern A

cc A/Prof Matt Golding
IFNHH
PN452

Prof Roger Lentle
IFNHH
PN452

Dr Lara Matia-Merino
IFNHH
PN452

Prof Richard Archer, HoI
IFNHH
PN452

Massey University Human Ethics Committee
Accredited by the Health Research Council
Research Ethics Office
Massey University, Private Bag 11222, Palmerston North 4442, New Zealand **T** +64 6 350 5573 +64 6 350 5575 **F** +64 6 350 5622
E humanethics@massey.ac.nz animalethics@massey.ac.nz gtc@massey.ac.nz **www.massey.ac.nz**

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

We need a sample of your breast milk to study how breast milk is digested in the infant stomach

We are looking for healthy mothers willing to donate 60 ml of breast milk. The mothers should be breast feeding babies who are between one month and three months old and live in Palmerston North.

All participants will receive a $20 voucher for 60 ml of breast milk.

If you are interested in participating in this study:
Please telephone Chalida Lueamsaisuk at Ph 3569099×81407, M 0211006525 or email: c.lueamsaisuk@massey.ac.nz

You will be asked some simple questions to check that you and baby are healthy and will be able to ask us questions if you have any queries about the study.

If this is all OK we will send you a consent form, a collection pack and a detailed information sheet about the study and arrange a time when you can provide a sample.
When you have produced the sample you will phone us and we will call and collect it from you.

Your name will not be used in any publications.
This study is approved by the Massey University Human Ethics Committee.

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

The Institute of Food, Nutrition and Human Health
Massey UniversityMassey University
Private Bag 11 222
Palmerston North 4442
New Zealand.
Ph +64-6-3569099x81407

Study: An examination of the structural and chemical changes in human milk during digestion

INFORMATION SHEET

Principal Investigators

- Chalida Lueamsaisuk is a PhD student in Food Technology, the Institute of Food, Nutrition and Human Health, Massey University.
- Prof. Roger Lentle is a qualified medical practitioner with experience in Clinical nutrition, and in Gastrointestinal Physiology, the Institute of Food, Nutrition and Human Health, Massey University.
- A/P Matt Golding is experienced in Food technology and Dairy science, the Institute of Food, Nutrition and Human Health, Massey University.
- Dr. Lara Matia-Merino is experienced in Emulsion technology and Dairy science, the Institute of Food, Nutrition and Human Health, Massey University.
- Dr. Alastair MacGibbon is experienced in lipid biochemistry and Dairy Science. Fonterra Research and Development Centre.

The purpose of the project is to investigate the digestion behaviour of human breast milk and infant formula emulsions in the model stomach. The information from this

study pertains to develop protein membrane covering lipid droplets. This will help Fonterra Research and Development Centre's synthesis a milk that is more digestible than currently formulation and suitable for infant who feed to adequate breast milk.

Project Description and Invitation

During the first year of life, breast milk is the best source of nutrition for infants. Breast milk provides several health benefits for both the mother and infant beyond the benefits of adequate nutrition. If breastfeeding is not the chosen method for feeding, formula feeding is a common substitute though it is well known that there are differences between human breast milk and infant formula. These differences can potentially affect the way milk is digested. The properties of breast milk and formula emulsions affect the efficiency of digestion of each type of milk. Previous human studies have indicated that infants digest and absorb formula milk more slowly than breast milk. The data obtained from breast milk in this study is intended to provide both, description of the properties and a baseline for comparison with the behaviour of formula feeds. The outcome benefits from this research will help to better understand about the digestion of lipids in the digestive system and how it affects the health properties of infants.

We would like to invite you to participate in this research project. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

Participant Identification and Recruitment

We wish to collect samples of human breast milk from approximately 25 healthy mothers who are breast feeding babies between 1 month and up to 3 months of age, by screening and selecting from healthy mothers who are breastfeeding their own babies, and have more milk than they need, so they are able to donate surplus milk.

We will collect information from donors on their general health and medical history, the age, health, and nutritional status of their baby. There is a need for participants to be exclusively breastfeeding. Likely cause of complementary feeding is shortage of breast milk. It would not be ethical to sample breast from mothers who are experience such shortage as it would in affect be taking breast milk from the baby. This study does not in any way imply that there is anything wrong with breast feeding.

At the end of the project, we will give you NZD $20 voucher.

Project Procedures

If you are interested in hearing more about the study, we will contact you to run through a brief screening questionnaire. We will provide you with a questionnaire to fill in, ask you to complete a consent form and provide you with sample sterilised containers to collect 50 mL of breast milk. We will return to your home at a later date to collect the breast milk samples.

We should advise you to collect a single sample of surplus breast milk by using a breast pump (either one owned by the mother or supplied by us) or can be expressed by hand if preferred, the sample being taken at half part between two feeding times. If the mother wants the breast pump, it will be delivered to mother's house by the researcher. The pump collection parts and the collection containers will be cleaned and sterilised beforehand, according to the instruction manual. If you are unable to produce the 50 mL of milk on one occasion, the sample can be collected over two occasions within one day. After collected the breast milk, should store breast milk in the fridge (4°C) only in sterilised containers and it should be transported to the lab as soon as possible. The samples will be collected at home and subsequently refrigerated. The researcher (Chalida) will go to mother's house to get breast milk after the mother has finished collected breast milk and call the researcher.

The characteristics of lipid in human breast milk in the model stomach will be determined. This data will form a basis for comparison with the behaviour of formula feeds with a view to improving the digestibility and acceptability of the

latter. The sample will be analysed by the Institute of Food, Nutrition and Human Health at Massey University and Fonterra Research and Development Centre, Fitzherbert Dairy Farm Road, Palmerston North, New Zealand. After analysis, the whole sample will be disposed.

Compensation for Injury

If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim.

Data Management

All information you give us is confidential. Each questionnaire and breast milk information will be entered into a database using ID numbers and the information will be stored in a locked cabinet in such a way that only the researcher will be able to gain access to it. No individual information or names will be published. At the end of the project any personal information will be destroyed immediately.

The breast milk samples and information will not be used in any other studies and the portion of milk used in the tests will be disposed of. Questionnaires will be seen by named researchers only, and when the study is completed all questionnaires will be locked away in filing cabinets.

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 13/08. If you have any concerns about the conduct of this research, please contact Dr Brian Finch, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 350 5799 x 84459, email humanethicsoutha@massey.ac.nz.

Participant's Rights

You are under no obligation to accept this invitation. If you decide to participate you have the right to:

- decline to answer any particular question;
- ask any questions about the study at any time during participation;
- provide information on the understanding that your name will not be used unless you give permission to the researcher;
- be given access to a summary of the project findings when it is concluded

Completion and return of the questionnaire implies consent. You have the right to decline to answer any particular question.

Please contact us by e-mail at C.Lueamsaisuk@massey.ac.nz, by phone at +64 (0)6 356-9099 x81407 by post at the Institute of Food, Nutrition and Human Health, Massey University, private bag 11 222 to discuss any queries or concerns about the study.

Thank you very much for your time in considering this study.

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

Dear Sir or Madam,

My name is Chalida Lueamsaisuk. I am a PhD student in Food Technology, the Institute of Food, Nutrition and Human Health, Massey University. I am studying the structural and chemical changes in human breast milk during digestion. This research will help to better understand the digestion of lipids in the infant gut and how it affects the health of infants.

The purpose of this letter is to ask you to post the attached advertisement on your community notice board to help us make contact with mothers who are willing to donate one sample of fresh breast milk when their baby is about one to three month old. Interested mother will need to ring the telephone number given on the poster to join the study.

I have also included the information sheet for the mothers.

Yours sincerely,

Chalida Lueamsaisuk

The Institute of Food, Nutrition and Human Health
Massey UniversityMassey University
Private Bag 11 222
Palmerston North 4442
New Zealand.
Ph +64-6-3569099x81407

Code.....
Date of collection.....

Breast Milk Screening Form

Donor name: .....

Address: .....

Phone: .....**Email address:** .....

Mother's age: .....**Baby's birth date:** .....**Sex of your infant**.....

ETHNICITY *(tick all that apply)* ☐ **NZ European** ☐ **Maori** ☐ **Samoan**
☐ **Cook Island Maori** ☐ **Niuean** ☐ **Chinese**
☐ **Indian** ☐ **Tongan**
☐ **Other**.....***(specify)***

Question	**Reply** (Yes, No)
1. Are you generally healthy?	
2. Do you have any general health concerns?	
3. Do you have any infectious blood borne diseases?	
4. Have you ever had any serious illness in the past year?	
5. Is your baby gaining weight and growing well?	
6. Do you normally produce an excess of milk even after feeding?	
7. Is your baby totally breastfed?	
8. Can you always breastfeed your baby without using formula as a supplement?	
9. Have you had any difficulty feeding in the past?	
10. Would you mind collecting your breast milk in between two feeding times?	
11. Was your baby a full term baby?..... If no, how many weeks were you over or under your due date?	
* full-term infant is one born in the interval from the thirty-seventh completed week to the forty-second completed week of gestation; 259 days to 293 days, inclusive.	
12. Have you breastfed any previous children? a. If yes, did you have any problems? please describe:.....	
13. Have you expressed and stored milk before?..... a. If yes, what method did you use? ..... b. If you use a pump, what type of pump do you use? ..... c. Would you like us to supply the breast milk?	
14. Have you ever had a breast infection? ..... If yes, please describe and give date(s):	
15. Are you on any special diet? e.g., low salt, low dairy products, vegetarian, diabetic, weight loss, etc.?..... If yes, please explain:	
16. Do you take any medications on a regular basis, if so please provide details in the space below	

I hereby certify to the best of my knowledge I understand and have answered all questions truthfully.

Signature: .....**Date**.....

Contact: Chalida Lueamsaisuk, E-mail: C.Lueamsaisuk@massey.ac.nz, Phone: +64 (0)6 356-9099 x81407,
Address: the Institute of Food, Nutrition and Human Health, Massey University, private bag 11 222

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

The Institute of Food, Nutrition and Human Health
Massey UniversityMassey University
Private Bag 11 222
Palmerston North 4442
New Zealand.
Ph +64-6-3569099x81407

Study: An examination of the structural and chemical changes in human milk during digestion

PARTICIPANT CONSENT FORM

I wish/do not wish to participate in the study (please delete one)

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I agree to participate and understand I have the right to withdraw from the study at any time and to decline to answer any particular questions.

I agree to participate in this study under the conditions set out in the Information Sheet.

Signature: ..... **Date:** .....

Full Name - printed .....

Bibliography

Abrahamse, E., Minekus, M., van Aken, G. A., van de Heijning, B., Knol, J., Bartke, N., Oozeer, R., van der Beek, E. M. and Ludwig, T. (2012) 'Development of the digestive system-experimental challenges and approaches of infant lipid digestion', *Food Digestion,* 3(1-3), pp. 63-77.

Ahmed, F. U. and Traistaru, C. N. (2009) *Non-chlorinated concentrated all-in-one acid detergent and method for using the same* Patent no. 7501027. [Online]. Available at: http://www.freepatentsonline.com/7501027.html.

Ahn, C. J. and Kim, Y. J. (1963) 'Acidity and volume of gastric contents in the first week of life.', *Journal of the Korean Medical Association,* 6, pp. 72.

Allen, A. and Flemström, G. (2005) 'Gastroduodenal mucus bicarbonate barrier: protection against acid and pepsin', *American Journal of Physiology: Cell Physiology,* 57(1), pp. C1-C19.

Almaas, H., Cases, A.-L., Devold, T. G., Holm, H., Langsrud, T., Aabakken, L., Aadnoey, T. and Vegarud, G. E. (2006) 'In vitro digestion of bovine and caprine milk by human gastric and duodenal enzymes', *International Dairy Journal,* 16, pp. 961-968.

Aloulou, A. and Carrière, F. (2008) 'Gastric lipase: an extremophilic interfacial enzyme with medical applications', *Cellular and Molecular Life Sciences,* 65(6), pp. 851-854.

Andersson, Y., Savman, K., Blackberg, L. and Hernell, O. (2007) 'Pasteurization of mother's own milk reduces fat absorption and growth in preterm infants', *Acta Paediatrica,* 96, pp. 1445-1449.

Anema, S. G. (2009) 'The whey proteins in milk: thermal denaturation, physical interactions and effects on the functional properties of milk', in Thompson, A., Boland, M. & Singh, H. (eds.) *Milk Proteins: From Expression to Food*. New York: Elsevier.

Argov, N., Wachsmann-Hogiu, S., Freeman, S. L., Huser, T., Lebrilla, C. B. and German, J. B. (2008) 'Size-dependent lipid content in human milk fat globules', *Journal of Agricultural and Food Chemistry,* 56(16), pp. 7446-7450.

Armand, M., Hamosh, M., Mehta, N. R., Angelus, P. A., Philpott, J. R., Henderson, T. R., Dwyer, N. K., Lairon, D. and Paul, H. (1996) 'Effect of human milk or formula on gastric function and fat digestion in the premature infant 1', *Pediatric Research,* 40(3), pp. 429-437.

Armand, M., Pasquier, B., André, M., Borel, P., Senft, M., Peyrot, J., Salducci, J., Portugal, H., Jaussan, V. and Lairon, D. (1999) 'Digestion and absorption of 2 fat emulsions with different droplet sizes in the human digestive tract', *The American Journal of Clinical Nutrition,* 70, pp. 1096-1106.

Arslanoglu, S., Moro, G. E. and Ziegler, E. E. (2009) 'Preterm infants fed fortified human milk receive less protein than they need', *Journal of Perinatology,* 29(7), pp. 489-492.

Atkinson, S. A. and Lonnerdal, B. (eds.) (1989) *Protein and non-protein nitrogen in human milk*. Florida: CRC Press.

Baba, T., Downs, D., Jackson, K. W., Tang, J. and Wang, C. S. (1991) 'Structure of human milk bile salt activated lipase', *Biochemistry,* 30(2), pp. 500-510.

Beer, H. D., Wohlfahrt, G., Schmid, R. D. and McCarthy, J. E. G. (1996) 'The folding and activity of the extracellular lipase of Rhizopus oryzae are modulated by a prosequence', *Biochemical Journal,* 319(2), pp. 351-359.

Berenhauser, A. C., Pinheiro do Prado, A. C., da Silva, R. C., Gioielli, L. A. and Block, J. M. (2012) 'Fatty acid composition in preterm and term breast milk.', *International Journal of Food Sciences and Nutrition,* 63(3), pp. 318-325.

Bernback, S., Blackberg, L. and Hernell, O. (1989) 'Fatty acids generated by gastric lipase promote human milk triacylglycerol digestion by pancreatic colipase-dependent lipase', *Biochimica et Biophysica Acta,* 1001, pp. 286-293.

Bernbäck, S., Hernell, O. and Bläckberg, L. (1987) 'Bovine pregastric lipase: a model for the human enzyme with respect to properties relevant to its site of action', *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism,* 922(2), pp. 206-213.

Berseth, C., Mitmesser, S., Ziegler, E., Marunycz, J. and Vanderhoof, J. (2009) 'Tolerance of a standard intact protein formula versus a partially hydrolyzed formula in healthy, term infants', *Nutrition Journal,* 8(1), pp. 27.

Bolanos-Garcia, V. M. and Miguel, R. N. (2003) 'On the structure and function of apolipoproteins: more than a family of lipid-binding proteins', *Progress in Biophysics and Molecular Biology,* 83(1), pp. 47-68.

Borgström, B. (1974) 'Fat digestion and absorption', in Smyth, D.H. (ed.) *Biomembranes*. New York: Plenum Press, pp. 555-560.

Borgström, B. and Patton, J. S. (2011) 'Luminal events in gastrointestinal lipid digestion', *Comprehensive Physiology*, pp. 475-504.

Bovo, P., Cataudella, G., Di Francesco, V., Vaona, B., Filippini, M., Marcori, M., Montesi, G., Rigo, L., Frulloni, L., Brunori, M. P., Andreaus, M. C. and Cavallini, G. (1995) 'Intraluminal gastric pH in chronic pancreatitis', *Gut,* 36(2), pp. 294-298.

Bracco, U. (1994) 'Effect of triglyceride structure on fat absorption', *American Society for Clinical Nutrition,* 60(Suppl), pp. 1002S-9S.

Brans, G., Schroen, C. G. P. H., Sman, R. G. M. v. d. and Boom, R. M. (2004) 'Membrane fractionation of milk: state of the art and challenges', *Journal of Membrane Science,* 243, pp. 263–272.

Britten, M. and Giroux, H. J. (1993) 'Interfacial properties of milk protein-stabilized emulsions as influenced by protein concentration', *Journal of Agricultural and Food Chemistry,* 41(8), pp. 1187-1191.

Brzozowski, A. M., Derewenda, U., Derewenda, Z. S., Dodson, G. G., Lawson, D. M., Turkenburg, J. P., Bjorkling, F., Huge-Jensen, B., Patkar, S. A. and Thim, L. (1991) 'A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex', *Nature,* 351(6326).

Campos, L. A. and Sancho, J. (2003) 'The active site of pepsin is formed in the intermediate conformation dominant at mildly acidic pH', *FEBS Letters,* 538(1-3), pp. 89-95.

Cano-Ruiz, M. E. and Richter, R. L. (1997) 'Effect of Homogenization Pressure on the Milk Fat Globule Membrane Proteins', *Journal of Dairy Science,* 80(11), pp. 2732-2739.

Caro, J. D., Sias, B., Grandval, P., Ferrato, F., Halimi, H., Carriere, F. and Caro, A. D. (2004) 'Characterization of pancreatic lipase-related protein 2 isolated from human pancreatic juice', *Biochimica et Biophysica Acta,* 1701, pp. 89-99.

Carr, A. J. (1999) *The functional properties of milk protein concentrates.* Doctor of philisophy, Massey University, Palmerston North.

Carver, J. D. (2003) 'Advances in nutritional modifications of infant formulas', *American Society for Clinical Nutrition,* 77(suppl), pp. 1550S-4S.

Cavaletto, M., Giuffrida, M. and Conti, A. (2008) 'Milk Fat Globule Membrane Components–A Proteomic Approach', in Bösze, Z. (ed.) *Bioactive Components of Milk Advances in Experimental Medicine and Biology*. New York: Springer, pp. 129-141.

Chahinian, H., Snabe, T., Attias, C., Fojan, P., Petersen, S. B. and Carrière, F. (2005) 'How gastric lipase, an interfacial enzyme with a Ser-His-Asp catalytic triad, acts optimally at acidic pH', *Biochemistry,* 45(3), pp. 993-1001.

Chatterton, D. E. W., Rasmussen, J. T., Heegaard, C. W., Sørensen, E. S. and Petersen, a. 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, pp. 373-383.

Chen, J. D., Co, E., Liang, J., Pan, J., Sutphen, J., Torres-Pinedo, R. B. and Orr, W. C. (1997) 'Patterns of gastric myoelectrical activity in human subjects of different ages', *Am. J. Physiol. Gastrointest. Liver Physiol.,* 272(5), pp. GT1022-GT1027.

Christensen, B., Fink, J., Merrifield, R. B. and Mauzerall, D. (1988) 'Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes.', *Proc. Natl. Acad. Sci. USA. ,* 85(14), pp. 5072-5076.

Christie, W. W. (1995) 'Composition and structure of milk lipids', in Fox, P.F. (ed.) *Advanced dairy chemistry-2: Lipids*. UK: Chapman&Hall.

Ciron, C. I. E., Gee, V. L., Kelly, A. L. and Auty, M. A. E. (2010) 'Comparison of the effects of high-pressure microfluidization and conventional homogenization of milk on particle size, water retention and texture of non-fat and low-fat yoghurts', *International Dairy Journal,* 20, pp. 314-320.

Crelin, E. S. (1973) *Function Anatomy of the Newborn.* London: Yale University Press.

Dael, P. V., Kastenmayer, P., Clough, J., Jarret, A. R., Barclay, D. V. and Maire, J. C. (2005) 'Substitution of casein by *b*-casein or of whey protein isolate by *α*-lactalbumin does not affect mineral balance in growing rats', *The journal of nutrition,* 135(6), pp. 1438-1443.

Dalgleish, D. G. (1992) 'Bovine milk protein properties and the manufacturing quality of milk', *Livestock Production Science,* 35, pp. 75-93.

Damodaran, S., Parkin, K. L. and Fennema, O. R. (2008) *Fennema's Food Chemistry.* CRC Press.

Danthine, S., Blecker, C., Paquot, M., Innocente, N. and Deroanne, C. (2000) 'Évolution des connaissances sur la membrane du globule gras du lait : synthèse bibliographique', 80(2), pp. 209-222.

Darragh, A. (1995) *The amino acid composition of human milk towards determining the amino acid requrements of the human infant.* Doctor of Philosophy, Massey University, Palmerston North.

Darragh, A. and Lönnerdal, B. (2011) 'Milk | Human Milk', in Fuquay, J.W. (ed.) *Encyclopedia of Dairy Sciences (Second Edition)*. San Diego: Academic Press, pp. 581-590.

de Belle, R. C., Vaupshas, V., Vitullo, B. B., Haber, L. R., Shaffer, E., Mackie, G. G., Owen, H., Little, J. M. and Lester, R. (1979) 'Intestinal absorption of bile salts: Immature development in the neonate', *The Journal of Pediatrics,* 94(3), pp. 472-476.

Dean, P. N. (1998) 'Confocal microscopy: principles and practices', in Robinson, J.P. (ed.) *Current protocols in cytometry*. New York: John Wiley & Sons.

Derewenda, U., Brzozowski, A. M., Lawson, D. M. and Derewenda, Z. S. (1992) 'Catalysis at the interface: The anatomy of a conformational change in a triglyceride lipase', *Biochemistry,* 31(5), pp. 1532-1541.

Dewettinck, K., Rombaut, R., Thienpont, N., Le, T. T., Messens, K. and Camp, J. V. (2008) 'Nutritional and technological aspects of milk fat globule membrane material', *International Dairy Journal,* 18, pp. 436–457.

Dickinson, E. (1993) 'Towards more natural emulsifiers', *Trends in Food Science & Technology,* 4(10), pp. 330-334.

Dickinson, E. (1997) 'Properties of emulsions stabilized with milk proteins: overview of some recent developments', *Journal of Dairy Science,* 80(10), pp. 2607-2619.

Dickinson, E. (2010) 'Flocculation of protein-stabilized oil-in-water emulsions', *Colloids and Surfaces B: Biointerfaces,* 81(1), pp. 130-140.

Donma, M. M. and Donma, O. (1989) 'Apolipoproteins: Biochemistry, methods and clinical significance', *Biochemical Education,* 17(2), pp. 63-68.

Dukhin, S. S., Mishchuk, N. A., Loglio, G., Liggieri, L. and Miller, R. (2003) 'Coalescence coupling with flocculation in dilute emulsions within the primary and/or secondary minimum', *Advances in Colloid and Interface Science,* 100–102(0), pp. 47-81.

Dukhin, S. S., Sjöblom, J., Wasan, D. T. and Sæther, Ø. (2001) 'Coalescence coupled with either coagulation or flocculation in dilute emulsions', *Colloids and Surfaces A: Physicochemical and Engineering Aspects,* 180(3), pp. 223-234.

Early, R. (1998) 'Liquid milk and cream', in Early, R. (ed.) *Technology of Dairy Products.* 2nd ed. London, United Kingdom: Springer.

Ebers, D. W., Smith, D. I. and Gibbs, G. E. (1956) 'Gastric acidity on the first day of life', *Pediatrics,* 18, pp. 800.

Evers, J. M. (2004) 'The milkfat globule membrane—methodologies for measuring milkfat globule (membrane) damage', *International Dairy Journal,* 14, pp. 747-760.

Favé, G., Coste, T. C. and Armand, M. (2004) 'Physicochemical properties of lipids: new strategies to manage fatty acid bioavailability', *Cellular and Molecular Biology,* 50, pp. 815-883.

Fink, N. H. (2013) *Fatty Acids in Nutrition Sources for Preterm Infants.* Master of Science, McMaster University, Hamilton, Ontario [Online] Available at: http://digitalcommons.mcmaster.ca/opendissertations/8124.

Fletcher, K., Catchpole Owen, J., Grey John, B. and Pritchard, M. (2007) *Beta-serum dairy products, neutral lipid-depleted and/or polar lipid-enriched dairy products, and processes for their production*. [Online]. Available at: http://europepmc.org/abstract/PAT/CN101090635.

Forsman, C. L. and Schwertfeger, K. L. (2013) 'Mammary gland development and structure: an overview', in Zibadi, S., Watson, R.R. & Preedy, V.R. (eds.) *Handbook of dietary and nutritional aspects of human breast milk*. Wageningen, The Netherlands: Wageningen Academic Publishers, pp. 15-34.

Forsyth, J. S. (1998) 'Lipids and infant formulas', *Nurrition Research Reviews,* 11, pp. 255-278.

Fox, P. F. and Brodkorb., A. (2008) 'The casein micelle: Historical aspects, current concepts and significance', *International Dairy Journal,* 18, pp. 677-684.

Fox, P. F. and McSweeney, P. L. H. (1998) *Dairy chemistry and biochemistry.* New York: Kluwer Academic/Plenum Publishers, p. 479.

Fox, P. F. and McSweeney, P. L. H. (eds.) (2006) *Advanced dairy chemistry* 3edn. New York: Springer.

Frayn, K. N. (2010) *Metabolic regulation: a human perspective.* 3 edn. Oxford: Wiley-Blackwell.

Freer, Y. and Lyon, A. (2005) 'Nasogastric tube aspirate pH values associated with typical enteral feeding patterns in infants admitted to an NICU', *Journal of Neonatal Nursing,* 11, pp. 106-109.

Author (2011) *F2011C00547 Australia New Zealand Food Standards Code - Standard 2.9.1 - Infant Formula Products*: Commonwealth of Australia Gazette.

Gallier, S., Gragson, D., Jiménez-Flores, R. and Everett, D. (2010) 'Using confocal laser scanning microscopy to probe the milk fat globule membrane and associated proteins', *Journal of Agricultural and Food Chemistry,* 58(7), pp. 4250-4257.

Gallier, S., Zhu, X. Q., Rutherfurd, S. M., Ye, A., Moughan, P. J. and Singh, H. (2013) 'In vivo digestion of bovine milk fat globules: Effect of processing and interfacial structural changes. II. Upper digestive tract digestion', *Food Chemistry,* 141(3), pp. 3215-3223.

Garcia, C., Antona, C., Robert, B., Lopez, C. and 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(0), pp. 494-504.

Ghosh, J. (2006) *Fundamental Concepts Of Applied Chemistry.* New Delhi: S. Chand Company Pvt Ltd.

Golding, M. and Wooster, T. J. (2010) 'The influence of emulsion structure and stability on lipid digestion', *Current Opinion in Colloid & Interface Science,* 15(1–2), pp. 90-101.

Golding, M., Wooster, T. J., Day, L., Xu, M., Lundin, L., Keogh, J. and Clifton, P. (2011) 'Impact of gastric structuring on the lipolysis of emulsified lipids', *Soft Matter,* 7(7), pp. 3513-3523.

Groh-Wargo, S. and Sapsford, A. (2009) 'Enteral nutrition support of the preterm infant in the neonatal intensive care unit', *Nutrition in Clinical Practice,* 24(3), pp. 363-376.

Guo, M. R., Hendricks, G. M. and Kindstedt, P. S. (1998) 'Component Distribution and Interactions in Powdered Infant Formula', *International Dairy Journal,* 8(4), pp. 333-339.

Guo, M. R., Hendricks, G. M., Kindstedt, P. S., Flynn, A. and Fox, P. F. (1996) 'Nitrogen and mineral distribution in infant formulae', *International Dairy Journal,* 6(10), pp. 963-979.

Haddad, I., Mozzon, M. and Frega, N. G. (2012) 'Trends in fatty acid positional distribution in human colostrum, transitional and mature milk', *European Food Research and Technology,* 235(2), pp. 325-332.

Hambidge, K. M. and Krebs, N. F. (1991) 'Current pediatric diagnosis and treatment', in Hathaway, W.E., Groothius, J.R., Hay, W.W. & Paisley, J. (eds.) *Normal childhood nutrition and its disorders.* 10 ed. East Norwalk, CT: Appleton & Lange.

Hamosh, M. (1990a) 'Lingual and gastric lipases', *Nutrition,* 6(6), pp. 421-428.

Hamosh, M. (1990b) 'Lingual and gastric lipases: their role in fat digestion', in Hamosh, M. (ed.). Michigan: CRC Press, pp. 239.

Hamosh, M. (1996) 'Digestion in the newborn', *Clinics in perinatology,* 23(2), pp. 191-208.

Hamosh, M. (2006) 'Enteral lipid digestion and absorption', in Thureen, P.J. & Hay, W.W. (eds.) *Neonatal Nutrition and Metabolism.* 2 ed: Cambridge University Press, pp. 350-378.

Hamosh, M., Bitman, J., Fink, C. S., Freed, L. M., York, C. M., Wood, D. L., Mehta, N. R. and Hamosh, P. (1985a) 'Lipid composition of preterm human milk and its digestion by the infant', in Schaub, J. (ed.) *Composition and physiological properties of human milk*. New York: Elsevier.

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

Hamosh, M. and Hamosh, P. (1996) 'Selectivity of Lipases: Developmental Physiology Aspects', in MalcataFX (ed.) *Engineering of/with Lipases*. Dordrecht: Kluwer Academic Publishers.: NATO ASI Series E, Applied Sciences, pp. 31-49.

Hamosh, M. and Hamosh, P. (1999) 'Development of digestive enzyme secretion', in Sanderson, I.R. & Walker, W.A. (eds.) *Development of the gastrointestinal tract*. Canana: B. C. Decker.

Hamosh, M., Scanlon, J. W., Ganot, D., Likel, M., Scanlon, K. B. and Hamosh, P. (1981) 'Fat Digestion in the Newborn: Characterization of lipase in gastric aspirates of premature and term infants', *Journal of Clinical Investigation,* 67, pp. 838-846.

Haumont, D. (2010) 'Lipid infusion and intravenous access in newborn infants', *Chinese Medical Journal,* 123(20), pp. 2766-2768.

Heine, W. E., Klein, P. D. and Reeds, P. J. (1991) 'The Importance of α-Lactalbumin in Infant Nutrition', *he Journal of Nutrition,* 121(3), pp. 277-283.

Hendricks, G. M., Guo, M. R. and Kindstedt, P. S. 'Characterization of protein-lipid interaction and redistribution of minerals in infant formula by CTEM and EDS'. 188-189.

Hiol, A., Jonzo, M. D., Rugani, N., Druet, D., Sarda, L. and 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(5–6), pp. 421-430.

Huppertz, T., Fox, P. F., Kruif, K. G. d. and Kelly, A. L. (2006) 'High pressure-induced changes in bovine milk proteins: A review', *Biochimica et Biophysica Acta,* 1764, pp. 593-598.

Hur, S. J., Decker, E. A. and McClements, D. J. (2009) 'Influence of initial emulsifier type on microstructural changes occurring in emulsified lipids during in vitro digestion.', *Food Chemistry,* 114(1), pp. 253-262.

Husvéth, F. (2011) *Physiological and reproductional aspects of animal production*: Debreceni Egyetem, Nyugat-Magyarországi Egyetem, Pannon Egyetem. Available at:

http://www.tankonyvtar.hu/en/tartalom/tamop425/0010_1A_Book_angol_05_termeleselettan/adatok.html.

IUPAC 2007. Definitions of terms relating to the structure and processing of sols, gels, networks, and inorganic-organic hybrid materials. *In:* McNaught, A.D. & Wilkinson, A. (eds.) *Compendium of Chemical Terminology (the "Gold Book").* 2 ed. Oxford Blackwell Scientific Publications.

Jensen, R. G. (1989) *The lipids of human milk.* Florida: CRC Press.

Jensen, R. G. (2002) 'The Composition of Bovine Milk Lipids: January 1995 to December 2000', *Journal of Dairy Science,* 85(2), pp. 295-350.

Jensen, R. G., Blanc, B. and Patton, S. (1995) 'B - Particulate Constituents in Human and Bovine Milks', in Jensen, R.G. (ed.) *Handbook of Milk Composition*. San Diego: Academic Press, pp. 50-62.

Jensen, R. G., Clark, R. M., deJong, F. A., Hamosh, M., Liao, T. H. and Mehta, N. R. (1982) 'The lipolytic triad human lingual, breast milk, and pancreatic lipases: Physiological implications of their characteristics in digestion of dietary fats', *Journal of Pediatric Gastroenterology and Nutrition,* 1(2), pp. 243-255.

Jensen, R. G., Ferris, A. M. and Lammi-Keefe, C. J. (1992) 'Lipids in human milk and infant formulars', *Annuual Review of Nutrition,* 12, pp. 417-441.

Jensen, R. G., Ferris, A. M., Lammi-Keefe, C. J. and Henderson, R. A. (1990) 'Lipids of bovine and human milks: a comparison', *Journal of Dairy Science,* 73(2), pp. 223-240.

Keenan, T. W. and Mather, I. H. 2002. Milk Fat Globule Membrane. *In:* Roginski, H., Fuquay, J.W. & Fox, P.F. (eds.) *Encyclopedia of dairy sciences.* London: Academic Press.

Keenan, T. W. and Patton, S. (1995) 'The Milk Lipid Globule Membrane', in Jensen, R.G. (ed.) *Handbook of milk composition*. New York: Academic Press, pp. 5-62.

Kempley, S. T., Sinha, A. K. and Thomas, M. R. (2005) 'Which milk for the sick preterm infant?', *Current Paediatrics,* 15, pp. 390-399.

Keowmaneechai, E. (2002) *Influence of Protein-Mineral Interactions on Physicochemical Properties of Model Nutritional Beverage Emulsions.* Doctor of Philosophy, University of Massachusetts Amherst.

Khan, S., Hepworth, A. R., Prime, D. K., Lai, C. T., Trengove, N. J. and Hartmann, P. E. (2013) 'Variation in fat, lactose, and protein composition in breast milk over 24 hours: Associations with infant feeding patterns', *Journal of Human Lactation,* 29(1), pp. 81-89.

Kierszenbaum, A. L. and Tres, L. (2012) *Histology and Cell Biology: An Introduction to Pathology.* 3 edn.: Elsevier.

Kim, E. H.-J., Chen, X. D. and Pearce, D. (2009) 'Surface composition of industrial spray-dried milk powders. 2. Effects of spray drying conditions on the surface composition', *Journal of Food Engineering,* 94, pp. 169-181.

Kontkanen, H., Rokka, S., Kemppinen, A., Miettinen, H., Hellström, J., Kruus, K., Marnila, P., Alatossava, T. and Korhonen, H. (2010) 'Enzymatic and physical modification of milk fat: A review', *International Dairy Journal*.

Koopman, R., Crombach, N., Gijsen, A. P., Walrand, S., Fauquant, J., Kies, A. K., Lemosquet, S., Saris, W. H., Boirie, Y. and van Loon, L. J. (2009) 'Ingestion of a protein hydrolysate is accompanied by an accelerated in vivo digestion

and absorption rate when compared with its intact protein', *The American Journal of Clinical Nutrition,* 90(1), pp. 106-115.

Kremer, F., Lagaly, G., Kawasaki, K., Lindman, B., Okabayashi, H., O'Connor, C. J. and Manuel, R. D. (1997) 'Calf pregastric lipase catalyzed hydrolysis of short and medium chain-length monoacid triglycerides: Temperature, pH and lipid concentration effects', pp. 188.

Lacroix, M., Bon, C., Bos, C., Leonil, j., Benamouzig, R., Luengo, C., Fauquant, J., D, T. and Gaudichon, C. (2008) 'Ultra high temperature treatment, but not pasteurization, affects the postprandial kinetics of milk proteins in Humans1,2.', *The Journal of Nutrition,* 138(12), pp. 2342-7.

Lai, D. T., Mackenzie, A. D., O'Connor, C. J. and Turner, K. W. (1997) 'Hydrolysis characteristics of bovine milk fat and monoacid triglycerides mediated by pregastric lipase from goats and kids', *Journal of Dairy Science,* 80(10), pp. 2249-2257.

Lapillonne, A., Groh-Wargo, S., Lozano Gonzalez, C. H. and Uauy, R. (2013) 'Lipid Needs of Preterm Infants: Updated Recommendations', *The Journal of Pediatrics,* 162(3, Supplement), pp. S37-S47.

Lawrence, R. A. (1999) *Breastfeeding: A Guide for the Medical Profession.* St. Louis: Mosby, Inc.

Le, T. T., Van de Wiele, T., Do, T. N. H., Debyser, G., Struijs, K., Devreese, B., Dewettinck, K. and Van Camp, J. (2012) 'Stability of milk fat globule membrane proteins toward human enzymatic gastrointestinal digestion', *Journal of Dairy Science,* 95(5), pp. 2307-2318.

Lentle, R. G. and Janssen, P. W. M. (2011) *The Physical Processes of Digestion.* Springer.

Lentle, R. G., Janssen, P. W. M., Goh, K., Chambers, P. and 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), pp. 3349-3360.

Lesmes, U. and McClements, D. J. (2012) 'Controlling lipid digestibility: Response of lipid droplets coated by β-lactoglobulin-dextran Maillard conjugates to simulated gastrointestinal conditions', *Food Hydrocolloids,* 26(1), pp. 221-230.

Leung, A. K. C. and Sauve, R. S. (2003) 'Whole cow's milk in infancy', *Paediatrics and Child Health,* 8(7), pp. 419-421.

Liao, T. H., Hamosh, P. and Hamosh, M. (1983) 'Gastric lipolysis in the developing rat ontogeny of the lipases active in the stomach ', *Biochimica et Biophysica Acta,* 754, pp. 1-9.

Lindquist, S. and Hernell, O. (2010) 'Lipid digestion and absorption in early life: An update', *Current Opinion in Clinical Nutrition and Metabolic Care,* 13(3), pp. 314-320.

Livney, Y. D. (2010) 'Milk proteins as vehicles for bioactives', *Current Opinion in Colloid & Interface Science,* 15, pp. 73-83.

Lopez, C. (2005) 'Focus on the supramolecular structure of milk fat in dairy products', *Reproduction Nutrition Development,* 45(4), pp. 497-511.

Lopez, C. (2010) 'Lipid domains in the milk fat globule membrane: Specific role of sphingomyelin', *Lipid Technology,* 22(8), pp. 175.

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), pp. 391-404.

Lopez, C. and Ménard, 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), pp. 29-41.

Loren, N., Langton, M. and Hermansson, A. M. (2007) 'Confocal fluorescence microscopy (CLSM) for food structure characterization', in McClements, D.J. (ed.) *Understanding and controlling the micro structure of complex foods*. Abington, Cambridge, England: Woodhead Publishing Limited, pp. 232-257.

Lueamsaisuk, C., Lentle, R. G., MacGibbon, A. K. H., Matia-Merino, L. and Golding, M. (2014a) '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*.

Lueamsaisuk, C., Lentle, R. G., MacGibbon, A. K. H., Matia-Merino, L. and Golding, M. (2014b) 'Factors influencing the dynamics of emulsion structure during neonatal gastric digestion in an in vitro model', *Food Hydrocolloids,* 36(0), pp. 162-172.

Lundin, L., Golding, M. and Wooster, T. J. (2008) 'Understanding food structure and function in developing food for appetite control', *Nutrition and Dietetics,* 65 (Suppl. 3), pp. S79-S85.

Luyten, H., Jonkman, M., Kloek, W. and van Vliet, T. (1993) 'Creaming behaviour of dispersed particles in dilute xanthan solutions', in Dickinson, E. & Walstra, P. (eds.) *Food Colloids and polymers: Stability and Mechanical Properties*. Cambridge: Royal Society of Chemistry, pp. 224.

MacGregor, J. (2008) *Introduction to the Anatomy and Physiology of Children: A guide for students of nursing, child care and health.* 2 edn. New York: Routledge, p. 255.

Mackie, A. and Macierzanka, A. (2010) 'Colloidal aspects of protein digestion', *Current Opinion in Colloid & Interface Science,* 15, pp. 102-108.

Mahmoudi, S. R., Adamiak, K. and Castle, G. S. P. 'On the Thermodynamic Description of Electrohydrodynamic Flows'. *The 2012 Electrostatics Joint Conference*, Cambridge, ON, Canada.

Malacarne, M., Martuzzi, F., Summer, A. and Mariani, P. (2002) 'Protein and fat composition of mare's milk: some nutritional remarks with reference to human and cow's milk', *International Dairy Journal,* 12, pp. 869–877.

Malaki Nik, A., Wright, A. J. and Corredig, M. (2011) 'Impact of interfacial composition on emulsion digestion and rate of lipid hydrolysis using different in vitro digestion models', *Colloids and Surfaces B: Biointerfaces,* 83(2), pp. 321-330.

Malbon, K. (ed.) (2006) *Neonatal nutrition and metabolism.* 2 edn. New York: Cambridge University Press.

Maldonado, J., Gil, A., Narbona, E. and Molina, J. A. (1998) 'Special formulas in infant nutrition: a review', *Early Human Development,* 53, Supplement 1(0), pp. S23-S32.

Manson, W. G., Coward, W. A., Harding, M. and Weaver, L. T. (1999) 'Development of fat digestion in infancy', *Archives of Disease in Childhood - Fetal and Neonatal Edition,* 80, pp. F183-F187.

Manson, W. G. and Weaver, L. T. (1997) 'Fat digestion in the neonate', *Archives of Disease in Childhood,* 76, pp. F206-F211.

Mansson, H. L. (2008) *Fatty acids in bovine milk fat*, Lund, Sweden: Swedish Dairy Association.

Manuel, R. D. (1999) *Calf pregastric lipase-A kinetic study.* PhD, University of Auckland, Auckland.

Marciani, L., Wickham, M., Wright, J., Bush, D., Faulks, R., Fillery-Travis, A., Gowland, P. and Spiller, R. C. (2003) 'Magnetic resonance imaging (MRI) insights into how fat emulsion stability alters gastric emptying', *Gastroenterology,* 124(4, Supplement 1), pp. A581.

Martini, S. and Marangoni, A. G. (2007) 'Microstructure of Dairy Fat Products', in Tamime, A. (ed.) *Structure of dairy products*. UK: Blackwell Publishing Ltd.

Mason, S. (1962) 'Some Aspects of Gastric Function in the Newborn', *Archives of Disease in Childhood,* 37(194), pp. 387-391.

Mather, I. H. (2011a) 'Mammary Gland, Milk Biosynthesis and Secretion | Secretion of Milk Constituents', in Fuquay, J.W. (ed.) *Encyclopedia of Dairy Sciences (Second Edition)*. San Diego: Academic Press, pp. 373-380.

Mather, I. H. (2011b) 'Milk Lipids | Milk Fat Globule Membrane', in Fuquay, J.W. (ed.) *Encyclopedia of Dairy Sciences (Second Edition)*. San Diego: Academic Press, pp. 680-690.

Matia-Merino, L. (2003) *Interactions in acid casein gels and emulsion gels containing sugar.* Doctor of Philosophy, University of Leeds.

Matsumiya, K., Takahashi, W., Inoue, T. and Matsumura, Y. (2010) 'Effects of bacteriostatic emulsifiers on stability of milk-based emulsions', *Journal of Food Engineering,* 96, pp. 185-191.

McCance, K. L. and Huether, S. E. (2005) *Pathophysiology: The Biologic Basis for Disease in Adults And Children.* 5 edn. St. Louis: Mosby.

McClements, D. J. (1999) *Food Emulsions: Principles, Practice and Techniques.* Boca Raton: CRC Press LLC, p. 378.

McClements, D. J. (2005) *Food Emulsions: Principles, Practice, and Techniques.* 2 nd. edn. Boca Raton: CRC Press LLC.

McClements, D. J. and Decker, E. A. (2009) *Designing functional foods : measuring and controlling food structure breakdown and nutrient absorption. Woodhead publishing in food science and technology* Boca Raton, FL [etc.]: CRC [etc.].

McClements, D. J. and Li, Y. (2010) 'Review of in vitro digestion models for rapid screening of emulsion-based systems', *Food & Function,* 1, pp. 32-59.

McManaman, J. L. and Neville, M. C. (2003) 'Mammary physiology and milk secretion', *Advanced Drug Delivery Reviews,* 55(5), pp. 629-641.

McManaman, J. L. and Neville, M. C. (2005) 'LACTATION | Physiology', in Caballero, B. (ed.) *Encyclopedia of Human Nutrition (Second Edition)*. Oxford: Elsevier, pp. 99-106.

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

Menéndez, B., David, C. and Nistal, A. M. n. (2001) 'Confocal scanning laser microscopy applied to the study of pore and crack networks in rocks', *Computers & Geosciences,* 27(9), pp. 1101-1109.

Michalski, M. C. (2013) 'Lipids and milk fat globule properties in human milk', in Zibadi, S., Watson, R.R. & Preedy, V.R. (eds.) *Handbook of Dietary and Nutritional Aspects of Human Breast Milk.* The Netherlands: Wageningen Academic Publishers, pp. 315-334.

Michalski, M. C., Briard, V. and Juaneda, P. (2005a) 'CLA profile in native fat globules of different sizes selected from raw milk', *International Dairy Journal,* 15, pp. 1089-1094.

Michalski, M. C., Briard, V., Michel, F., Tasson, F. and Poulain, P. (2005b) 'Size Distribution of Fat Globules in Human Colostrum, Breast Milk, and Infant Formula', *Journal of Dairy Science,* 88, pp. 1927-1940.

Michalski, M. C. and Januel, C. (2006) 'Does homogenization affect the human health properties of cow's milk?', *Trends in Food Science & Technology,* 17, pp. 423-437.

Michalski, M. C., Michel, F., Sainmont, D. and Briard, V. (2002) 'Apparent ζ-potential as a tool to assess mechanical damages to the milk fat globule membrane', *Colloids and Surfaces B: Biointerfaces,* 23(1), pp. 23-30.

Miled, N., Canaan, S., Dupuis, L., Roussel, A., Rivière, M., Carrière, F., de Caro, A., Cambillau, C. and Verger, R. (2000) 'Digestive lipases: From three-dimensional structure to physiology', *Biochimie,* 82(11), pp. 973-986.

Mitchell, D. J., McClure, B. G. and Tubman, T. R. J. (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), pp. 273-276.

Monks, J. and McManaman, J. L. (2013) 'Secretion and fluid transport mechanisms in the mammary gland', in Zibadi, S., Watson, R.R. & Preedy, V.R. (eds.) *Handbook of dietary and nutritional aspects of human breast milk*. Wageningen, The Netherlands: Wageningen Academic Publishers, pp. 35-56.

Montagne, D. H., Dael, P. V., Skanderby, M. and Hugelshofer, W. (2009) 'Infant Formulae-Powders and Liquids', in Tamime, A. (ed.) *Dairy powders and concentrated milk products*. UK: Wiley-Blackwell, pp. 294-331.

Moreno, F. J., Mackie, A. R. and Mills, E. N. C. (2005) 'Phospholipid Interactions Protect the Milk Allergen α-Lactalbumin from Proteolysis during in Vitro Digestion', *Journal of Agricultural and Food Chemistry,* 53(25), pp. 9810-9816.

Mukherjee, M. (2003) 'Human digestive and metabolic lipases—a brief review', *Journal of Molecular Catalysis B: Enzymatic,* 22, pp. 369-376.

Mulder, H. and Walstra, P. (1974) *The milk fat globule : emulsion science as applied to milk products and comparable foods / [by] H. Mulder, and P. Walstra ; [translated from the Dutch ms.]. Technical communication of the Commonwealth Bureau of Dairy Science and Technology ; no. 4*: Slough : Commonwealth Agricultural Bureaux, 1974.

Newton, J. L. (2004) 'Changes in upper gastrointestinal physiology with age', *Mechanisms of Ageing and Development,* 125, pp. 867-870.

Norman, A., Strandvik, B. and Ojamäe, Ö. (1972) 'Bile acids and pancreatic enzymes during absorption in the newborn', *Acta Pædiatrica,* 61(5), pp. 571-576.

Olivecrona, T. and Bengtsson, G. (1984) 'Lipases in milk', in Borgström, B. & Brockman, H.L. (eds.) *Lipases*. Amsterdam, The Netherlands: Elsevier, pp. 205-261.

Ollivier-Bousquet, M. (2002) 'Milk lipid and protein traffic in mammary epithelial cells: Joint and independent pathways', *Reproduction Nutrition Development,* 42(2), pp. 149-162.

Oosting, A., Kegler, D., Wopereis, H. J., Teller, I. C., Bj, Verkade, H. J. and Em (2012) 'Size and phospholipid coating of lipid droplets in the diet of young mice modify body fat accumulation in adulthood', *Pediatric Research,* 72(4), pp. 362-369.

Osano, J. (2010) *Emulsifying properties of a novel polysaccharide extracted from the seeds of basil (Ocimum basilicum L.)* Master of Technology in Food Technology, Massey University, Palmerston North, New Zealand.

Packard, V. S. (1982) *Human and infant formula.* New York: Academic Press, p. 269.

Pal, A., Indireshkumar, K., Schwizer, W., Abrahamsson, B., Fried, M. and Brasseur, J. G. (2004) 'Gastric flow and mixing studied using computer simulation', *Proceedings. Biological sciences / The Royal Society,* 271(1557), pp. 2587-2594.

Paul Van der, M., Jan, C. and Jan, V. (2004) 'Surface Charge Analysis', *Handbook of Food Analysis, Second Edition -3 Volume Set Food Science and Technology*: CRC Press, pp. 1825-1836.

Pearson, J. P. and Hutton, D. 2005. Gastrointestinal Tract:Structure and Function of the Stomach *Encyclopedia of Human Nutrition.*

Poulsen, K. R., Snabe, T., Petersen, E. I., Fojan, P., Neves-Petersen, M. T., Wimmer, R. and Petersen, S. B. (2005) 'Quantization of pH: evidence for acidic activity of triglyceride lipases', *Biochemistry,* 44(34), pp. 11574-80.

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

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

Raikos, V., Kapolos, J., Farmakis, L., Koliadima, A. and Karaiskakis, G. (2009) 'The use of sedimentation field-flow fractionation in the size characterization of bovine milk fat globules as affected by heat treatment', *Food Research International,* 42, pp. 659–665.

Ramírez, M., Amate, L. and Gil, A. (2001) 'Absorption and distribution of dietary fatty acids from different sources', *Early Human Development,* 65(SUPPL. 2), pp. S95-S101.

Reis, P., Holmberg, K., Watzke, H., Leser, M. E. and Miller, R. (2009) 'Lipases at interfaces: A review', *Advances in Colloid and Interface Science,* 147–148(0), pp. 237-250.

Richmond, V., Tang, J., Wolf, S., Trucco, R. E. and Caputto, R. (1958) 'Chromatographic isolation of gastricsin, the proteolytic enzyme from gastric juice with pH optimum 3.2', *Biochimica et Biophysica Acta,* 29(2), pp. 453-454.

Rogers, I. S., Emmett, P. M. and Golding, J. (1997) 'The growth and nutritional status of the breast-fed infant', *Early Human Development,* 49(Suppl.), pp. S157-S174.

Roman, C., Carriere, F., Villeneuve, P., Pina, M., Millet, V., Simeoni, U. and 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), pp. 83-88.

Roussel, A., Canaan, S., Egloff, M. P., Rivière, M., Dupuis, L., Verger, R. and Cambillau, C. (1999) 'Crystal structure of human gastric lipase and model of lysosomal acid lipase, two lipolytic enzymes of medical interest', *Journal of Biological Chemistry,* 274(24), pp. 16995-17002.

Ruby, M. V., Davis, A., Link, T. E., Schoof, R., Chaney, R. L., Freeman, G. B. and 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), pp. 2870-2877.

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

Rumsey, R. D. E. 2005. Gastrointestinal Tract: Structure and Function of the Small Intestine. *Encyclopedia of Human Nutrition.*

Sarkar, A. (2010) *Behaviour of milk protein-stabilized oil-in-water emulsions in simulated physiological fluids.* Massey University, Palmerston North, New Zealand.

Sarkar, A., Goh, K. K. T., Singh, R. P. and Singh, H. (2009) 'Colloidal stability and interactions of milk-protein-stabilized emulsions in an artificial saliva', *Food Hydrocolloids,* 23, pp. 1563-1569.

Schubert, H. (1993) 'Instantization of powdered food products', *International Journal of Chemical Engineering,* 33, pp. 28-45.

Serpero, L. D., Frigiola, A. and Gazzolo, D. (2012) 'Human milk and formulae: Neurotrophic and new biological factors', *Early Human Development,* 88, Supplement 1(0), pp. S9-S12.

Sharma, A., Jana, A. H. and Chavan, R. S. (2012) 'Functionality of milk powders and milk-based powders for end use applications-A review.', *Comprehensive Reviews in Food Science and Food Safety,* 11, pp. 518-528.

Shi, Y. and Burn, P. (2004) 'Lipid metabolic enzymes: Emerging drug targets for the treatment of obesity', *Nature Reviews Drug Discovery,* 3, pp. 695-710.

Shields, B. (2003) 'Principles of Newborn and Infant Drug Therapy', in Kenner, C. & Lott, J.W. (eds.) *Comprehensive Neonatal Nursing: A Physiologic.* 3 ed. USA: Elsevier Science.

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

Splinter, W. M. and Schreiner, M. S. (1999) 'Preoperative Fasting in Children', *Anesth Analg,* 89, pp. 80-89.

Srinivasan, M., Singh, H. and Munro, P. A. (2000) 'The effect of sodium chloride on the formation and stability of sodium caseinate emulsions', *Food hydrocolloids-Oxford,* 14(5), pp. 497-507.

Storti, F. and Balsamo, F. (2010) 'Particle size distributions by laser diffraction: sensitivity of granular matter strength to analytical operating procedures', *Solid Earth,* (1), pp. 25.

Thiebaud, M., Dumay, E., Picart, L., Guiraud, J. P. and Cheftel, J. C. (2003) 'High-pressure homogenisation of rawbovine milk. Effects on fat globule size

distribution and microbial inactivation', *International Dairy Journal,* 13, pp. 427-439.

Thomas, N., Holm, R., Rades, T. and Müllertz, A. (2012) 'Characterising lipid lipolysis and its implication in lipid-based formulation development', *AAPS Journal,* 14(4), pp. 860-871.

Thompkinson, D. K. and Kharb, S. (2007) 'Aspects of Infant Food Formulation', *Comprehensive Reviews in Food Science and Food Safety,* 6(4), pp. 79-102.

Thompson, J. M. (ed.) (1998) *Nutritional requirements of infants and young children practical guidelines*. Oxford: Blackwell Science.

Uauy, R., Hoffman, D. R., Mena, P., Llanos, A. and 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 Suppl), pp. S17-S25.

Untersmayr, E. and Jensen-Jarolim, E. (2008) 'The role of protein digestibility and antacids on food allergy outcomes', *Journal allergy and clinical immunology,* 121(1301-1308).

Valivullah, H. M., Bevan, D. R., Peat, A. and Keenan, T. W. (1988) 'Milk Lipid Globules: Control of Their Size Distribution', *Proceedings of the National Academy of Sciences of the United States of America,* (23), pp. 8775.

van Aken, G. A. (2003) 'Competitive adsorption of protein and surfactants in highly concentrated emulsions: effect on coalescence mechanisms', *Colloids and Surfaces A: Physicochemical and Engineering Aspects,* 213(2–3), pp. 209-219.

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

van der Ven, C., Gruppen, H., de Bont, D. B. A. and Voragen, A. G. J. (2001) 'Emulsion Properties of Casein and Whey Protein Hydrolysates and the Relation with Other Hydrolysate Characteristics', *Journal of Agricultural and Food Chemistry,* 49(10), pp. 5005-5012.

Vanderghem, C., Bodson, P., Danthine, S., Paquot, M., Deroanne, C. and Blecker, C. (2010) 'Milk fat globule membrane and buttermilks: from composition to valorization', *Biotechnologie, Agronomie, Société et Environnement,* 14(3), pp. 485-500.

Verger, R. 1980. Enzyme kinetics of lipolysis.

Versantvoort, C. H. M., Kamp, E. v. d. and Rompelberg, C. J. M. 2004. Development and applicability of an in vitro digestion model in assessing the bioaccessibility of contaminants from food.

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

Wabel, C. T. (1998) *Influence of Lecithin on Structure and Stability of Parenteral Fat Emulsions.* Dissertation, Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany [Online] Available at: http://www2.chemie.uni-erlangen.de/services/dissonline/data/dissertation/Christoph_Wabel/html/ (Accessed.

Wade, T. and Beattie, J. K. (1997) 'Electroacoustic determination of size and zeta potential of fat globules in milk and cream emulsions', *Colloids and Surfaces B: Biointerfaces,* 10, pp. 73-85.

Walstra, P. (1995) 'Physical chemistry of milk fat globules', in Fox, P.F. (ed.) *Advanced Dairy Chemistry-2: Lipids*. London: Chapman & Hall, pp. 131-178.

Dairy Technology: Principles of Milk Properties and Processes (1999). Marcel Dekker.

Wells, J. C. K. (1996) 'Nutritional considerations in infant formula design', *Semin Neonatol,* 1, pp. 19-26.

Wiking, L., Graef, V. D., Rasmussen, M. and Dewettinck, K. (2009) 'Relations between crystallisation mechanisms and microstructure of milk fat', *International Dairy Journal,* 19, pp. 424-430.

Wright, K., Coverston, C., Tiedeman, M. and Abegglen, J. A. (2006) 'Formula supplemented with docosahexaenoic acid (DHA) and arachidonic acid (ARA): a critical review of the research', *Journal for Specialists in Pediatric Nursing,* 11(2), pp. 100-112.

Yao, N. and Wang, Z. L. (2005) *Handbook of Microscopy for Nanotechnology.* Boston: Kluwer Academic Publishers.

Ye, A., Anema, S. G. and Singh, H. (2007) 'Behaviour of homogenized fat globules during the spray drying of whole milk', *International Dairy Journal,* 17, pp. 374-382.

Ye, A., Cui, J. and Singh, H. (2010) 'Effect of the fat globule membrane on in vitro digestion of milk fat globules with pancreatic lipase', *International Dairy Journal*.

Yu, Z., Palkovicova, L., Drobna, B., Petrik, J., Kocan, A., Trnovec, T. and Hertz-Picciotto, I. (2007) 'Comparison of organochlorine compound concentrations in colostrum and mature milk', *Chemosphere,* 66, pp. 1012–1018.

Zoppi, G., Andreotti, G., Pajno-Ferrara, F., Njai, D. M. and Gaburro, D. (1972) 'Exocrine Pancreas Function in Premature and Full Term Neonates', *Pediatr Res,* 6(12), pp. 880-886.