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DIGESTIBILITY AND STRUCTURAL CHANGES OF INGREDIENTS IN INFANT FORMULAE DURING THE GASTROINTESTINAL DIGESTION

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Abstract

Although mothers' milk is the ideal food for babies, infant formula has become an alternative when breastfeeding is not possible or inadequate for babies. To design a proper formula for babies, it is essential to understand the digestibility of macronutrients and their bio-accessibility in the infant gastrointestinal tract. Because i*n vivo* gastrointestinal studies on human infants are restricted by ethical constraint, cost issues, and intensive resource, *in vitro* models could be a better replacement. I*n vitro* models offer advantages with low cost, easy sampling accessibility and no ethical issues. This thesis aims to assess the digestibility of each ingredient proteins, lipids, and carbohydrates in infant formulation then compare with mothers' milk. A static bench-top *in vitro* model for infant digestion was set up with infant gastric pH (4.0-4.5) and the activity of simulated digestive enzymes suitable for human infants with 60 minutes of gastric phase and 120 minutes of intestinal phase.

Popular protein sources of caseins, whey, and soy proteins were employed in infant formulations. The *in vitro* digestion of these proteins in infant formulations was studied in the presence of enzyme proteases only (without lipolytic enzymes). Obtained results showed around 20% of caseins and no components of whey were hydrolysed after 60 minutes in the simulated stomach. In the simulated intestinal phase, 8% of α–lactalbumin was hydrolysed while caseins and β–lactoglobulin were completely digested immediately and 30 minutes respectively after addition of intestinal digestive proteases. Overall, soy proteins indicated lower level of hydrolysis than dairy proteins during *in vitro* infant digestion as observed by SDS-PAGE. The soy protein fractions glycinin and β-conglycinin were partially hydrolysed during the gastrointestinal phase. The observed pH drop confirms that caseins are easily digested in the intestinal phase compared to whey and soy protein. Gastric digestion resulted in a decrease of the particle size of protein aggregates, but no fat coalescence was observed during both gastric and intestinal digestion in the given conditions.

The *in vitro* digestion of hydrolysed and non-hydrolysed dairy (casein and whey proteins) was studied under conditions without lipolytic enzymes. Results show hydrolysed proteins were completely digested in the small intestine while non-hydrolysed proteins (caseins, α-lactalbumin, β-lactoglobulin, conglycinin, glycinin) were only partially digested in the simulated gastrointestinal tract. Although observed pH-drop for non-hydrolysed protein formulations was lower, significantly higher levels of ninhydrin-reactive amino nitrogen in hydrolysed proteins suggested higher digestibility of hydrolysed proteins than their non-hydrolysed counterparts. Only formulations containing caseins showed a

decrease in particle size of protein aggregates during gastric digestion. No fat globule coalescence was observed during both gastric and intestinal digestions in the given conditions.

Lipid digestion of infant formula emulsions based on both hydrolysed and non-hydrolysed proteins (dairy and soy) with vegetable oils was studied under an *in vitro* gastrointestinal environment (with and without proteases). The size and distribution of oil droplets, released free fatty acids, and micro-structure of the digesta were monitored over the digestion period. Oil droplet coalescence was observed during gastric phase but not in the intestinal phase for most of formulations in both the matrices. Higher rate of lipolysis in infant formula emulsion stabilized by hydrolysed proteins was noted. The obtained results suggested that digestive proteases had a limited impact on lipolysis of these particular infant formula systems.

The *in vitro* digestion of carbohydrate in infant formulations and control formulations (solution of carbohydrate without proteins and vegetable oils) suggests infant formulations with precooked starch or locust bean gum have a higher viscosity than other formulation without thickening agents. No carbohydrate was digested in stomach phase. Precooked starch is well digested in the simulated intestine, but locust bean gum in infant formula resisted *in vitro* digestion. Higher amount of released glucose were observed in the digesta of the formulations with lactose than in the formulations with glucose syrup.

The *in vitro* digestion of mothers' milk and infant formulation based on bovine proteins and vegetable oils in the presence of all the digestive enzymes showed caseins digested quicker than whey proteins in the gastrointestinal tract. Lipolysis of mothers' milk releases free fatty acids with medium carbon chain from C_{10} to C_{14}, which are very little in infant formulation. However, similar amount of total free fatty acids was obtained from the digestion of the fat in mothers' milk and in the infant formulation. Lactose in mothers' milk or in infant milk formulae behaved the same in the *in vitro* infant digestion as the same type of lactose was used which is in water soluble state without any effect of pH, thus is easily accessible to enzyme.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Keywords

in vitro digestibility, caseins; whey proteins; soy protein isolate; proteolysis; lipolysis, confocal microscopy; particle size, free fatty acids, glucose.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATION USED IN THE THESIS

A_{253}-absorption at wavelength 253nm

A_{280}- absorption at wavelength 280nm

AA-arachidonic acid

ANOVA- Analysis of Variance

ATP-adenosine triphosphate

BAEE-Nα-Benzoyl-L-arginine Ethyl Ester

BCAA-branched-chain amino acids

BSSL-bile salt simulated lipase

BTEE-N-Benzoyl-L-Tyrosine Ethyl Ester

CC-calcium caseinate

CLSM-Confocal Laser Scanning Microscopy

DHA-docosahexanenoic acid

EC-Enzyme Commission

eHF-Extensively hydrolysed formula

FFA-free fatty acid

FID-flame-ionization detector

GOS-galactose-oligosaccharides

HCP-hydrolysed casein protein

HK assay-Hexokinase/glucose-6-phosphate-dehydrogenase assay

HSPI-hydrolysed soy protein isolate

HWP-hydrolysed whey protein

LA- linoleic acids

LBG-Locust bean gum

LCPUFA-long-chain polyunsaturated fatty acid

MFGM-milk fat globule membrane

PDCAAS-Protein Digestibility Corrected Amino Acid Scores

pHF-Partially hydrolysed formula

PLA2-phospholipase A2

PLRP 1, 2-pancreatic related to protein 1,2

PTL-pancreatic triglyceride lipase

SDS-PAGE- Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SPI-soy protein isolate

TCA-Trichloroacetic acid

WPI- whey protein isolate

CHAPTER 1 GENERAL INTRODUCTION

1.1 Overview

Mothers' milk is well known the perfect food for human infants as it provides the ideal nutrition for infants' growth and development. With the well-balanced nourishment, growth factors, and immune components that have valuable impacts on infants' digestion, immune system, and cognitive development, mothers' milk is recommended for infants at least up to the age of 6 months (Hernell, 2011; Lönnerdal, 2013). However, when mothers' milk is not available, infant formula becomes the best second option. Most of the infant formulas are based on bovine milk or soymilk (Martin, Ling, & Blackburn, 2016). Due to the differences in composition of proteins, fats, and carbohydrates between human milk, bovine milk, and soymilk, infant formula has been designed to be closer to mothers' milk as much as possible (Goedhart & Bindels, 1994; Martin et al., 2016). Many attempts has been done to increase the quality of infant formula such as: the whey:casein ratio in infant formula has been adjusted according to mothers' milk, α-lactalbumin and lactoferrin has been added to improve the amino acid profile and enhance the immune effect, a mixture of vegetable oils has been used as a lipid source in infant formula (Lien, 2003; Lönnerdal, 2014; Martin et al., 2016). Therefore, there is a need to study the digestibility of the various ingredients supplemented in infant formula.

To understand the digestibility, structural changes, and kinetics of food under a closely simulated physiological conditions in the human gastrointestinal tract, *in vitro* digestion models have been applied (Hur, Lim, Decker, & McClements, 2011). It is clear that *in vitro* models cannot imitate fully the complicated digestion process in the human gut, especially the composition and subsequent digestive secretion, digestion and absorption, and the interaction between the host, the food, and micro-bacteria in the digestive system (Coles, Moughan, & Darragh, 2005). However, *in vitro* models are able to offer great advantages compared to in vivo models with no ethical issues, low cost, and easy sampling accessibility (Sopade & Gidley, 2009). Both dynamic and static models have been employed in *in vitro* digestion using simulated gastrointestinal fluids including enzymes, bile salts, and other surfactants. The main advantages of the dynamic models are they can imitate the dynamic digestion in the human gastrointestinal tract such as the gastric emptying, peristaltic movements, pH change in the stomach, enzyme, and fluid secretion (Guerra et al., 2012). However, the dynamic models are costly equipment and require a complex operation; meanwhile static models are simple,

low cost, and easy to run. Also, there are some differences between the physical gastrointestinal digestion of adults and infants that need to be taken into account when setting up an *in vitro* digestion models for infants. Liquid milk are the main food for babies aged 0-6 months so the digestion does not start at the oral phase due to the very short transit time through the mouth, pharynx and oesophagus (10-15 s) (Arvedson & Brodsky, 2002). The digestion of infants primarily happens in the gastric and intestinal phases. Also, the availability of some digestive enzymes, their concentration, and gastric pH are different between infants and adults (Bourlieu et al., 2014).

1.2 Objectives

It is clear that there are requirements to understand the digestion of baby food via the *in vitro* models, but limited systematic studies focus on the digestion and microstructure changes of all the ingredients in infant formula during their passage through the simulated infant digestive tract. This study aimed to assess the digestibility of each ingredient in infant formulation then compare with mothers' milk. For this purpose, the thesis has been divided into 6 research chapters as follows:

1. Develop and validate a simple bench-top digestion unit for the routine investigation of *in vitro* infant digestion experiments (chapter 3).

2. Assess and compare the digestibility of non-hydrolysed dairy proteins and soy proteins which are used infant formulas under the *in vitro* infant digestion model (chapter 4)

3. Assess and compare the digestibility of hydrolysed dairy proteins and hydrolysed proteins which are used infant formulas under the *in vitro* infant digestion model (chapter 5)

4. Study the *in vitro* lipid digestion of infant formulation under the effect of protease hydrolysis (chapter 6).

5. Assess and compare the digestibility of different carbohydrate sources: lactose, corn starch, locust bean gum (carob bean gum) and glucose syrup which are commonly added to infant formula (chapter 7)

6. Compare the digestibility of all the ingredients in mothers' milk with infant formulations based on bovine milk and soymilk under the presence of all the digestive enzymes (chapter 8).

1.3. References

Arvedson, J. C., & Brodsky, L. (2002). Pediatric swallowing and feeding: Assessment and management. Clifton Park, NY Delmar Cengage Learning.

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

Coles, L., Moughan, P., & Darragh, A. (2005). In vitro digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals. *Animal Feed Science and Technology*, 123, 421-444.

Goedhart, A. C., & Bindels, J. G. (1994). The composition of human milk as a model for the design of infant formulas: recent findings and possible applications. *Nutrition Research Reviews*, 7, 1-24.

Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., & Alric, M. (2012). Relevance and challenges in modeling human gastric and small intestinal digestion. Trends in Biotechnology, 30(11), 591-600.

Hernell, O. (2011). Human milk vs. cow's milk and the evolution of infant formulas. Paper presented at the Milk and Milk Products in Human Nutrition, Copenhagen.

Hur, S. J., Lim, B. O., Decker, E. A., & McClements, D. J. (2011). In vitro human digestion models for food applications. *Food Chemistry*, 125(1), 1-12.

Lien, E. L. (2003). Infant formulas with increased concentrations of α-lactalbumin. *The American Journal of Clinical Nutrition*, 77(6), 1555S-1558S.

Lönnerdal, B. (2013). Bioactive proteins in breast milk. Journal of Paediatrics and Child Health, 49, 1-7. doi: 10.1111/jpc.12104

Lönnerdal, B. (2014). Infant formula and infant nutrition: bioactive proteins of human milk and implications for composition of infant formulas. *The American Journal of Clinical Nutrition*, 99(3), 712S-717S.

Martin, C. R., Ling, P.-R., & Blackburn, G. L. (2016). Review of infant feeding: key features of breast milk and infant formula. *Nutrients*, 8(5), 279.

Sopade, P. A., & Gidley, M. J. (2009). A rapid In-vitro digestibility assay based on glucometry for investigating kinetics of starch digestion. *Starch-Stärke*, 61(5), 245-255.

CHAPTER 2 LITERATURE REVIEW

2.1. Introduction

Infants are the people under the age of 12 months and infant formula is the product presented as mothers' milk substitute, which satisfies the nutritional requirements of infants up to four to six months of age (Australian Government, 2000). Mothers' milk is the best food for adequate growth and development of infants as it contains a balance of essential nutrients and specific bioactive components such as growth factors, immune factors, enzymes etc. that are explicitly available only in mothers' milk (Alles, Scholtens, and Bindels, 2004). Infant formula forms a substitute only when breast milk is inadequate or ceases for some reason. At present, due to the advances in food technology and engineering, the main targets of current infant formula have been supposedly met from the point of view of safety for infants and the composition in macro-nutrients (protein, fat, and carbohydrates) and micro-nutrients (vitamins and minerals) comparable to mothers' milk (Hernell, 2011). However, there can be differences in outcomes in growth and development patterns between breast-fed infants and formula-fed infants in both the short and long term. For instance, infants who are fed with infant formula gain weight faster and have more body fat from 3 months of age; have different gut microbiota; and also have higher concentration of serum amino acids, insulin, blood urea nitrogen compared to breast-fed infants. These factors are related to higher risk of obesity, diabetes, and cardiovascular disease (Lönnerdal, 2014). Ideally, both breast-fed and formula-fed infants should show similar growth and development patterns (Lönnerdal, 2014). To achieve this goal, modifications of nutrients in infant formula with clinical trials are being carried out (Lönnerdal, 2014). Alongside this, there is a need to study the digestibility of various ingredients supplemented in infant formula to better understand the degradation mechanism of these components as well as the bio-accessibility of the digested nutrients in the gastrointestinal tract. Application of *in vitro* models to simulate digestion through the gastrointestinal tract has become widely more popular than obtaining data from *in vivo* experiments due to no ethical restrictions, low cost, and less time requirements. The *in vitro* models help observe the digestibility, structural changes, and the release of nutrients under simulated gastrointestinal digestion (Hur, Lim, Decker, & McClements, 2011; Kamstrup, Berthelsen, Sassene, Selen, & Müllertz, 2016).

2.2. Digestion in infants with comparison to adults

Mothers' milk and infant formula, the main food for infants, are a rich source of proteins, fats and carbohydrates. The digestion of these ingredients provides the essential nutrients for the growth and development of babies. The knowledge of infant gastrointestinal function plays an important role in infant feeding application and has advanced rapidly over the past few decades (Friedt and Welsch, 2013; Lebenthal, Lee, and Heitlinger, 1983).

Digestion process in infants aged between 0-6 months who exclusively consume liquid milk does not happen at oral phase due to the very short transit time through mouth, pharynx and oesophagus (10-15 seconds) (Arvedson and Brodsky 2002). Therefore, infant digestion of macronutrients mainly occur in gastric and intestinal phases. Although it is clear that the gastrointestinal system is quite mature in full-term newborns (newborns are human infants in the first 28 days of life, WHO), the availability of some digestive enzymes, their concentration, and gastric pH are different between infants and adults (Bourlieu et al., 2014; Poquet & Wooster, 2016). The digestive enzymes are salivary amylase secreted by salivary gland, pepsin and gastric lipase secreted by human gastric mucosa, pancreatic enzymes, and brush border mucosal enzymes (Hamosh, 1996; Moreau, Laugier, Gargouri, Ferrato, and Verger, 1988). The pancreatic enzymes contain proteases (trypsin, chymotrypsins, etalastase, carboxypeptidases), lipases (colipase-dependent lipase, carboxyester lipase, pancreatic lipase related proteins, bile salt dependent lipase). Brush border mucosal enzymes contain lactase, glucoamylase, sucrase, isomaltase which hydrolyse carbohydrates (Hamosh, 1996). Table 1 summarises and compares the activities of the digestive enzymes found in the gastrointestinal tract of both adults and infants.

Infant gastric pH is less acidic compared to adults (Fig 2.1 A and 2.1 B). It has been reported that gastric pH in pre-term infant varied from 3.2 to 3.5 before feeding and raised to 6.0-6.5 immediately after having a meal (Bourlieu et al., 2014). In an earlier study Nagita et al. (1996) observed a gastric pH of 3.0-4.0 in newborns (under 28 days old) and 1.5-3.0 in infants (under 12 months old) during fasting. Fig 2.1A shows that the pH in infant's stomach increases from 3.5 to 6.4 before and after 30 minutes of feeding with mothers' milk and then decreases to above pH 3 after 180 min of gastric digestion (Roman et al., 2007; Mason, 1962). Cavell (1983) also observed a decrease in pH of infant gastric content 6.0 (after 30 minutes of feeding) and further decreased to pH 5.2 (after two hours of feeding). The corresponding pH figures in adult stomach is 1.5-1.8 (Mitchell, McClure, and Tubman,

2001; Shani-Levi, Levi-Tal, and Lesmes, 2013). Thus based on the above study it is clear that after two hours of feeding, gastric pH in the infant stomach remains between 4-5, while the pH for adults is lower than 2 which has also been reported by Li-Chan and Nakai, (1989).

Table 2.1 Gastrointestinal enzymes in infants and their activity compared to adults. Adapted from Lebenthal et al.(1983), Hamosh (1996), and Lindquist & Hernell (2010)

Enzymes	Contribution to infant digestion	Activity (% of adult)
Protein digestion		
Pepsin	Low	<10
Trypsin	Adequate	10-60
Chymotrypsin	Adequate	10-60
Elastases	Low	NA
Carboxypeptidases (A and B)	Adequate	NA
Lipid digestion		NA
Gastric lipase	Important	100
Pancreatic triglyceride lipase	Low	5-10
Bile salt dependant lipase	Moderate	NA
Pancreatic lipase-related to protein 2	Important	NA
Carbohydrate digestion		NA
Salivary α-amylase	Moderate	10
Pancreatic α-amylase	Absent in infants < 6 months	0
Glucoamylase	High	50-100
Lactase	High	>100
Sucrase-Isomaltase	High	100

NA: not availble

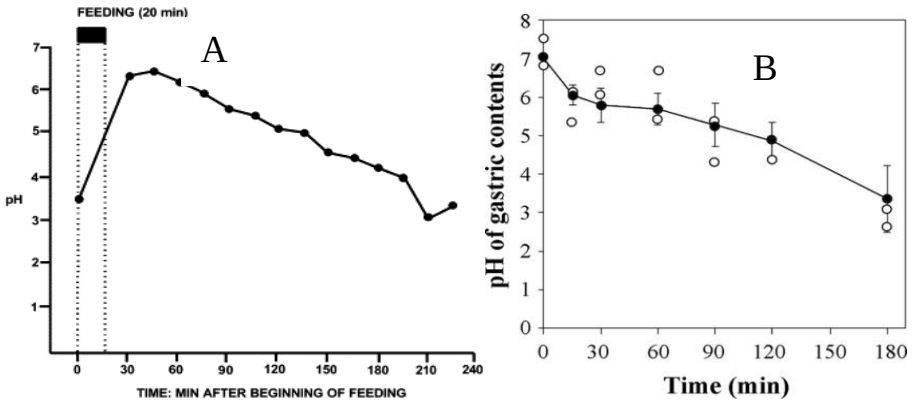


Fig 2.1 Gastric pH during feeding of infants - mean values of pH of the stomach contents A (as presented in Chatterton et al., 2004) and B (as presented in Roman et al., 2007).

Table 2 summarises the pH change after one hour of feeding for infants of different ages. In the intestinal phase, both adults and infants have similar pH in the small intestine (Andrea, and Nikoletta, 2010).

Table 2.2 Gastric pH of 39 infants (one hour after feeding). Adapted from (Miller, 1942)

Age in month	Number of babies	pH range	pH average
2-3	8	4.6-5.2	4.7
4-6	19	3.5-5.5	4.4
7-9	11	4.0-5.2	4.5
12	1	3.8	3.8

2.2.1. Digestion of proteins in infants

Digestion of proteins in infants involve proteases in the stomach, luminal proteases and brush border peptidases in the small intestine (Dallas, Underwood, Zivkovic, and German, 2012). The gastric and intestinal digestion of proteins is described in the following sections.

2.2.1.1. Gastric proteolysis

Pepsin is the protease responsible for digestion of protein in the stomach at an optimal pH 2. In full-term-infants, the high gastric pH and low output of pepsin restricts digestion of milk protein in the infant stomach compared to that in adults (Mason, 1962). The infants' gastric pH is higher than the optimal pH required for secretion of the pepsin enzyme (Hamosh, 1996) and this results in minimal

protein hydrolysis in the stomach of babies below 3 months of age because of very low pepsin secretion and high gastric pH, (Agunod, Yamaguchi, Lopez, and Glass, 1969). Berfenstam, Jagenburg, and Mellander (1955) also detected only traces of hydrolysed protein in the stomach of newborn infants. Conversely, full-term-infants from 3 months of age can have a level of pepsin similar to that of older children and adults while pre-term infants have only 50% of the pepsin level found in full-term infants (DiPalma et al., 1991).

In stomach of newborns within 6-8 hours of postpartum, Henschel, Newport, and Parmar (1987) detected a protease of highly hydrolysed milk protein that resembles chymosin found in calf. However, this protease disappears from the gastric fluid at 10 days of postpartum, and is not found in adult gastric fluid (Dallas et al., 2012). In their researches, Holton et al. (2014) and Dallas et al. (2012, 2014) used peptidomic analysis to study *in vivo* proteolysis of mother's milk in infant stomach. They compared the activity of protease in mother's milk before and after 2 hours of ingestion and detected a significantly higher level of peptides in digested samples than in mother's milk. It is likely that proteases from mother's milk continue to be active in infant stomach and is responsible for protein hydrolysis not the gastric proteases secreted in the infant stomach. To understand gastric protein hydrolysis, more thorough studies are required to be done.

2.2.1.2. Intestinal proteolysis

Following digestion in the stomach by pepsin, the protein is further hydrolysed into peptides by pancreatic proteases (trypsin, α-chymotrypsin, elastase, peptidases, carboxypeptidases A, and carboxypeptidases B) in the intestine (Boisen and Eggum, 1991). The peptides are further broken down by peptidases in the intestinal brush border. Trypsin is the most vital digestive proteases and accounts for up to 20% of the protein in pancreatic fluids (Hamosh, 1996). Borgstrom, Lindquist, and Lundh (1960) suggested both pre-term and full-term infants have similar concentrations of trypsin as in adults, while the levels of chymotrypsins and carboxypeptidases B just account for about 10% to 60% of the activity present in adults (Lebenthal and Lee, 1980a).

It is widely accepted that brush border and cytosolic peptidases (excluding amino-peptidases) completely hydrolyse peptides into amino acids, even in premature infants (Auricchio, Stellato, and De Vizia, 1981). As per Lebenthal, Lee & Heitlinger (1983) (cited in Hiranta & Matusuo, 1969), ten-

day-old babies can absolutely digest and absorb 1.3% cow milk protein and four to six-month-old babies can absolutely digest and absorb 2.5% cow milk protein.

2.2.2. Digestion of lipids in infants

Lipids account for around half of the total energy content in breast milk and formulas and contain *n-6* and *n-3* fatty acids such as linoleic acid (C18:2, *n-6*) and α-linoleic acid (C18:3, *n-3*) crucial for brain and eye development of infants (Hermoso et al., 2010, Joeckel and Phillips, 2009). They are the transporters of essential fat-soluble vitamins. Thus, adequate digestion and absorption of dietary fats in infants is paramount. The major difference in lipid digestion and absorption between infants and adults is the lipid intake per kilogram of bodyweight, which is much higher (three to five times) in infants than adults (Andersson, Hernell, Bläckberg, Fält, and Lindquist, 2011). Also, the activity and function of digestive lipases varies between infants and adults.

2.2.2.1. Gastric lipolysis

Gastric lipolysis plays a more important role in fat digestion in infants than in adults. Enzyme gastric lipase digests the milk fat in the infant diet. It is well known that both lingual lipase and gastric lipase are present in rodent infants (Hamosh, 1990 and Hamosh, 1994). However, so far there is no evidence of existence of lingual lipase in humans (N'Goma et al., 2012; Moreau et al., 1988). Gastric lipase is active over a wide range of pH levels (1.5-7.0), does not require bile salts as the cofactor, is not inhibited by milk fat globule membranes (Ville, Carrière, Renou, Laugier, 2002; Hamosh, 1996; Hernell et al., 1988) and is capable of hydrolysing the triglyceride within milk fat globules (Bourlieu et al., 2015; Bernbäck, Bläckberg, and Hernell 1990; Plucinski, Hamosh; Hamosh, 1979; Cohen, Morgan, and Hofmann, 1971). Conversely pancreatic triglyceride lipase and bile salt stimulated lipase cannot hydrolyse the core of triglycerides because of their inability to penetrate into milk fat globules (Roman et al., 2007; Cohen, Morgan, & Hofmann, 1971). Thus, gastric lipase is able to act properly in the infant stomach. Besides, fatty acids produced in gastric phase encourages the activity of pancreatic lipase due to the better interface between fat globules and the aqueous environment (Bernbäck, Bläckberg, and Hernell, 1989). Thus, fat hydrolysis in the stomach may quantitatively be more important for infants than in adults (Carey, Small, and Bliss, 1983; Hamosh et al., 1981; Murphy and Signer, 1974).

It has been shown that the level of gastric lipase in infants is similar to the level found in adults (Sarles Moreau, and Verger, 1992). Commare and Tappenden (2007) suggested a rise in gastric lipase activity from 26 and 35 weeks of gestation, which then reaches adult levels when babies are born full term. Some studies (Armand et al., 1996; Armand et al., 1995) also reported gastric lipase activity in full-term infants was much higher than in adults, with four-week-old infants having gastric lipase activity 50% higher than adults' levels. DiPalma et al. (1991) examined the activity of gastric lipase in humans from different age groups (5-19 months, 2-4 years, 6-10 years, 11-13 years, and 15-26 years). They observed the gastric lipase activity to be in the range of 1.8-5.3 U/mg protein (1U is 1 µmol oleic acid released from triolein per minute), and no significant difference in the lipase activity between the studied age groups. The high level of gastric lipase may compensate for the low amount of pancreatic lipases and explains why infants can consume a high dietary fat (Armand et al 1996, Hamosh, 2006). Armand et al (1996) also observed rapid gastric lipolysis of mother's milk compared to infant formula due to the significant amount of lipase present in mothers' milk.

In adults, gastric lipase hydrolyses 10-25% of lipids in the stomach and the remainder fat hydrolysis takes place in the duodenum with the help of pancreatic lipase (Gallier, and Singh, 2012; Hamosh, 1990). In healthy infants, due to the clinical invasive procedures such as the employment of nasogastric and nasoduodenal tubes or the drawing of blood samples, very limited data about physical digestion is known so far. Meanwhile, preterm infants are usually fed via a tube that allows to collect the samples (Abrahamse et al., 2012). It has been reported that up to 25-60% of fat digestion may happen in the stomach of animal infants depending upon species (Abrahamse et al., 2012; Hamosh, 2006). In preterm infants, gastric lipolysis accounted for 25% of fat digestion for mothers' milk and 14% for infant formula (Ruegg and Blanc, 1982). Similar results have been reported by Hamosh, Sivasubramanian, Salzman-Mann, and Hamosh, (1978) and Hernell et al., (1988) who observed a significant hydrolysis of dietary fat in the preterm infant stomach.

Substrate selectivity is also an important function of gastric lipase. Gastric lipase has high specificity to *sn-3* position of the triglycerides (Hamosh, 1996; Hamosh, Iverson, Kirk, and Hamosh, 1994). As a result, long-chain polyunsaturated fatty acids in mother milk and short to medium chain fatty acids in bovine milk are efficiently released in the infant stomach because they are primarily settled at *sn-3* position (Hamosh, 2006). However, an *in vivo* digestion study by Roman et al. (2007) with infant formula enriched with 25% of medium chain triglycerides (octanoic and decanoic acids), shows the profile of released fatty acids was dominated by palmitic acids and oleic acids, not the medium chain

ones. This suggests gastric lipase mainly hydrolyses long chain fatty acids as this enzyme has a higher affinity towards *sn-3* position.

The other important function of gastric lipase is working in conjunction with pancreatic lipases in the duodenum (Carriere, Barrowman, Verger, and Laugier, 1993 and Bernbäck et al., 1989). Gastric lipase can penetrate into the core of milk fat globules (while pancreatic triglyceride lipase and bile salt stimulated lipase cannot) due to its hydrophobic nature and inability to hydrolyse the acyl bond of phospholipids (Bourlieu et al., 2015). Hence, pancreatic triglyceride lipase and bile salt stimulated lipase uses partially hydrolysed milk fat globules from the stomach as the substrate to perform its activity (Hamosh, 1996; Bernbäck, Bläckberg, & Hernell, 1990).

2.2.2.2. Intestinal lipolysis

Pancreatic triglyceride lipase (PTL), pancreatic lipase-related to protein 2 (PLRP 2), and bile salt-stimulated lipase (BSSL) are the principle lipases involved in the intestinal digestion of lipids. Pancreatic lipase-related to protein 1 (PLRP 1) was detected in the small intestine of human newborns, but has no lipase activity (Berton, Sebban-Kreuzer, Rouvellac, Lopez, and Crenon, 2009; Roussel et al., 1998). Lipids need to be emulsified by bile salts first to enable hydrolysis by pancreatic lipases.

The activity of pancreatic lipases and the concentration of bile salt in infants are very low (Lebenthal et al., 1983; Lindquist and Hernell, 2010) compared to adults. The concentration of pancreatic lipase and bile salts in mature infants are approximately 5-10% and 50% of adults' figures, respectively (Lebenthal et al., 1983), while the corresponding for preterm infants were much lower (Hernell, Blackberg, and Bernback, 1988; Lebenthal et al., 1983).

While in adults, PTL is the principle lipolytic enzyme in the small intestine, PLRP2 and BSSL are predominant during lipid digestion in infants (Andersson et al., 2011; Lindquist & Hernell, 2010). The exact activity of PLRP2 and bile salt-stimulated lipase in infants is still not clear (Andersson et al., 2011). It is believed breast-fed infants are able to digest lipids in the small intestine better than formula-fed infants because of significant activity of BSSL present in mothers' milk (Hamosh, 1996; Formon, Ziegler, Thomas, Jensen, and Filer, 1970).

2.2.3. Digestion of carbohydrates in infants

Carbohydrate intake accounts for 35-55% of total energy in the infant diet. There are three stages of carbohydrate consumption in the early stages of human life starting from newborn to childhood. In the first stage of life, lactose from mothers' milk or formulas is the main source of carbohydrates without any solid food. The next stage introduces the presence of different polysaccharides such as maltodextrin, carob bean gum, guar gum, that are thickening agents added to mother milk and infant formula (Cichero, Nicholson, and September, 2013). The last phase is dominated by polysaccharides with solid food (Lebenthal et al., 1983).

Lactose and sucrose are hydrolysed by lactase and sucrase enzymes into monosaccharide components at birth for full-term infants. Hence, full-term infants are able to digest lactose and sucrose that comes from mothers' milk or infant formulas during the neonatal period. However, a low lactase activity is found in pre-term babies born at 28-34 weeks of gestation while maltase and isomaltase are detected at high levels at that time (70% level of full term). Consequently, infants born at 34 weeks of gestation can well tolerate maltose, sucrose, and isomaltose but not lactose (Lebenthal et al., 1983). However, clinical lactose intolerance is uncommon in preterm infants despite low lactase levels (Patole, 2013).

Polysaccharides need a group of enzymes to complete digestion. The digestion of starch depends on salivary amylase, pancreatic amylase, glucoamylase, maltase, and isomaltase for complete digestion. The salivary and pancreatic amylase are classified as α-amylase. Very low levels of α-amylase are found in the saliva of infants (within the first month), which is less than 25% the amount found in adults. However, due to the lack of pancreatic amylase, salivary amylase contributes to a significant amount of starch digestion in infants (Sibley, 2004). Lebenthal et al. (1983) have found evidence of very low or no α-amylase activity in the duodenal fluid of babies less than 4 months of age. For breast-fed infants, there is a significant supply of α-amylase from mothers' milk. In mothers' milk, the highest activity of α-amylase is in colostrum and declines rapidly during the course of lactation (Dewit, Dibba, and Prentice, 1990).

Glucoamylase (or amyloglucosidase) is a brush border enzyme that can digest starch directly to glucose. In the small intestinal mucosa of newborns and infants, glucoamylase activity has been reported to be above 50% that of adults (Lebenthal et al, 1983). Therefore, although pancreatic amylase is absent in newborn babies, they can digest a reasonable amount of starch because

glucoamylase becomes an alternate enzyme for starch digestion in infants (Lebenthal and Lee, 1980b). Lee, Werlin, Trost, and Struve (2004) examined the activity of enzymes responsible for carbohydrate hydrolysis in 214 subjects aged from 1 month to 20 years including 11 infants and observed no significant difference with age in the activity of these enzymes.

2.3. Difference in composition between mothers' milk and infant formula and their digestibility

Mothers' milk is the most complete food for human infants at least up to the age of 6 months (Agostoni et al., 2008; Eidelman and Feldman-Winter, 2005; World Health Organization, 2003). Mothers' milk provides the ideal nourishment for infants' growth and development because of the well-balanced nutrition, growth factors, and immune components that have beneficial effects on infants' digestion and immune system (Hernell, 2011; Agostoni et al., 2009; Alles, Scholtens, & Bindels, 2004). Table 3 provides a comparison of the major nutrients, their amount, and function present in mothers' milk and bovine milk.

Although the composition of mothers' milk has been reported as being variable during lactation and among mothers (Flack and Shaw, 2003; Goedhart and Bindels, 1994), it is still considered as a guide to establish the composition of infant formulas (O'Callaghan, O'Mahony, Ramanujam, & Burgher, 2011; Floris, Lambers, Alting, and Kiers, 2010; Aggett et al., 2001; Ben, 2008). Most of the infant formulas are based on cow's milk and a minority use soy protein isolate as a protein source. The differences in composition between human milk and bovine milk led to the modification of the infant formula contents, to be closer to human milk as much as possible (Goedhart & Bindels, 1994).

The sections below describe the main components of mothers' milk: proteins, fats and carbohydrates that is taken into consideration while designing infant formulas.

2.3.1. Proteins

Mothers' milk contains a wide range of proteins that play unique roles in the growth and development of infants. Many of them are well digested to provide a balanced source of amino acids, others take responsibility for assisting nutrient digestion and absorption (α-amylase, bile salt simulated lipase, lactoferrin, β-casein, α-lactalbumin), protecting newborns from illness and bacterial infection

(immunoglobulins, lactoferrin, lysozyme, κ-casein, α-lactalbumin, and lactoperoxidase) (Lönnerdal, 2003). The concentratrions and functions of ingredients of mothers' milk is desctibed in table 2.3.

Table 2.3 Function of the principle nutrients of human milk in infants. Adapted from Shah (2000), Haug, Hostmark & Harstad, (2007), Lönnerdal and Darragh (2011), Landers and Hartmann (2013).

Nutrients	**Concentration (g/L)**		**Function**
	Human	**Cow**	
Protein			
Total whey protein	6.7	6.3	
Immunoglobulins (slgA, IgM and IgG)	1.3	0.7	Immune protection
Lactoferrin	1.5	0.1	Anti-infective, iron carrier
α-Lactalbumin	1.9	1.2	Ion carrier (Ca^{2+}), part of lactose synthase
Total caseins	2.7	26	Ion carrier, inhibits microbial adhesion to mucosal membranes
Carbohydrate			
Lactose	67	53	Energy source
Oligosaccharides	0.05-0.2	-	Microbial ligands
Fat	32-36	33	
Triglyceride	97-98%	97%	Energy source

It is well known that protein content in human milk is around 0.8-1.3 g/100 mL (Bosscher et al., 2000; Jensen, 1995), much lower than in cow milk that has about 3.4 g of protein/100 mL (Jensen, 1995). While the ratio between whey and casein in mature mothers' milk is 60:40, the proportion in cow milk is about 20:80 (Hernell, 2011; Gurr, 1981). In addition, the proportions of whey and casein subclasses between the two milks are very different and that is discussed in detail in the below sections.

2.3.1.1. Whey protein

(a) α-lactalbumin: α-lactalbumin is the main protein in human milk and accounts for 41% of whey and 17-28% of total protein. However, in bovine milk α-lactalbumin accounts for only 3.0-3.5% of total protein (Heine, Klein, and Reeds, 1991; Gurr, 1981). Because in human milk, α-lactalbumin accounts for 63.2% of total essential amino acids with a high content of lysine and cysteine and a remarkably high content of tryptophan (5.9% of total amino acids), the problem with infant formulas based on cow milk is the low level of tryptophan and cysteine (Heine et al., 1991). This is the reason why the protein content in infant formula is adjusted to ≥ 15 g of protein/L compared to mother milk 9-11 g of protein/L to compensate for the difference in essential amino acids between mother milk and infant formula (Davis, Harris, Lien, Pramuk, & Trabulsi, 2008; Elgar, Evers, Holroyd, Johnson, & Rowan, 2016; Heine, Radke, Wutzke, Peters, & Kundt, 1996; Lien, 2003). Therefore, infant formulas were supplemented with α-lactalbumin to improve protein quality, reduce total protein concentration, and make amino acid composition similar to that in mothers' milk (Sandström, Lönnerdal, Graverholt, and Hernell, 2008; Heine et al., 1991). α-lactalbumin concentration in current formulas is 0.14 g/100 mL and 0.22 g/100 mL for α-lactalbumin based infant formula (Lien, 2003).

Some past researchers have reported limited digestion of α-lactalbumin in cow's milk, human milk, and infant formula under simulated gastric digestion using human gastric juices or commercial porcine pepsin (Chatterton, Rasmussen, Heegaard, Sørensen, & Petersen, 2004; Sakai et al., 2000; Jakobsson, Lindberg, and Benediktsson, 1982). Jakobsson et al. (1982) observed that only 1 mg of α-lactalbumin as opposed to 30 mg of casein was digested under the same condition at pH 4.5-5.0 (normal gastric pH of infants) or even pH 1.5-2.0 which is optimal for pepsin. Sakai et al. (2000) studied the *in vitro* gastric digestibility of α-lactalbumin of commercial infant formula at pH 1.5-4.0. α-lactalbumin hydrolysed at pH 1.5-2.5, but it was resistant to proteolysis at pH above 3.0. Similar results were obtained during human infants *in vivo* digestion study by Chatterton et al. (2004) with mothers'milk, and cow's milk. Their inference was, α-lactalbumin significantly resists digestion, and it is likely that α-lactalbumin in both human and cow's milk have the same *in vitro* digestibility pattern.

However, it is likely that during *in vivo* digestion, α-lactalbumin is well digested into small peptides in the upper part of the gastrointestinal tract such as stomach and duodenum and then act as bioactive peptides in later part of the gastrointestinal tract (Lönnerdal, 2014). Davidson and Lönnerdal (1987)

and Donovan, Atkinson, Whyte, and Lönnerdal (1989) observed that no intact α-lactalbumin was detected in stool samples of preterm and term infants fed on mothers' milk. Heine, Radke, Wutzke, Peters, & Kundt (1996) also observed the similar content of plasma tryptophan (tryptophan is high proportion in α-lactalbumin) in infants fed on mothers' and formula enriched with α-lactalbumin. In addition, Lien, Davis, Euler, and Multicentre Study Group (2004) reported that the growth rates and serum albumin content were comparable between the infants' group feeding standard formula and enriched α-lactalbumin formula. The reason for the difference of α-lactalbumin digestibility *in vitro* and *in vivo* is possibly the full enzyme system *in vivo* as compared to *in vitro* conditions.

(b) Lactoferrin: Lactoferrin is the second highest whey protein in mothers' milk with an average amount of 1.4 mg/mL (Mao et al., 2017; O'Callaghan, O'Mahony, Ramanujam, & Burgher, 2011) and is considered to have more immune function than nutritional value. It plays an important role as an iron transport protein, mucosal proliferation stimulant and has antibacterial effect (Chierici and Vigi, 1994; Iyer and Lönnerdal, 1993; Davidson & Lönnerdal, 1987). It is worth noting that both lactoferrin in mothers' milk and cow milk are highly resistant to hydrolysis by proteinases (Lönnerdal, 2016; Lönnerdal, 2014; Goedhart & Bindels, 1994).

Because lactoferrin content in cow milk is very low, varying between 0.15 - 485.63 μg/mL (Adlerova, Bartoskova, and Faldyna, 2008), lactoferrin was the first supplement added to infant formula in 1986 (Ben, 2008; Tomita, Wakabayashi, Yamauchi, Teraguchi, and Hayasawa, 2002). Clinical studies indicate lactoferrin enriched formulas help infants increase haematocrits and reduce the incidence of respiratory illnesses (O'Callaghan et al., 2011). Therefore, the European Food Safety Authority recommended 0-6 month-old-infants could take 200 mg of lactoferrin per kg bodyweight or 1.2 g bovine lactoferrin per day without adverse effects (Tetens, 2012).

However, it was reported that infant formula enriched with lactoferrin does not improve iron absorption because bovine lactoferrin is not recognized by human lactoferrin receptors (Aly, Ros, and Frontela, 2013; Jovani, Barbera, and Farré, 2003; Jovani, Barbera, and Farré, 2001). In addition, due to the high cost of this ingredient and the difficulty in preserving the bioactive function of lactoferrin during infant formula production, the application of lactoferrin in commercial infant formulas are still limited (O'Callaghan et al., 2011).

(c) β-lactoglobulin: β-lactoglobulin is the dominant whey protein in cow milk with approximately 50% of total bovine whey protein, but it is completely absent in human whey (Gurr, 1981). β-lactoglobulin is thought of as an allergen (Wal, 2004), and the disulphide (S-S) bonds may be responsible for the allergic reaction (Matsumoto, 2011). Therefore, removing β-lactoglobulin from cow's whey or using hydrolysed whey were suggested in order to make infant formulas closer to mothers' milk (Eugenia Lucena, Alvarez, Menéndez, Riera, and Alvarez, 2006; Floris et al., 2010).

(d) Immunoglobulins: The main immunoglobulins in human milk are secretory IgA (sIgA), IgG1, IgG2 and IgM, in which sIgA makes up the largest proportion with over 90% in human milk, at around 0.1-0.2 g/100 mL. The highest concentration of sIgA is found in human colostrum with 0.9 g/100 mL (Lönnerdal, 2013; Goldman, Goldblum, Atkinson, and Lönnerdal, 1989; Harzer and Bindels, 1985). Human colostrum contains approximately 100-fold higher concentration of immunoglobulins than that in cow milk (Floris, Lambers, Alting, & Kiers, 2010; Gurr, 1981). Immuglobulins play an important part in protecting the newborns against infections from intestinal tract diseases (Feng, Fuerer, & McMahon, 2017; Floris et al., 2010; Uruakpa, Ismond, and Akobundu, 2002; Xu, 1996).

While sIgA is the dominant immunoglobulin in mothers' milk, IgG1 is the major one in bovine milk. In spite of the difference in their structure, they seem to have the same function. Attempts have been made to elevate the concentration of immunoglobulins in infant formulas by adding isolated immunoglobulins from bovine's milk. However, whether bovine colostrum is acceptable to be added to infant formulas is questionable. Some clinical studies showed that cow colostrum enriched formula is beneficial for defence from rotavirus (Davidson et al., 1989; Ebina et al., 1985) or necrotising enterocolitis resistance in preterm piglets (Moller et al., 2011). Recent studies suggested bovine colostrum may be a relevant alternative to mothers' milk with preterm infants when mothers' milk is not available (Jensen et al., 2013). However, other studies demonstrated contradictory results, for example, Turner and Kelsey (1993) concluded that bovine milk antibodies could prevent illnesses related to rotavirus but not rotavirus infection. Aunsholt et al. (2012) also reported that although bovine colostrum has been shown to support intestinal development in the newborn pigs, diets including bovine colostrum, did not improve intestinal function in children from 13 to 169-months of age.

2.3.1.2. Caseins

In human milk, β-casein is the main casein, making up over 70% of total casein (O'Callaghan et al., 2011), the remaining amount is for α_{s1}-casein and κ-casein. α_{s2}-casein is not present in mothers' milk. In cow's milk, both β-casein and α_{s1}-casein are the predominant casein. The whey/casein ratio in human milk changes during the lactation course, from about 90:10 in the early lactation, 60:40 in mature milk, and 50:50 in the late lactation (Kunz and Lönnerdal, 1992). However, in cow's milk the whey/casein ratio is 80:20 in colostrum and around 20:80 in mature milk (Zhang and Carpenter, 2013; Fomon, 1993).

(a) β-casein: β-casein is a highly phosphorylated protein that supplies nutrition and has bioactive function. When being broken down in the gastrointestinal tract, smaller casein phosphopeptides are formed which facilitate calcium and zinc absorption (Lönnerdal, 2013; Sato, Noguchi, and Naito, 1986). This may lead to the better absorption of calcium from mother's milk which has a high percentage of β-casein than infant formula. Commercial β-casein with high-purity is available, that may be substituted to increase this protein content in infant formulas. However, clinical studies related to β-casein enriched-formulas are still limited (O'Callaghan et al., 2011).

β-casein was instantly and completely digested during the gastric phase of *in vitro* digestion models for adults (Astwood, Leach, and Fuchs, 1996; Fu, Abbott, and Hatzos, 2002; Pinto et al., 2014; Su et al., 2017) but remained almost stable in infants' model (Dupont et al., 2010b). A similar profile of digested products was observed with *in vitro* digestion by commercial enzymes and by human fluids using SDS page but digestion with human fluids was quicker (Benedé et al., 2014).

(b) κ-casein: κ-casein is heavily glycosylated and is present in very small amounts in mothers' milk. This casein subunit is considered to stimulate the growth of probiotic bacteria and inhibit the adhesion of bacteria to the gastric mucosa (López, 2007; Stromqvist et al., 1995).
(c) α_{s2}-casein: Not present in mothers' milk, and only present at a very small proportion in cow milk.

Heat treatment during processing is also a factor that affects digestibility of milk proteins due to protein aggregation as well as the Maillard reaction that modifies protein structure. Dupont et al. (2010d) reported heat processing during milk powder manufacture causes caseins to aggregate thereby increases its resistance to *in vitro* digestion. Recent studies applied proteomic techniques to

compare the modification to proteins during different heat treatment (Wada and Lönnerdal, 2014). It was found that lactulosyllysine, a Maillard reaction product is an indicator of digestibility, a high level corresponds to low protein digestibility as observed with in-can sterilized and UHT milk. This suggests heat treatment decreases protein digestibility (Wada & Lönnerdal, 2014).

2.3.1.3. Soy protein isolate

Soy-based infant formula contains protein from plant (soybean), used for babies suffering from galactosemia (cow's milk protein intolerance) or lactose intolerance (Joeckel & Phillips, 2009; Thompkinson and Kharb, 2007). However, proteins from soybean are not easy to digest due to the structure of soy protein and heat treatment effects. Anti-nutritional factors in legumes such as proteases inhibitors, tannins or phytates are minimized in soybean products with proper technological treatments (Carbonaro, Maselli, & Nucara, 2012). Heat processing promotes aggregation of β-sheet structure in soy proteins that provides resistance to its digestion (Carbonaro, Maselli, & Nucara, 2014; Carbonaro et al, 2012). Other significant concern has been raised relating to the effect of phytoestrogenic isoflavone content in soy based infant formula on nutritional adequacy and sexual development during infancy and later life. Many researchers proved the safety of isoflavones and concluded that soy based infant formulas can be an option for term infants (Vandenplas, De Greef, Devreker, and Hauser, 2011; Badger et al., 2009; Perry et al., 2007; Merritt and Jenks, 2004; Strom et al., 2001; Klein, 1998). Nowadays, soy based formulas have become prevalent, accounting for approximately 25% of infant formula sold in the United States and 13% in New Zealand (Agostoni et al., 2006; Klein, 1998; Lönnerdal, 1994).

Not many studies have investigated the digestibility of soybased infant formula. However, there are suggestions to pre-treat soy protein isolate by proteases to increase the number of soy protein hydrolysates, which will improve soy protein digestibility. (Li, Zhu, Zhou, Peng, and Guo, 2013; El-Agamy, 2007; Terracciano, Isoardi, Arrigoni, Zoja, and Martelli, 2002).

2.3.2. Lipids

Mothers' milk contains 3.0-4.5% fat that constitutes the main energy source, providing approximately 50% total energy for the growth of infants (Alles et al., 2004; Flack & Shaw, 2003). Fat in mothers'

milk is comprised of 98% triglycerides , 1% phospholipids and 0.5% cholesterols and cholesterol esters (Lapillonne, Groh-Wargo, Lozano Gonzalez, and Uauy, 2013; Picciano, 2001).

The major differences between the lipid in humans' milk and infant formulas are their content of long-chain polyunsaturated fatty acids (LCPUFAs) with 20-22 carbon atoms, which is crucial for structural component of cell membrane phospholipids of the central nervous system and retinal photoreceptors (Foundation, 1992). Therefore, LCPUFAs are essential for the retina and brain development, and the functional outcome of these (Bindels, 1992). Both n-3 and n-6 LCPUFAs are present in humans' milk, which do not exist in infant formulas without supplements (O'Callaghan et al., 2011). Mothers' milk supplies a rich source of the essential LCPUFAs such as linoleic acids (LA), α-linoleic acids (C18:3, n-3) (ALA), docosahexanenoic acid (C22:6, n-3) (DHA), AA (arachidonic acid, C20:4, n-6) and other LCPUFAs (Hermoso et al., 2010; Koletzko, Thiel, and Abiodun, 1992). The level of LCPUFAs in humans' milk is inconsistent due to the dietary content undertaken by mothers (Thompkinson & Kharb, 2007) and LCPUFAs level is found much higher in colostrum than in mature milk (Renneberg and Skåra, 1992).

In an attempt to formulate infant formula similar to mothers' milk, many studies have worked on the influence of infant formulas enriched with DHA and AA on visual and cognitive development during infancy. However, there have been very inconsistent results from these studies. Some studies concluded that DHA and AA supplemented infant formulas may improve the visual resolution of preterm and term infants (Koletzko et al., 2001; San Giovanni, Berkey, Dwyer, and Colditz, 2000; Souza et al., 2017). Also, adding DHA individually or in combination with AA resulted in similar levels of essential fatty acids in the red blood cells of breastfed infants, and this supplementation has significant effect on visual function in infants (Hoffman et al, 2000; Neumann, Simmer, and Gibson, 2000). However, Neumann et al. (2000) observed that infants fed on formulas supplemented with DHA and AA did not lead to any expected influence on visual evoked potential, mental development, and psychomotor development, while their breastfed counterparts had significantly higher corresponding indexes. A report by Lucas et al. (1999) advocated that there was no significant difference in cognitive development between infants feeding with or without enriched LCPUFAs. In contrast, Willatts, Forsyth, DiModugno, Varma, and Colvin (1998) concluded LCPUFAs could increase the intelligence of babies who received LCPUFAs elevated formula.

In addition to the inconsistent impact of LCPUFAs enriched formula on infant development, it is widely known that full-term infants can synthesize LCPUFAs such as DHA and AA from precursors (Uauy, Mena, Wegher, Nieto, and Salem, 2000). Therefore, a question was raised: should LCPUFAs be added to infant formula or not? (Fanaro and Vigi, 2012; Ben, 2008; Alles et al., 2004). However, according to Lauritzen, Hansen, Jorgensen, and Michaelsen (2001), the endogenous synthesis may not meet the infants' demand of DHA and AA. In addition, the levels of DHA in plasma lipids, in red blood cell membrane phospholipids, and in cerebral cortex was significantly higher in infants fed on the DHA supplement as compared with the non-supplement DHA. This finding supports a strong rationale for adding LCPUFAs in infant formulas. Indeed, ALA, DHA and AA were recommended to be added to infant formulas, but individually DHA or AA supplement was not recommended because these compounds need to work together (Abayomi, 2005). In addition, high consumption of ALA could lead to the rise of lipid peroxidation, product rancidification, and influence the stability of the formula (Koletzko et al., 2005). The recommended amount of LCPUFAs by European Commission (2003) and the Coordinated International Expert Group of European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (Koletzko et al., 2005) is summarised in Table 2.4.

Cow's milk fat is not relevant for infant formulas because it contains more short-chain and saturated fatty acids (over 50% of milk fatty acids), and almost no LCPUFAs (Haug, Hostmark, and Harstad, 2007; Jensen, Ferris, Lammi-Keefe, and Henderson, 1990). Fat in cow's milk also has limited absorption by newborns (Bindels, 1992). Therefore, vegetable oils are currently added to infant formulas.

In term of digestibility, the fat content in breast milk is much more efficiently digested and absorbed than the fat in bovine milk. This is because not only does breast milk contain a significant amount of bile salt-simulated lipase, but also a high proportion (over 70%) of triglycerides with palmitic acid located at sn-2 position (Jensen, 1999; Lien, Yuhas, and Boyle, 1993). The 2-monoglycerides with palmitic acids at the sn-2 position are easier to absorb by infants than free fatty acids (Sidnell and Greenstreet, 2011; Thompkinson & Kharb, 2007). The triglyceride structure also strongly influences the fat absorption, the longer the chain and higher the saturation of the fatty acids, the less it is well absorbed (Tomarelli, Meyer, Weaber, and Bernhart, 1968), (Bracco, 1994).

Table 2.4 ESPGHAN recommendation about components in infant formula. Adapted from Koletzko et al. (2005).

	Component	Unit	Minimum	Maximum
Protein	Cow's milk protein	g/100 kcal	1.8	3
	Soy protein isolates	g/100 kcal	2.25	3
	Hydrolysed cow's milk protein	g/100 kcal	1.8	3
Lipids	Total fats	g/100 kcal	4.4	6.0
	Linoleic acids	g/100 kcal	0.3	1.2
	α-linoleic acids	mg/100 kcal	50	Not specified
	Ratio linoleic acids/α-linoleic acids		5:1	15:1
Carbohydrates	Total carbohydrates	g/100 kcal	9.0	14
	Starches	g/100ml		2
	Glucose, sucrose and fructose should not be added to infant formula			

The size of fat globules may have a significant effect on digestibility in infants. Michalski, Briard, Michel, Tasson, and Poulain (2005) observed a difference in the sizes of fat globules between mothers' milk and infant formula. The droplets were much larger in colostrum (9μm) and mature mother milk (4μm) compared to infant formulas (0.4μm). Some recent studies have reported, homogenised fat droplets were digested to a larger extent in both *in vivo* gastric and small intestine digestion (Bourlieu et al., 2015; Gallier et al., 2013). It is suggested that mother's milk may protect infants against obesity and this raised a considerable concern to formula fed infants. Recently, Oosting et al. (2014) reported evidence of long-term effects of early diet of physical structural lipids on fat accumulation and metabolism in mice. This confirmation provides support for the emerging consideration that dietary lipid structure in early life is related to later-life obesity risk. However, there is still limited study on the effect of fat globule size on digestibility (Bourlieu et al., 2015).

2.3.3 Carbohydrates

Carbohydrates are the second most important source of energy for infant after lipids and make up to about 35-55% of the total energy of the infant diet (Fanaro & Vigi, 2012; Lebenthal et al., 1983).

Although human milk contains both digestible (lactose) and indigestible carbohydrates (oligosaccharides such as gluco-oligosaccharides and maltodextrin-like oligosaccharides) (Engfer, Stahl, Finke, Sawatzki, and Daniel, 2000), only digestible carbohydrates are permitted to be added to infant formula (Thompkinson & Kharb, 2007). According to European Union (2006) only the following carbohydrates can be used in infant formula: lactose, maltose, sucrose, maltodextrins, glucose syrup or dried glucose syrup, precooked starch and gelatinised starch which are naturally free of gluten.

2.3.3.1. Oligosaccharides

Oligosaccharides are molecules that contain a small number (between 2 to 10) of monosaccharide residues connected by glycosidic linkages (International Union of Pure and Applied Chemistry, International Union of Biochemistry, 1982). The main difference between carbohydrate content in human and cow milk is the amount of oligosaccharides. While only humans' milk is a rich source of oligosaccharides at 5-20 g/L (mature milk), this content is at very low level in cow's milk (data not reported) (Bode, 2012; Engfer et al., 2000; Rudloff and Kunz, 1997). Most of the oligosaccharides in human milk are resistant to digestion and absorption within the small intestine and act as prebiotics in the infant's colon (Engfer et al., 2000; Gnoth, Kunz, Kinne-Saffran, and Rudloff, 2000). Indeed, oligosaccharides promote the growth of bifidus flora in the gut (Flack & Shaw, 2003; Rudloff and Kunz, 1997; Goedhart & Bindels, 1994) and inhibit bacterial adhesion to epithelial surfaces (Kunz, Rudloff, Baier, Klein, and Strobel, 2000) thereby preventing gastrointestinal infection in breast-fed infants.

Based on their good effect on the infant gastrointestinal tract, oligosaccharides were expected to be included in infant formulas. However, with over 100 types of oligosaccharide structures present in mother's milk, this makes it hard to choose the appropriate form of oligosaccharide to add to infant formula (Kunz and Rudloff, 1993). Recently, Ben (2008) showed that oligosaccharides in mothers' milk contain 70-90% galactose-oligosaccharides (GOSs) and 10-30% fructose-oligosaccharides (FOSs) in the first few months of lactation. The author further reported that commercial infant formulas have been supplemented with GOSs at 0.2-0.4 g/100 mL and with FOSs at 0.05-0.1 g/100 mL, although the recommended amount for oligosaccharide supplementation are still unavailable (Ben, 2008).

2.3.3.2. Lactose

Lactose is the primary fraction of carbohydrates in milk with around 6-7 g/100 mL in mothers' milk (Bosscher et al., 2000; Jensen, 1995; Bindels, 1992) and around 4.5 g/100 mL in cow's milk (Fox and McSweeney, 1998). Lactose can be used as a sole carbohydrate source in infant formula and the amount of lactose supplement should not exceed the recommended total carbohydrates for infant formula (Ben, 2008).

Lactose is a slow digestible sugar in the small intestine. The remaining lactose continues to be fermented in the large intestine that contributes towards maintaining the acidic pH 5.5-6.0, that is beneficial for protecting babies from infection (Thompkinson & Kharb, 2007). Lactose also helps increase the absorption of some minerals in the human body such as calcium, sodium, and iron (Thompkinson & Kharb, 2007; Koletzko et al., 2005).

2.3.3.3. Glucose

Only a small amount of glucose is present in both mothers' and cow's milk (Whitnah, 1931). A very low level of glucose (0.2-0.3 g/L) is added in some commercial infant formulas to improve the taste. The glucose addition should be limited to under 2.0 g/100 kcal because glucose content offers no bioactivity over other sugar sources and could unnecessarily increase the osmolality of formula (Ben, 2008; Thompkinson & Kharb, 2007). According to Koletzko et al. (2005) 1g glucose contributes an increase of osmolality by 58 mOsm/kg. So far, the ESPGHAN does not recommend adding glucose to infant formulas.

2.3.3.4. Sucrose and fructose

In mothers' milk, there is currently no available information about sucrose content and fructose is absent (Stephen et al., 2012). Sucrose and fructose are much sweeter than lactose. This is the reason why infants tend to take higher volumes of formula containing sucrose than lactose (Thompkinson & Kharb, 2007). Normally sucrose is supplemented (up 20% of total carbohydrate content) in infant formulas based on hydrolysed protein to disguise the bitter taste of protein hydrolysates (European Commission, 2003). However, consumption of formula supplemented with fructose and sucrose may result in a detrimental impact on newborns who have hereditary fructose intolerance (Koletzko et al.,

2005; Mock, Perman, Thaler, and Morris Jr, 1983). In addition, high intake of fructose could lead to intolerance in infants and should be the reason why fructose is not suggested as an additive to infant formula (Stephen et al, 2012; Nobigrot, Chasalow, and Lifshitz, 1997). Therefore, ESPGHAN recommended sucrose and fructose should not be supplemented in infant formulas, especially for babies below 4-6 months (Koletzko et al., 2005).

2.3.3.5. Maltose, maltodextrins, and corn-starch syrup solids

Maltose, maltodextrin, and corn-starch syrup are the products of corn-starch hydrolysis. Maltose, maltodextrin are permitted to be added to infant formulas due to the sufficiency of maltase and glucoamylase in infants and they do not raise the osmolality of the formula (Fanaro & Vigi, 2012; Koletzko et al., 2005; Lebenthal, 1983). To add in infant formula, maltodextrins with 5-9 glucose units should be a good option because human glucoamylase has specificity on the chain length of maltodextrin (European Commission, 2003). However, chain length of maltodextrins has not been regulated, so current commercial infant formulas contained maltodextrins formed from 1-30 glucose units (Coppa et al., 1993).

2.3.3.6. Starches

Only a small amount of starches are recommended as additives to infant formula due to insufficient amylase enzymes during infancy (Koletzko et al., 2005). Compared with native starches, modified starches are preferred to be used in infant formula because they do not retrograde during storage and can prolong the shelf-life of infant formula (Filer 1971). In addition, unretrogradation is able to improve the digestibility of modified starches (Filer 1971). Thus, precooked starch and gelatinised starch (naturally free of gluten) are preferred in infant formula (Thompkinson & Kharb, 2007). The recommended amount of these starches added in infant formulas is no more than 2 g/100 mL or no more than 30% of total carbohydrates (Koletzko et al., 2005).

2.4. *In vitro* infant digestion models

In vitro digestion models have been increasingly applied to understand digestibility, structural changes, and kinetics of digestion under closely simulated physiological conditions in the human gastrointestinal tract (Hur, Lim, Decker, & McClements, 2011). Although *in vitro* models cannot

mimic exactly the whole complex digestion process in the human gut, especially the composition and subsequent digestive secretion, digestion and absorption, and the interaction between the host, the food and micro-bacteria in the digestive system (Coles, Moughan, and Darragh, 2005), they offer significant advantages compared to *in vivo* models as there are no ethical issues, low cost, and easy sampling accessibility (Sopade and Gidley, 2009). The commonly applied *in vitro* digestion models are static and dynamic models that are discussed in the sections below.

2.4.1. Static models

Static or *biochemical* models are defined as the ones, in which the final digestive products remain in reaction vessels during the digestion process, and other physical movements like shear, mixing, falling of gastric pH, and absorption process are not employed (Wickham, Faulks, & Mills, 2009). Hur, Lim, Decker, & McClements (2011) have reviewed many static models widely applied in the study of digestibility of food ingredients, bioavailability of individual nutrients, allergenicity, antioxidant, and bio-accessibility of toxic metals from soil (Daly et al., 2010; Argyri, Birba, Miller, Komaitis, and Kapsokefalou, 2009; Oomen et al., 2003; Kitabatake & Kinekawa, 1998). The most common model is a conical flask or beaker placed in a shaking water bath set at 60-250 rpm and a temperature of 37°C similar to human body temperature (Fabek, Messerschmidt, Brulport, and Goff, 2014; Hur et al., 2011; Nik, Corredig, and Wright, 2010). In terms of gastric pH, static models are not able to recreate the dynamic pH changes during the ingestion period. The mean of fasting gastric pH varied between 1.5-2 and 3-7 for fed condition (N'Goma, Amara, Dridi, Jannin, Carrière, 2012; Charman, Porter, Mithani, and Dressman, 1997, Dressman et al., 1990). Depending upon the purpose of research, simulated model for adults chose gastric pH from 1.07 to 2.5 (Pinto et al., 2014; Gallier, Tate, and Singh, 2012; Oomen et al., 2003). A more exhaustive justification of static *in vitro* digestion method being close to physiological condition was recently produced by Minekus et al. (2014). The international consensus advises the use of pH 3 for *in vitro* gastric pH in adults. Hence, for infant simulated digestion, pH in the stomach should be higher than that in adults (Fig 2.1). In addition, the other critical consideration in the digestibility study is the concentration of various gastrointestinal fluids like enzymes, bile salts and other surfactants. In some recent series of studies on *in vitro* infant simulated protein digestion, 22.75 U/mg of pepsin was added for *in vitro* stomach digestion (pH 3.0), 0.04 U/mg protein of α-chymotrypsin and 3.45 U/mg of trypsin for *in vitro* intestinal digestion (pH 6.5) (Dupont et al., 2010a; Dupont et al., 2010b; Dupont et al., 2010c) that are similar to the physiological amount found in infants.

The advantages of static models are their simplicity, low cost and easy cleaning.

2.4.2. Dynamic models

The main disadvantages of the static models are that they cannot imitate the dynamic digestion process taking place in the human gastrointestinal tract that are the gastric emptying, peristaltic movements, pH change in the stomach, enzyme and fluid secretion during digestion. These difficulties are overcome in a dynamic model. The two popular dynamic models are the TIM1 and TIM2. Schematic representation of a static and several different dynamic models has been presented in the review article by Guerra et al., 2012.

TIM1 (TNO gastro-intestinal model 1) consists of the gastrointestinal tract with stomach, and three other components for the small intestine (duodenum, jejunum, ileum), and the large intestine. They were replicated by six vessels controlled by a computer (Minekus, Marteau, and Havenaar, 1995). TIM1 takes into account most of the key parameters such as human temperature, pH change in the gastric, gastric and pancreatic automatic secretion, gastric emptying, gastric and intestinal transit times, peristalsis movements, nutrient absorption in the intestine by a dialysis system (Guerra et al., 2012).

TIM 2 was developed from TIM1 and additionally can imitate the microbiota (Yoo & Chen, 2006). All these parameters in TIM-1 and TIM-2 are controlled to mimic the digestion in human body at different life stages from infant, adults, and elderly (Blanquet et al., 2004). TIM-1 was applied to study the behaviour of oral drug dosage under *in vitro* infant digestion (Blanquet et al., 2004). Blanquet et al. (2004) suggested that TIM-1 is an effective instrument to see the changes and availability of drugs in infant (and adult) gastrointestinal conditions.

However, TIM-1 is very expensive for commercial product, and complicated for cleaning and handling (Ménard et al., 2014). Ménard et al. (2014) designed a simpler dynamic digestion system for infants, which contain two successive chambers for simulated stomach and small intestine. Each chamber has a water jacket connected to a water-bath set at 39^0C to mimic the piglet body temperature. The flows of ingested food, digestive enzymes, bile salts and other chemicals are controlled by various pumps. The whole system is controlled and monitored by a computer program.

This dynamic model showed a high correlation for proteolysis between *in vitro* and *in vivo* models from piglets, but not for the lipolysis (Ménard et al., 2014).

2.4.3. Commercially available enzymes for *in vitro* infant digestion study

The commercially available enzymes that are employed in the *in vitro* digestion studies closely resemble the functionality of the digestive enzymes naturally excreted in the gastrointestinal tract. The characteristics, and enzyme concentration are the crucial parameters for *in vitro* digestion models. Single and purified enzymes or biological mixture has been suggested to be used for standardization among these models and to enable comparisons between researchers (Coles, Moughan & Darragh, 2005). The other advantage of single enzymes is for forecasting the digestibility of single ingredients in food such as protein, starch, or lipids (Boisen & Eggum, 1991). However, the hydrolysis of a specific bond relies on the approach of the enzyme to the substrate, so it seems more relevant to the real digestion when using the biological mixture of enzymes instead of using individual enzymes (Boisen & Eggum, 1991).

The physiological activities of digestive enzymes in infants as compared to that for adults are summarized in Table 2.1. It is clear that the activity of most enzymes such as α-amylase, pepsin, pancreatic triglyceride lipase are present at very low levels in infants compared to their activity in adults, with the exception of gastric lipase and lactase (Armand et al., 1996; Armand et al., 1995; Lebenthal et al., 1983). Hence, it is recommended to reduce the concentration of digestive enzymes when infant digestion experiments are conducted. For instance, Dupont et al. (2010b) reduced the pepsin concentration employed in infant models by 8 times; bile salt concentration by 4 times; phospholipid vesicle, trypsin and chymotrypsin concentration by 10 times as compared to the corresponding figure for adult models. Similarly, Böttger, Etzel, and Lucey (2013) employed one-tenth of the pancreatin used in adults for infant digestion models. Recently, Amara et al. (2014) conducted *in vitro* digestion of lipid under infant condition and employed pancreatic lipase that was reduced by 17 times compared to adult value (Minekus et al., 2000).

The source of the enzymes used in the digestion studies are as described below:

2.4.3.1 Proteases

Proteases are comprised of three main enzymes responsible for breakdown of dietary protein and peptides into smaller peptides and amino acids. They are pepsin in stomach, trypsin, and chymotrypsins in the small intestine (Hur, Lim, Decker, & McClements, 2011). For *in vitro* digestion study, usually pepsin from porcine mucosa is used for gastric proteolysis while trypsin and chymotrypsin of porcine or bovine origin are used for protein hydrolysis in the intestine. Some researchers also recommend using pancreatic proteases (pancreatin) to mimic digestion in the intestinal phase. Pancreatin contains both trypsin, and chymotrypsin as well as pancreatic amylase and lipase.

All the individual proteases enzymes such as pepsin, trypsin, chymotrypsin or pancreatin sourced from mammals are commercially available for *in vitro* digestion of infants.

2.4.3.2. Lipases

Gastric lipase is the only lipase involved in the lipolysis of ingested fat in the stomach. Some *in vitro* studies used human gastric juice or purified human gastric lipase (Carrière et al, 2001; Carrière et al., 2000). However, due to the ethical issues and clinical invasive procedures, using human gastric lipase in simulated digestion studies is very limited. Other sources of analogue gastric lipase have been applied such as recombinant dog gastric lipase (Amara et al., 2014; Fernandez et al., 2013), rabbit gastric lipase (Bourlieu et al., 2015; Capolino et al., 2011; Oliveira, Bourlieu, et al., 2016; Oliveira, Deglaire, et al., 2016; Vors et al., 2012;) and fungal lipase (Ménard et al., 2014; Mandalari et al., 2008). Although mammal gastric lipase closely resembles human gastric lipase than fungal lipases, its use is restricted because it is not commercially available (Bourlieu et al., 2014). Only fungal lipases are commercially available, but fungal lipases expose a different specificity compared to human gastric lipase. Fungal lipases has high specificity to *sn-1* and *sn-3* position of triglyceride, whereas mammal gastric lipase prefer only *sn-3* (Ménard et al., 2014). However, no commercial analogue gastric lipase is better than fungal lipase up to now (Ménard et al., 2014).

In the small intestine, the lipid enzyme system is more complicated than that in the gastric with pancreatic triglyceride lipase (PTL), PLRP 1, 2, phospholipase A2 (PLA2), BSSL or cholesterol esterase. Therefore, during *in vitro* lipid digestion, porcine pancreatin is employed as the most popular

lipases as it contains a mixture of all enzymes secreted by the pancreas (Larsen, Sassene, and Müllertz, 2011). However, the chemical composition and enzyme activity in pancreatin rely upon its biological origin, isolation, and purification process. Hence, this leads to significant variation in pancreatin from supplier to supplier, and even batch to batch (Löhr et al., 2009) though cheaper than purified pancreas lipases. Commercial purified pancreas lipases are consistent because of good purification (McClements and Li, 2010). In addition, lipase derived from bacteria (non-pancreatic lipase source) has also been employed (de María, Fernández-Álvaro, ten Kate, and Bargeman, 2009). These non-pancreatic lipases are highly pure and cheaper than purified pancreas lipases. However, due to the bacterial origin, the behaviour of these lipases may be different from those, which has been isolated from mammals. The lipase activity also depends on its history, solution, and environmental conditions with not very long shelf-life (McClements & Li, 2010).

Pancreatic lipase is dominant in intestinal lipolysis in adults, but this enzyme in infants presents at very low levels. In contrast, PLRP 1, 2 and BSSL are the key lipases in intestinal lipid digestion in infants (Andersson et al., 2011; Lindquist & Hernell, 2010). However, not only are gastric lipase and PLRP 2 not commercially available, the crucial information such as concentration of these enzymes in the small intestine of infants has not yet been published. It has been suggested that *in vitro* lipid digestion study for infants is a big challenge because to mimic infant lipid digestion, gastric lipase, pancreatic lipase, pancreatic lipase related protein 2 and bile salt stimulated lipase should be present (Abrahamse et al., 2012). Therefore, there are limited studies on *in vitro* lipolysis in infants.

2.4.3.3 Carbohydrases

Carbohydrases represent a group of enzymes that help in digestion of starch including α-amylase in the mouth and α-amylase and glucoamylase in the intestine. Salivary α-amylase begins the starch digestion in the mouth. However, due to the very short residence time of starch in adults' mouth, the role of salivary α-amylase in starch digestion in the mouth phase is usually ignored (Wolter, Hager, Zannini, and Arendt, 2013; Kaur, Sandhu, and Lim, 2010; Wong et al., 2009). In addition, a high portion of salivary amylase is inactivated by the acidic gastric environment of adults. However, it could remain a minor activity in the poorly acidified infants' stomach (Bourlieu et al., 2014). Therefore, the digestion of starch in the stomach of infants should be considered due to the level of α-amylase in the small intestine of infants is very low.

Regarding oligosaccharides and disaccharides digestion, the enzymes responsible to digest this type of carbohydrates are not secreted into the intestinal fluid. They are bound to the intestinal mucosa. Thus, to examine the digestibility of human milk oligosaccharides, Gnoth, Kunz, Kinne-Saffran, & Rudloff (2000) and Engfer et al. (2000) employed the intestinal brush border membranes from humans and pigs.

2.5. Conclusions

Although commercially available infant formula has been designed to be close to mothers' milk, there are still differences in composition such as content of β-lactoglobulin, α-lactalbumin, lactoferrin, α_{s2}-casein, LCPUFAs, oligosaccharides, etc. that could result in different composition of formula and its subsequent effect on growth and developmental pattern of infants.

In the current review, the key differences in infant physiology of the gastrointestinal tract have been elucidated. They are gastric pH, the concentration range of digestive enzymes, and bile salts. These basic parameters can be applied to simulate infant digestion of mother's milk and infant formula. *In vitro* models can be a good alternative to *in vivo* digestion to obtain data in structural changes, rheology, digestibility, and bioavailability of infant foods, although they are unable to present exactly the *in vivo* digestive condition in infants. In addition, simulated digestive enzymes such as human gastric lipase, PLRP2, and BSSL have no commercial availability and their activities in the infant gastrointestinal tract remain to be elucidated.

2.6. References

Abayomi, J. (2005). Infant formula-evaluating the safety of new ingredients. *Journal of Human Nutrition and Dietetics, 18*(3), 226-226.

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

Adlerova, L., Bartoskova, A., & Faldyna, M. (2008). Lactoferrin: a review. *Veterinarni Medicina, 53*(9), 457-468.

Aggett, P. J., Agostini, C., Goulet, O., Hernell, O., Koletzko, B., Lafeber, H. L., et al. (2001). The nutritional and safety assessment of breast milk substitutes and other dietary products for

infants: a commentary by the ESPGHAN Committee on Nutrition. *Journal of Pediatric Gastroenterology and Nutrition, 32*(3), 256-258.

Agostoni, C., Axelsson, I., Goulet, O., Koletzko, B., Michaelsen, K. F., Puntis, J., et al. (2006). Soy protein infant formulae and follow-on formulae: a commentary by the ESPGHAN Committee on Nutrition. *Journal of Pediatric Gastroenterology and Nutrition, 42*(4), 352-361.

Agostoni, C., Braegger, C., Decsi, T., Kolacek, S., Koletzko, B., Michaelsen, K. F., et al. (2009). Breast-feeding: A Commentary by the ESPGHAN Committee on Nutrition. *Journal of Pediatric Gastroenterology and Nutrition, 49*(1), 112-125.

Agostoni, C., Decsi, T., Fewtrell, M., Goulet, O., Kolacek, S., Koletzko, B., et al. (2008). Complementary Feeding: A Commentary by the ESPGHAN Committee on Nutrition. *Journal of Pediatric Gastroenterology and Nutrition, 46*(1), 99-110.

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

Alles, M. S., Scholtens, P. A., & Bindels, J. G. (2004). Current trends in the composition of infant milk formulas. *Current Paediatrics, 14*(1), 51-63.

Aly, E., Ros, G., & Frontela, C. (2013). Structure and Functions of Lactoferrin as Ingredient in Infant Formulas. *Journal of Food Research*, *2*(4), 25-36.

Amara, S., Patin, A., Giuffrida, F., Wooster, T. J., Thakkar, S. K., Bénarouche, A., ... & Carrière, F. (2014). In vitro digestion of citric acid esters of mono-and diglycerides (CITREM) and CITREM-containing infant formula/emulsions. *Food & Function*, *5*(7), 1409-1421.

Andersson, E.-L., Hernell, O., Bläckberg, L., Fält, H., & Lindquist, S. (2011). BSSL and PLRP2: key enzymes for lipid digestion in the newborn examined using the Caco-2 cell line. *Journal of Lipid Research, 52*(11), 1949-1956.

Andrea, N.E., & Nikoletta, F. (2010). Oral drug absorption in Paediatric population. In Jennifer, B.D., & Christos, R. (Eds.), *Oral drug absorption: prediction and assessment* (pp.108-123)

Argyri, K., Birba, A., Miller, D., Komaitis, M., & Kapsokefalou, M. (2009). Predicting relative concentrations of bioavailable iron in foods using in vitro digestion: New developments. *Food Chemistry, 113*(2), 602-607.

Armand, M., Hamosh, M., DiPalma, J. S., Gallagher, J., Benjamin, S. B., Philpott, J. R., et al. (1995). Dietary fat modulates gastric lipase activity in healthy humans. *The American Journal of Clinical Nutrition, 62*(1), 74-80.

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

Arvedson, J. C., & Brodsky, L. (2002). *Pediatric swallowing and feeding: Assessment and management*. Clifton Park, NY Delmar Cengage Learning.

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

Aunsholt, L., Jeppesen, P. B., Lund, P., Sangild, P. T., Ifaoui, I. B. R., Qvist, N., et al. (2012). Bovine colostrum to children with short bowel syndrome a andomized, double-blind, crossover pilot study. *Journal of Parenteral and Enteral Nutrition, 38*(1), 99-106.

Auricchio, S., Stellato, A., & De Vizia, B. (1981). Development of brush border peptidases in human and rat small intestine during fetal and neonatal life. *Pediatric Research, 15*(7), 991-995.

Australia Government, Commonwealth Law. (2000). Australia New Zealand Food Standards Code - Standard 2.9.1 - Infant Formula Products. Retried from http://www.comlaw.gov.au/Series/F2008B00658

Badger, T. M., Gilchrist, J. M., Pivik, R. T., Andres, A., Shankar, K., Chen, J.-R., et al. (2009). The health implications of soy infant formula. *The American Journal of Clinical Nutrition, 89*(5), 1668S-1672S.

Ben, X.-M. (2008). Nutritional management of newborn infants: Practical guidelines. *World Journal of Gastroenterology: WJG, 14*(40), 6133.

Benedé, S., López-Expósito, I., Giménez, G., Grishina, G., Bardina, L., Sampson, et al. (2014). In vitro digestibility of bovine β-casein with simulated and human oral and gastrointestinal fluids. Identification and IgE-reactivity of the resultant peptides. *Food Chemistry, 143*(0), 514-521.

Berfenstam, R., Jagenburg, R., and Mellander, O. (1955). Protein Hydrolysis in the Stomachs of Premature and Full-term Infants. *Acta Paediatrica, 44*(4), 348-354.

Bernbäck, S., Bläckberg, 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 (BBA)-Lipids and Lipid Metabolism, 1001*(3), 286-293.

Bernbäck, S., Bläckberg, L., and Hernell, O. (1990). The complete digestion of human milk triacylglycerol in vitro requires gastric lipase, pancreatic colipase-dependent lipase, and bile salt-stimulated lipase. *Journal of Clinical Investigation, 85*(4), 1221.

Berton, A., Sebban-Kreuzer, C., Rouvellac, S., Lopez, C., & Crenon, I. (2009). Individual and combined action of pancreatic lipase and pancreatic lipase-related proteins 1 and 2 on native versus homogenized milk fat globules. *Molecular Nutrition & Food Research, 53*(12), 1592-1602.

Bindels, J. G. (1992). Artificial feeds for infants - human milk substitutes: current composition and future trends. *Current Paediatrics, 2*(3), 163-167.

Blanquet, S., Zeijdner, E., Beyssac, E., Meunier, J.-P., Denis, S., Havenaar, R., et al. (2004). A dynamic artificial gastrointestinal system for studying the behavior of orally administered drug dosage forms under various physiological conditions. *Pharmaceutical research, 21*(4), 585-591.

Bode, L. (2012). Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology, 22*(9), 1147-1162.

Boisen, S., & Eggum, B. (1991). Critical evaluation of *in vitro* methods for estimating digestibility in simple-stomach animals. *Nutrition Research Reviews, 4*, 141-162.

Borgstrom, B., Lindquist, B., & Lundh, G. (1960). Enzyme concentration and absorption of protein and glucose in duodenum of premature infants. *Archives of Pediatrics and Adolescent Medicine, 99*(3), 338.

Borgstrom, B., Lindquist, B., & Lundh, G. (1961). Digestive studies in children: studies under normal and pathological conditions. *Archives of Pediatrics and Adolescent Medicine, 101*(4), 454.

Bosscher, D., Van Caillie-Bertrand, M., Van Dyck, K., Robberecht, H., Van Cauwenbergh, R., & Deelstra, H. (2000). Thickening infant formula with digestible and indigestible carbohydrate: availability of calcium, iron, and zinc in vitro. *Journal of Pediatric Gastroenterology and Nutrition, 30*(4), 373-378.

Bourlieu, C., Ménard, O., Bouzerzour, K., Mandalari, G., Macierzanka, A., Mackie, A. R., et al. (2014). Specificity of infant digestive conditions: some clues for developing relevant in vitro models. *Critical Reviews in Food Science and Nutrition, 54*(11), 1427-1457.

Bourlieu, C., Ménard, O., De La Chevasnerie, A., Sams, L., Rousseau, F., Madec, M. N., ... & Dupont, D. (2015). The structure of infant formulas impacts their lipolysis, proteolysis and disintegration during in vitro gastric digestion. *Food Chemistry, 182*, 224-235.

Bracco, U. (1994). Effect of triglyceride structure on fat absorption. *The American Journal of Clinical Nutrition, 60*(6), 1002S-1009S.

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

Carey, M. C., Small, D., & Bliss, C. (1983). Lipid Digestion and Absorption. *Annual Review of Physiology, 45*(1), 651-677.

Carrière, F., Barrowman, J. A., Verger, R., & Laugier, R. (1993). Secretion and contribution to lipolysis of gastric and pancreatic lipases during a test meal in humans. *Gastroenterology, 105*(3), 876-888.

Carrière, F., Renou, C., Ransac, S., Lopez, V., De Caro, J., Ferrato, F., ... & Laugier, R. (2001). Inhibition of gastrointestinal lipolysis by Orlistat during digestion of test meals in healthy volunteers. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *281*(1), G16-G28.

Carver, J. D. (2003). Advances in nutritional modifications of infant formulas. *The American Journal of Clinical Nutrition, 77*(6), 1550S-1554S.

Cavell, B. (1983). Postprandial gastric acid secretion in infants. *Acta Paediatrica, 72*(6), 857-860.

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

Charman, W. N., Porter, C. J., Mithani, S., & Dressman, J. B. (1997). Physicochemical and physiological mechanisms for the effects of food on drug absorption: the role of lipids and pH. *Journal of Pharmaceutical Sciences*, *86*(3), 269-282.

Chen, X. D. GIT physicochemical modeling-a critical review. *International Journal of Food Engineering, 2*(4).

Chierici, R., & Vigi, V. (1994). Lactoferrin in infant formulae. *Acta Peadiatrica, 83,* 83-88.

Cichero, J. A., Nicholson, T. M., & September, C. (2013). Thickened milk for the management of feeding and swallowing issues in infants: A call for interdisciplinary professional guidelines. *Journal of Human Lactation*, *29*(2), 132-135.

Cohen, M., Morgan, R. G., & Hofmann, A. F. (1971). Lipolytic activity of human gastric and duodenal juice against medium and long chain triglycerides. *Gastroenterology, 60*(1), 1-15.

Coles, L., Moughan, P., & Darragh, A. (2005). In vitro digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals. *Animal Feed Science and Technology, 123*, 421-444.

Commare, C. E., & Tappenden, K. A. (2007). Development of the infant intestine: implications for nutrition support. *Nutrition in Clinical Practice, 22*(2), 159-173.

Commission, E. (2006). Directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae and amending Directive 1999/21/EC. *OJEU, 401*, 1-33.

Coppa, G. V., Gabrielli, O., Pierani, P., Catassi, C., Carlucci, A., & Giorgi, P. L. (1993). Changes in carbohydrate composition in human milk over 4 months of lactation. *Pediatrics, 91*(3), 637-641.

Daly, T., Ryan, E., Aisling Aherne, S., O'Grady, M. N., Hayes, J., Allen, P., et al. (2010). Bioactivity of ellagic acid, lutein or sesamol enriched meat patties assessed using an in vitro digestion and Caco-2 cell model system. *Food Research International, 43*(3), 753-760.

Dallas, D. C., Underwood, M. A., Zivkovic, A. M., & German, J. B. (2012). Digestion of protein in premature and term infants. *Journal of Nutritional Disorders & Therapy, 2*(3), 1-9.

Davidson, G., Daniels, E., Nunan, H., Moore, A., Whyte, P., Franklin, K., et al. (1989). Passive immunisation of children with bovine colostrum containing antibodies to human rotavirus. *The Lancet, 334*(8665), 709-712.

Davidson, L. A., & Lönnerdal, B. (1987). Persistence of human milk proteins in the breast-fed infant. *Acta Paediatrica, 76*(5), 733-740.

Davis, A. M., Harris, B. J., Lien, E. L., Pramuk, K., & Trabulsi, J. (2008). α-Lactalbumin-rich infant formula fed to healthy term infants in a multicenter study: plasma essential amino acids and gastrointestinal tolerance. *European Journal of Clinical Nutrition, 62*(11), 1294-1301.

Dewit, O., Dibba, B., and Prentice, A. (1990). Breast-milk amylase activity in English and Gambian mothers: effects of prolonged lactation, maternal parity, and individual variations. *Pediatric Research, 28*(5), 502-506.

DiPalma, J., Kirk, C. L., Hamosh, M., Colon, A. R., Benjamin, S. B., & Hamosh, P. (1991). Lipase and pepsin activity in the gastric mucosa of infants, children, and adults. *Gastroenterology, 101*(1), 116-121.

Donovan, S. M., Atkinson, S. A., Whyte, R. K., & Lönnerdal, B. (1989). Partition of nitrogen intake and excretion in low-birth-weight infants. *American Journal of Diseases of Children, 143*(12), 1485-1491.

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

Duncan, B., Ey, J., Holberg, C. J., Wright, A. L., Martinez, F. D., & Taussig, L. M. (1993). Exclusive breast feeding for at least 4 months protects against otitis media. *Pediatrics, 91*(5), 867-872.

Dupont, D., Boutrou, R., Menard, O., Jardin, J., Tanguy, G., Schuck, P., et al. (2010a). Heat treatment of milk during powder manufacture increases casein resistance to simulated infant digestion. *Food Digestion, 1*(1-2), 28-39.

Dupont, D., Mandalari, G., Molle, D., Jardin, J., Léonil, J., Faulks, R. M., et al. (2010b). Comparative resistance of food proteins to adult and infant in vitro digestion models. *Molecular Nutrition and Food Research, 54*(6), 767-780.

Dupont, D., Mandalari, G., Mollé, D., Jardin, J., Rolet-Répécaud, O., Duboz, G., et al. (2010c). Food processing increases casein resistance to simulated infant digestion. *Molecular Nutrition and Food Research, 54*(11), 1677-1689.

Ebina, T., Sato, A., Umezu, K., Ishida, N., Ohyama, S., Oizumi, A., et al. (1985). Prevention of rotavirus infection by oral administration of cow colostrum containing antihumanrotavirus antibody. *Medical Microbiology and Immunology, 174*(4), 177-185.

Eidelman, A., & Feldman-Winter, L. (2005). From the American academy of pediatrics: policy statement: breastfeeding and the use of human milk. *Pediatrics, 115*(2), 496-506.

Elgar, D., Evers, J. M., Holroyd, S. E., Johnson, R., & Rowan, A. (2016). The measurement of protein in powdered milk products and infant formulas: A review and recent developments. *Journal of AOAC International*, 99(1), 26-29.

El-Agamy, E. (2007). The challenge of cow milk protein allergy. *Small Ruminant Research, 68*(1), 64-72.

Engfer, M. B., Stahl, B., Finke, B., Sawatzki, G., & Daniel, H. (2000). Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. *The American Journal of Clinical Nutrition, 71*(6), 1589-1596.

Eugenia Lucena, M., Alvarez, S., Menéndez, C., Riera, F. A., & Alvarez, R. (2006). Beta-lactoglobulin removal from whey protein concentrates: Production of milk derivatives as a base for infant formulas. *Separation and Purification Technology, 52*(2), 310-316.

European Commission, Health, and Consumers Protectorate-General. (2003). Report of the scientific committee on food on the revision of essential requirements of infant formulae and follow-on formulae. Retried from http://ec.europa.eu/food/fs/sc/scf/out199_en.pdf

European Union. (2006). Commission Directive 2006/141/EC of 22th December 2006 on infant formulae and follow-on formulae, amending Directive 1999/21/EC. Offical Journal of

European Union, L 401/1, 2006. Retried from http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006L0141&from=EN

Fabek, H., Messerschmidt, S., Brulport, V., & Goff, H. D. (2014). The effect of in vitro digestive processes on the viscosity of dietary fibres and their influence on glucose diffusion. *Food Hydrocolloids, 35*(0), 718-726.

Fanaro, S., & Vigi, V. (2012). Feeding the term infant: human milk and formulas. *Neonatology* (pp. 290-297): Springer.

Feng, P., Fuerer, C., & McMahon, A. (2017). Quantification of whey protein content in Infant formulas by sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CGE): single-laboratory validation, first fction 2016.15. *Journal of AOAC International*, 100(2), 510-521.

Fernandez, S., Jannin, V., Chevrier, S., Chavant, Y., Demarne, F., & Carrière, F. (2013). In vitro digestion of the self-emulsifying lipid excipient Labrasol® by gastrointestinal lipases and influence of its colloidal structure on lipolysis rate. *Pharmaceutical Research, 30*(12), 3077-3087.

Filer, L. J. (1971). Modified food starches for use in infant foods. *Nutrition Reviews, 29*(3), 55-59.

Flack, S., & Shaw, V. (2003). Infants\ Breast and bottle feeding. In Benjamin, C., (Eds Second Edition), *Encyclopedia of Food Sciences and Nutrition.* (pp. 3288-3293). Oxford: Academic Press.

Floris, R., Lambers, T., Alting, A., & Kiers, J. (2010). Trends in infant formulas: a dairy perspective. In M. W. Griffiths (Ed.), *Improving the safety and quality of milk, Volume 2: Improving quality in milk products* (pp. 454-474).

Fomon, S. J. (1993). *Nutrition of normal infants*: Mosby-Year Book, Inc.

Formon, S. J., Ziegler, E. E., Thomas, L. N., Jensen, R. L., & Filer, L. (1970). Excretion of fat by normal full-term infants fed various milks and formulas. *The American Journal of Clinical Nutrition, 23*(10), 1299-1313.

Foundation, B. N. (1992). *Unsaturated Fatty Acids: Nutritional and Physiological Significance: the Report of the British Nutrition Foundation's Task Force*. Chapman and Hall.

Fox, P. F., & McSweeney, P. L. (1998). *Dairy Chemistry and Biochemistry*. Springer.

Friedt, M., & Welsch, S. (2013). An update on pediatric endoscopy. *European Journal of Medical Research, 18*(1), 24.

Fu, T.-J., Abbott, U. R., & Hatzos, C. (2002). Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid:a comparative study. *Journal of Agricultural and Food Chemistry, 50*(24), 7154-7160.

Gallier, S., Tate, H., & Singh, H. (2012). In vitro gastric and intestinal digestion of a walnut oil body dispersion. *Journal of Agricultural and Food Chemistry, 61*(2), 410-417.

Gallier, S., Zhu, X. Q., Rutherfurd, S. M., Ye, A., Moughan, P. J., & 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), 3215-3223.

Gnoth, M. J., Kunz, C., Kinne-Saffran, E., & Rudloff, S. (2000). Human milk oligosaccharides are minimally digested in vitro. *The Journal of Nutrition, 130*(12), 3014-3020.

Goedhart, A. C., & Bindels, J. G. (1994). The composition of human milk as a model for the design of infant formulas: recent findings and possible applications. *Nutrition Research Reviews, 7*, 1-24.

Goldman, A., Goldblum, R., Atkinson, S., & Lönnerdal, B. (1989). *Protein and Non-protein Nitrogen in Human Milk*. CRC Press, Inc.

Goldman, A. S., Garza, C., Nichols, B. L., & Goldblum, R. M. (1982). Immunologic factors in human milk during the first year of lactation. *The Journal of Pediatrics, 100*(4), 563-567.

Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., & Alric, M. (2012). Relevance and challenges in modeling human gastric and small intestinal digestion. *Trends in Biotechnology, 30*(11), 591-600.

Gurr, M. I. (1981). Review of the progress of dairy science: human and artificial milks for infant feeding. *Journal of Dairy Research, 48*, 519-554.

Hamosh, M. (1990). *Lingual and gastric lipases: their role in fat digestion*. CRC Press, Inc.

Hamosh, M. (1994). Gastric and lingual lipases. *Physiology of the Gastrointestinal Tract, 3*, 1239-1253.

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

Hamosh, M. (2006). Enteral lipid digestion and absorption. In William W. H (Eds.), *Neonatal nutrition and metabolism* (pp. 350-368). *Cambridge University Press, Cambridge*.

Hamosh, M., Iverson, S. J., Kirk, C. L., & Hamosh, P. (1994). Milk lipids and neonatal fat digestion: relationship between fatty acid composition, endogenous and exogenous digestive enzymes and digestion of milk fat. *World Review of Nutrition and Dietetics, 75*, 86-91.

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

Hamosh, M., Sivasubramanian, K., Salzman-Mann, C., & Hamosh, P. (1978). Fat digestion in the stomach of premature infants: I. Characteristics of lipase activity. *The Journal of Pediatrics, 93*(4), 674-679.

Harzer, G., & Bindels, J. (1985). Changes in human milk immunoglobulin A and lactoferrin during early lactation. *Compositional and physiological properties of human milk. Elsevier, Amsterdam*, 171-177.

Haug, A., Hostmark, A. T., & Harstad, O. M. (2007). Bovine milk in human nutrition-a review. *Journal of Lipids in Health and Disease, 6*(25), 1-16.

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

Henderson, T., Hamosh, M., Armand, M., Mehta, N., & Hamosh, P. (2001). Gastric proteolysis in preterm infants fed mother's milk or formula. In D. Newburg (Ed.), *Bioactive Components of Human Milk* (Vol. 501, pp. 403-408). Springer US.

Hermoso, M., Tabacchi, G., Iglesia-Altaba, I., Bel-Serrat, S., Moreno-Aznar, L. A., García-Santos, Y., et al. (2010). The nutritional requirements of infants. Towards EU alignment of reference values: the EURRECA network. *Maternal and Child Nutrition, 6*, 55-83.

Hernell, O. (2011). Human milk vs. cow's milk and the evolution of infant formulas. In R. A. Clemens, O. Hernell and K. M. Michaelsen (Eds.), *Milk and Milk Products in Human Nutrition* (Vol. 67, pp. 17-28).

Hernell, O., Blackberg, L., & Bernback, S. (1988). *Digestion and absorption of human milk lipids.* In Bristol-Myers nutrition symposia. USA

Hoffman, D. R., Birch, E. E., Birch, D. G., Uauy, R., Castañeda, Y. S., Lapus, M. G., et al. (2000). Impact of early dietary intake and blood lipid composition of long chain polyunsaturated fatty acids on later visual development. *Journal of Pediatric Gastroenterology and Nutrition, 31*(5), 540-553.

Hur, S. J., Lim, B. O., Decker, E. A., & McClements, D. J. (2011). In vitro human digestion models for food applications. *Food Chemistry, 125*(1), 1-12.

International Union of Pure and Applied Chemistry, International Union of Biochemistry, 1982. Abbreviated terminology of oligosaccharide chains. *Journal of Pure and Applied Chemistry, 54*(8), 1517-1522.

Iyer, S., & Lönnerdal, B. (1993). Lactoferrin, lactoferrin receptors and iron metabolism. *European Journal of Clinical Nutrition, 47*(4), 232.

Jakobsson, I., Lindberg, T., & Benediktsson, B. (1982). In vitro digestion of cow's milk proteins by duodenal juice from infants with various gastrointestinal disorders. *Journal of Pediatric Gastroenterology and Nutrition, 1*(2), 183-192.

Jensen, M. L., Sangild, P. T., Lykke, M., Schmidt, M., Boye, M., Jensen, B. B., et al. (2013). Similar efficacy of human banked milk and bovine colostrum to decrease incidence of necrotizing enterocolitis in preterm piglets. *American Journal of Physiology- Regulatory, Integrative and Comparative Physiology, 305*(1), R4-R12.

Jensen, R. G. (1995). *Handbook of milk composition*. Academic Press.

Jensen, R. G. (1999). Lipids in human milk. *Lipids, 34*(12), 1243-1271.

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

Joeckel, R. J., & Phillips, S. K. (2009). Overview of infant and pediatric formulas. *Nutrition in Clinical Practice, 24*(3), 356-362.

Kamstrup, D., Berthelsen, R., Sassene, P. J., Selen, A., & Müllertz, A. (2016). In vitro model simulating gastro-intestinal digestion in the pediatric population (neonates and young infants). *AAPS PharmSciTech*, 18(2), 317-329.

Kitabatake, N.,& Kinekawa, Y.I. (1998). Digestibility of bovine milk whey protein and β-lactoglobulin in vitro and in vivo. *Journal of Agricultural and Food Chemistry, 46*(12), 4917-4923.

Klein, K. O. (1998). Isoflavones, Soy-based infant formulas, and relevance to endocrine function. *Nutrition Reviews, 56*(7), 193-204.

Koletzko, B., Agostoni, C., Carlson, S., Clandinin, T., Hornstra, G., Neuringer, M., et al. (2001). Long chain polyunsaturated fatty acids (LC-PUFA) and perinatal development. *Acta Paediatrica, 90*(4), 460-464.

Koletzko, B., Baker, S., Cleghorn, G., Neto, U. F., Gopalan, S., Hernell, O., et al. (2005). Global standard for the composition of infant formula: recommendations of an ESPGHAN Coordinated International Expert Group. *Journal of Pediatric Gastroenterology and Nutrition, 41*(5), 584-599.

Koletzko, B., Thiel, I., & Abiodun, P. O. (1992). The fatty acid composition of human milk in Europe and Africa. *The Journal of Pediatrics, 120*(4), S62-S70.

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

Kunz, C., & Rudloff, S. (1993). Biological functions of oligosaccharides in human milk. *Acta Paediatrica, 82*(12), 903-912.

Kunz, C., Rudloff, S., Baier, W., Klein, N., & Strobel, S. (2000). Oligosaccharides in human milk: structural, functional, and metabolic aspects. *Annual Review of Nutrition, 20*(1), 699-722.

Landers, S., & Hartmann, B. T. (2013). Donor human milk banking and the emergence of milk sharing. *Pediatric Clinics of North America, 60*(1), 247-260.

Lapillonne, A., Groh-Wargo, S., Lozano Gonzalez, C. H., & Uauy, R. (2013). Lipid needs of preterm infants: updated recommendations. *The Journal of Pediatrics, 162*(3), S37-S47.

Lauritzen, L., Hansen, H. S., Jorgensen, M., & Michaelsen, K. (2001). The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina. *Progress in Lipid Research, 40*(1), 1-94.

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

Lebenthal, E., & Lee, P. (1980b). Glucoamylase and disaccharidase activities in normal subjects and in patients with mucosal injury of the small intestine. *The Journal of Pediatrics, 97*(3), 389-393.

Lebenthal, E., Lee, P., & Heitlinger, L. A. (1983). Impact of development of the gastrointestinal tract on infant feeding. *The Journal of Pediatrics, 102*(1), 1-9.

Lee, P., Werlin, S., Trost, B., & Struve, M. (2004). Glucoamylase activity in infants and children: normal values and relationship to symptoms and histological findings. *Journal of Pediatric Gastroenterology and Nutrition, 39*(2), 161-165.

Li-Chan, E., & Nakai, S. (1989). Enzymic dephosphorylation of bovine casein to improve acid clotting properties and digestibility for infant formula. *Journal Dairy Research, 56*(3), 381-390.

Li, H. J., Zhu, K. X., Zhou, H. M., Peng, W., & Guo, X. N. (2013). Comparative study about some physical properties, in vitro digestibility and immunoreactivity of soybean protein isolate for infant formula. *Plant Foods for Human Nutrition, 68*(2), 124-130.

Lien, E. L. (2003). Infant formulas with increased concentrations of alpha-lactalbumin. *American Journal of Clinical Nutrition, 77*(6), 1555S-1558S.

Lien, E. L., Yuhas, R. J., & Boyle, F. G. (1993). Corandomization of fats improves absorption in rats. *Journal of Nutrition, 123,* 1859-1867

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

Lönnerdal, B. (2016). Bioactive proteins in human milk-potential benefits for preterm infants. *Clinics in Perinatology*, 44(1), 179-191.

Lönnerdal, B. (2003). Nutritional and physiologic significance of human milk proteins. *Amerian Journal of Clinical Nutrition, 77*(6), 1537-1543.

Lönnerdal, B. (1994). Nutritional aspects of soy formula. *Acta Paediatrica, 83*(402), 105-108.

Lönnerdal, B and Darragh, 2011. Human milk. In Fuquay, J.W. (Eds). *Encyclopedia of Dairy Sciences* (pp.581-590). Academic Press.

Lönnerdal, B. (2013). Bioactive proteins in breast milk. *Journal of Paediatrics and Child Health, 49*, 1-7.

Lönnerdal, B. (2014). Infant formula and infant nutrition: bioactive proteins of human milk and implications for composition of infant formulas. *The American Journal of Clinical Nutrition, 99*(3), 712S-717S.

López, A. M. (2007). Proteins in human milk. *Breastfeeding review: professional publication of the Nursing Mothers' Association of Australia, 15*(1), 5-16.

Lucas, A., Stafford, M., Morley, R., Abbott, R., Stephenson, T., MacFadyen, U., et al. (1999). Efficacy and safety of long chain polyunsaturated fatty acid supplementation of infant formula milk: a randomised trial. *The Lancet, 354*(9194), 1948-1954.

Mao, K., Du, H., Bai, L., Zhang, Y., Zhu, H., & Wang, Y. (2017). Poly (2-methyl-2-oxazoline) coating by thermally induced immobilization for determination of bovine lactoferrin in infant formula with capillary electrophoresis. Talanta, 168, 230-239.

Mason, S. (1962). Some aspects of gastric function in the newborn. *Archives of Disease in Childhood, 37*(194), 387-391.

Matsumoto, T. (2011). Mitigation of the allergenic activity of beta-lactoglobulin by electrolysis. *Pediatric Allergy and Immunology, 22*(2), 235-242.

Ménard, O., Cattenoz, T., Guillemin, H., Souchon, I., Deglaire, A., Dupont, D., et al. (2014). Validation of a new in vitro dynamic system to simulate infant digestion. *Food Chemistry, 145*(0), 1039-1045.

Merritt, R. J., & Jenks, B. H. (2004). Safety of soy-based infant formulas containing isoflavones: the clinical evidence. *The Journal of Nutrition, 134*(5), 1220-1224.

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

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

Miller, R. (1942). Gastric acidity during the first year of life. *Archives of Disease in Childhood, 17*(92), 198-209.

Minekus, M., Marteau, P., & Havenaar, R. (1995). Multicompartmental dynamic computer-controlled model simulating the stomach and small intestine. *Alternatives to Laboratory Animals: ATLA, 23*, 197-209.

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

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

Mock, D. M., Perman, J. A., Thaler, M. M., & Morris Jr, R. C. (1983). Chronic fructose intoxication after infancy in children with hereditary fructose intolerance: a cause of growth retardation. *New England Journal of Medicine, 309*(13), 764-770.

Moller, H. K., Thymann, T., Fink, L. N., Frokiaer, H., Kvistgaard, A. S., & Sangild, P. T. (2011). Bovine colostrum is superior to enriched formulas in stimulating intestinal function and necrotising enterocolitis resistance in preterm pigs. *British Journal of Nutrition, 105*(01), 44-53.

Moreau, H., Laugier, R., Gargouri, Y., Ferrato, F., & Verger, R. (1988). Human preduodenal lipase is entirely of gastric fundic origin. *Gastroenterology*, *95*(5), 1221-1226.

Murphy, G., & Signer, E. (1974). Bile acid metabolism in infants and children. *Gut, 15*(2), 151-163.

Nagita, A., Amemoto, K., Yoden, A., Aoki, S., Sakaguchi, M., Ashida, K., et al. (1996). Diurnal variation in intragastric pH in children with and without peptic ulcers. *Pediatric Research, 40*(4), 528-532.

Neumann, M. A., Simmer, K., & Gibson, R. A. (2000). A critical appraisal of the role of dietary long-chain polyunsaturated fatty acids on neural indices of term infants: a randomized, controlled trial. *Pediatrics, 105*(1), 32-38.

Nielsen, S. S. (1991). Digestibility of legume proteins. *Food Technology, 45*, 112-114.

N'Goma, J. C. B., Amara, S., Dridi, K., Jannin, V., & Carrière, F. (2012). Understanding the lipid-digestion processes in the GI tract before designing lipid-based drug-delivery systems. *Therapeutic Delivery*, *3*(1), 105-124.

Nik, A. M., Corredig, M., & Wright, A. J. (2010). Changes in WPI-stabilized emulsion interfacial properties in relation to lipolysis and ß-carotene transfer during exposure to simulated gastric-duodenal fluids of variable composition. *Food Digestion*, *1*(1-2), 14-27.

Nobigrot, T., Chasalow, F., & Lifshitz, F. (1997). Carbohydrate absorption from one serving of fruit juice in young children: age and carbohydrate composition effects. *Journal of the American College of Nutrition*, *16*(2), 152-158.

O'Callaghan, D. M., O'Mahony, J. A., Ramanujam, K. S., & Burgher, A. M. (2011). Dehydrated Dairy Products \ Infant Formulae. In John, W.F (Ed.), *Encyclopedia of Dairy Sciences (Second Edition)* (pp. 135-145). Academic Press.

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

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

Oomen, A., Rompelberg, C., Bruil, M., Dobbe, C., Pereboom, D., & Sips, A. (2003). Development of an in vitro digestion model for estimating the bioaccessibility of soil contaminants. *Archives of Environmental Contamination and Toxicology*, *44*(3), 0281-0287.

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

Patole, S. (2013). Developmental physiology of the gastrointestinal tract and feed intolerance in preterm neonates. In Patole, S (Ed.). *Nutrition for the Preterm Neonate* (pp. 3-23). Springer Netherlands.

Perry, D. L., Spedick, J. M., McCoy, T. P., Adams, M. R., Franke, A. A., & Cline, J. M. (2007). Dietary soy protein containing isoflavonoids does not adversely affect the reproductive tract of male cynomolgus macaques (Macaca fascicularis). *The Journal of Nutrition, 137*(6), 1390-1394.

Picciano, M. F. (2001). Nutrient composition of human milk. *Pediatric Clinics of North America, 48*(1), 53-67.

Pinto, M. S., Léonil, J., Henry, G., Cauty, C., Carvalho, A. F., & Bouhallab, S. (2014). Heating and glycation of β-lactoglobulin and β-casein: aggregation and in vitro digestion. *Food Research International, 55*(0), 70-76.

Plucinski, T. M., Hamosh, M., & Hamosh, P. (1979). Fat digestion in rat: role of lingual lipase. *American Journal of Physiology, 237*(6), G541-G547.

Poquet, L., & Wooster, T. J. (2016). Infant digestion physiology & the relevance of in vitro biochemical models to test infant formula lipid digestion. *Molecular Nutrition & Food Research*, 60(8),1876-1895.

Renneberg, R., & Skåra, B. (1992). Essential fatty acids in human colostrum. *Acta Paediatrica, 81*(10), 779-783.

Roman, C., Carriere, F., Villeneuve, P., Pina, M., Millet, V., Simeoni, U., et al. (2007). Quantitative and qualitative study of gastric lipolysis in premature infants: do MCT-enriched infant formulas improve fat digestion? *Pediatric Research, 61*(1), 83-88.

Rudloff, S., & Kunz, C. (1997). Protein and nonprotein nitrogen components in human milk, bovine milk, and infant formula: quantitative and qualitative aspects in infant nutrition. *Journal of Pediatric Gastroenterology and Nutrition, 24*(3), 328-344.

Roussel, A., de Caro, J., Bezzine, S., Gastinel, L., de Caro, A., Carrière, F., Leydier, S., Verger.,R, Cambillau, C. (1998). Reactivation of the totally inactive pancreatic lipase RP1 by structure-predicted point mutations. *Proteins: Structure, Function, and Bioinformatics, 32*(4), 523-531.

Sakai, K., Yoshino, K., Satter, M. A., Ota, F., Nil, Y., Fututa, K., et al. (2000). Effects of pH variation and NaCl on in vitro digestibility of cow's milk proteins in commercially available infant formulas. *Journal of Nutritional Science and Vitaminology, 46*(6), 325-328.

Sandström, O., Lönnerdal, B., Graverholt, G., & Hernell, O. (2008). Effects of α-lactalbumin enriched formula containing different concentrations of glycomacropeptide on infant nutrition. *The American Journal of Clinical Nutrition, 87*(4), 921-928.

SanGiovanni, J. P., Berkey, C. S., Dwyer, J. T., & Colditz, G. A. (2000). Dietary essential fatty acids, long-chain polyunsaturated fatty acids, and visual resolution acuity in healthy fullterm infants: a systematic review. *Early Human Development, 57*(3), 165-188.

Sato, R., Noguchi, T., & Naito, H. (1986). Casein phosphopeptide (CPP) enhances calcium absorption from the ligated segment of rat small intestine. *Journal of Nutritional Science and Vitaminology, 32*(1), 67-76.

Shah, N. P. (2000). Effects of milk-derived bioactives: an overview. *British Journal of Nutrition, 84*(S1), 3-10.

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

Sibley, E. (2004). Carbohydrate digestion and absorption. In Johnson, L. R. (Ed.), *Encyclopedia of Gastroenterology* (pp. 275-278). New York: Elsevier.

Sidnell, A., & Greenstreet, E. (2011). Infant nutrition-review of lipid innovation in infant formula. *Nutrition Bulletin, 36*(3), 373-380.

Sopade, P. A., & Gidley, M. J. (2009). A rapid in vitro digestibility assay based on glucometry for investigating kinetics of starch digestion. *Starch-Stärke, 61*(5), 245-255.

Souza, C. O., Leite, M. E. Q., Lasekan, J., Baggs, G., Pinho, L. S., Druzian, J. I., . . . Costa-Ribeiro, H. (2017). Milk protein-based formulas containing different oils affect fatty acids balance in term infants: A randomized blinded crossover clinical trial. *Lipids in Health and Disease*, 16(1), 78.

Stephen, A., Alles, M., De Graaf, C., Fleith, M., Hadjilucas, E., Isaacs, E., et al. (2012). The role and requirements of digestible dietary carbohydrates in infants and toddlers. *European Journal of Clinical Nutrition, 66*(7), 765-779.

Strom, B. L., Schinnar, R., Ziegler, E. E., Barnhart, K. T., Sammel, M. D., Macones, G. A., et al. (2001). Exposure to soy-based formula in infancy and endocrinological and reproductive outcomes in young adulthood. *JAMA: the journal of the American Medical Association, 286*(7), 807-814.

Stromqvist, M., Falk, P., Bergstrom, S., Hansson, L., Lönnerdal, B., Normark, S., et al. (1995). Human milk kappa-casein and inhibition of helicobacter pylori adhesion to human gastric mucosa. *Journal Pediatrics Gastroenterol Nutrition, 21*(3), 288-296.

Su, M.-Y., Broadhurst, M., Liu, C.-P., Gathercole, J., Cheng, W.-L., Qi, X.-Y., . . . Haigh, B. (2017). Comparative analysis of human milk and infant formula derived peptides following in vitro digestion. *Food Chemistry,* 221, 1895-1903.

Terracciano, L., Isoardi, P., Arrigoni, S., Zoja, A., & Martelli, A. (2002). Use of hydrolysates in the treatment of cow's milk allergy. *Annals of Allergy, Asthma and Immunology, 89*(6), 86-90.

Tetens, I. (2012). *EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA); Scientific Opinion on bovine lactoferrin: EFSA-Q-2010-01269*. European Food Safety Authority.

Thompkinson, D., & Kharb, S. (2007). Aspects of infant food formulation. *Comprehensive Reviews In Food Science And Food Safety*, *6*(4), 79-102.

Tomarelli, R., Meyer, B., Weaber, J., & Bernhart, F. (1968). Effect of positional distribution on the absorption of the fatty acids of human milk and infant formulas. *The Journal of Nutrition*, *95*(4), 583-590.

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

Turner, R. B., & Kelsey, D. K. (1993). Passive immunization for prevention of rotavirus illness in healthy infants. *The Pediatric Infectious Disease Journal*, *12*(9), 718-721.

Uauy, R., Mena, P., Wegher, B., Nieto, S., & Salem, N. (2000). Long chain polyunsaturated fatty acid formation in neonates: effect of gestational age and intrauterine growth. *Pediatric Research*, *47*(1), 127-127.

Uruakpa, F. O., Ismond, M. A. H., & Akobundu, E. N. T. (2002). Colostrum and its benefits: a review. *Nutrition Research*, *22*(6), 755-767.

Vandenplas, Y., De Greef, E., Devreker, T., & Hauser, B. (2011). Soy infant formula: is it that bad? *Acta Paediatrica*, *100*(2), 162-166.

Ville, E., Carrière, F., Renou, C., & Laugier, R. E. (2002). Physiological study of pH stability and sensitivity to pepsin of human gastric lipase. *Digestion*,*65*(2), 73-81.

Vors, C., Capolino, P., Guérin, C., Meugnier, E., Pesenti, S., Chauvin, M. A., ... & Michalski, M. C. (2012). Coupling in vitro gastrointestinal lipolysis and Caco-2 cell cultures for testing the absorption of different food emulsions. *Food & Function*, *3*(5), 537-546.

Wal, J. M. (2004). Bovine milk allergenicity. *Annals of Allergy, Asthma and Immunology*, *93*(5), 2-11.

Whitnah, C. H. (1931). Indications of Glucose in Milk. *Journal of the American Chemical Society*, *53*(1), 300-304.

Wickham, M., Faulks, R., & Mills, C. (2009). In vitro digestion methods for assessing the effect of food structure on allergen breakdown. *Molecular Nutrition and Food Research*, *53*(8), 952-958.

Willatts, P., Forsyth, J. S., DiModugno, M. K., Varma, S., & Colvin, M. (1998). Effect of long-chain polyunsaturated fatty acids in infant formula on problem solving at 10 months of age. *The Lancet*, *352*(9129), 688-691.

World Health Organization, UNICEF. (2003). *Global strategy for Infant and Young child feeding*. Retried from http://www.who.int/nutrition/publications/infantfeeding/9241562218/en/

Xu, R. (1996). Development of the newborn GI tract and its relation to colostrum/milk intake: a review. *Reproduction, Fertility and Development, 8*(1), 35-48.

Yoo, J. Y., & Chen, X. D. (2006). GIT physicochemical podeling-a critical review. *International Journal of Food Engineering, 2*(4).

Zhang, Q., & Carpenter, C. J. (2013). Proteomics in milk and milk processing. In *Proteomics in Foods* (pp. 223-245). Springer US.

Chapter 3 DEVELOPMENT AND VALIDATION OF A SIMPLE MODEL FOR THE *IN VITRO* GASTROINTESTINAL DIGESTION OF INFANT FORMULA

3.1. Introduction

In this chapter, the main objective was to set up an easy to operate bench-top digestion unit for routine investigation of *in vitro* digestion experiments. The following tasks were undertaken:

- The *in vitro* digestive unit was set up and instrumented with water bath, overhead stirrer, pH meter to help measure digestibility.

- The *in vitro* digestive unit was studied using a sample infant formula to evaluate the operation of the pH meter. The digestibility was calculated for an infant formulae with whey protein isolates and calcium caseinate in the ratio 6:4.

3.2. Materials and method

Bench-top *in vitro* digestion unit

The bench-top *in vitro* digestion unit was developed at the School of Agriculture and Food Sciences, University of Queensland. The flow diagram of the unit is as shown in Fig 3.1.

This model comprises of two water-jacketed reaction vessels. The water jacket allowed constant circulation of warm water in and out of the reaction vessel from a water bath thereby maintaining a constant temperature of 37°C, the vessels were enclosed to prevent evaporation. Each of the reaction vessels was connected to a pH meter that recorded pH of the digesta at regular intervals throughout the digestion process. The pH meter used a PC-based data acquisition system (Horiba F-50 & D-50 Software) that allowed real time monitoring of pH data and generated data logs, which were used for analysis of digestibility in MS-Excel. A glass stirrer connected to an overhead stirrer continuously mixed the *in vitro* digesta at 250 rpm. The stirrer speed was maintained at a speed higher than the peristalsis movement in the human gastrointestinal tract (50 rpm), to ensure complete mixing of all the ingredients in the reaction vessel. In their studies Pérez et al., 2014 and Oomen et al., 2002 have also operated the stirrer at a speed range 200-250 rpm to achieve uniform mixing.

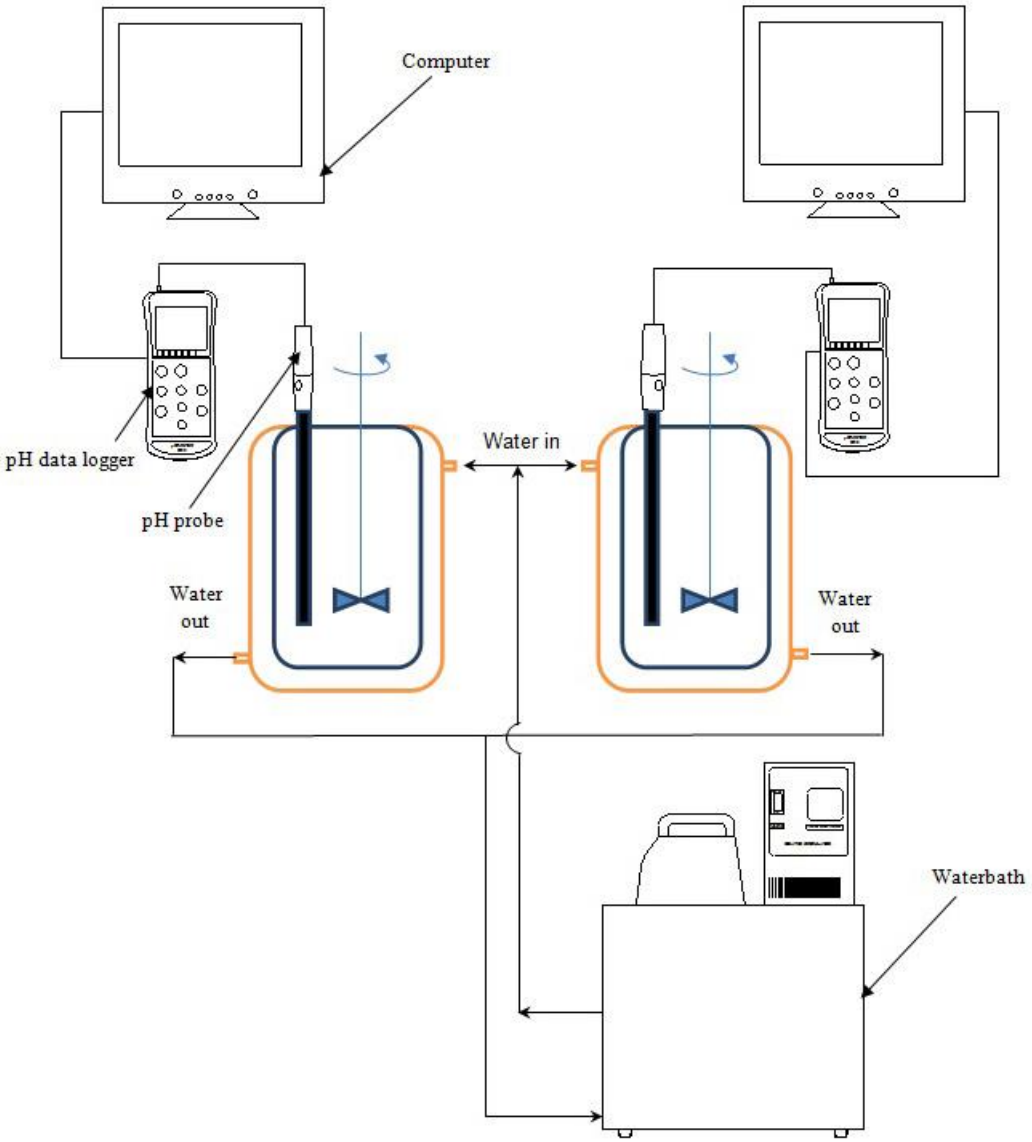


Fig 3.1 Flow diagram of the *in vitro* digestion unit

Digestibility Measurement

The pH of digesta after 10 min of digestion in the intestinal phase (X_1) was recorded to calculate the digestibility of protein in infant milk formula. The digestibility was calculated using the equation developed by Hsu, Vavak, Satterlee, and Miller (1977):

$$Digestibility = 210.46 - 18.10X_1 \qquad \text{equation (1)}$$

In vitro digestive unit preparation

Before starting an *in vitro* digestion experiment, the water bath was set to 37^0C, followed by circulating water in the water-jackets of the reaction vessels to maintain a constant temperature of 37^0C. The two pH meters were calibrated with pH indicators 4.0 and 7.0. The cables connecting the pH data logger to the computer was checked and then operated before starting the software to log the pH data.

Cleaning of the *in vitro* digestive unit

After finishing the digestion experiments, remaining digestion solution was discarded. The reaction vessels and the glass stirrer were soaked with 0.01M NaOH to remove the fat and protein from the vessel. The vessels were then rinsed with distilled water several times and then left to dry before the next experiment.

3.3. Results and Discussion

Evaluation of the *in vitro* digestive model was carried out with an infant formula containing whey protein isolates and calcium caseinate in the ratio 6:4.

The water bath was set to 37°C and within around 15 min, the temperature of the formula in the reaction vessel reached 37°C. The pH data logger and the software were all initiated to log the pH data. The total capacity of the reaction vessel is 150mL and the least volume that can be accommodated for a digestive study is 40mL. Samples were collected with ease from the reaction vessel at various stages of digestion. The pH dropped during the *in vitro* digestion trial with the experimental infant formula and the logger recorded the data successfully. The pH drop is as shown in Fig 3.2. With the experimental infant formula containing an WPI:CC (whey protein isolate:calcium caseinate) ratio of 6:4, the pH dropped from 8.0 to 7.63 after 10 min of the intestinal digestion. The protein digestibility calculated using equation (1) is 72.26.

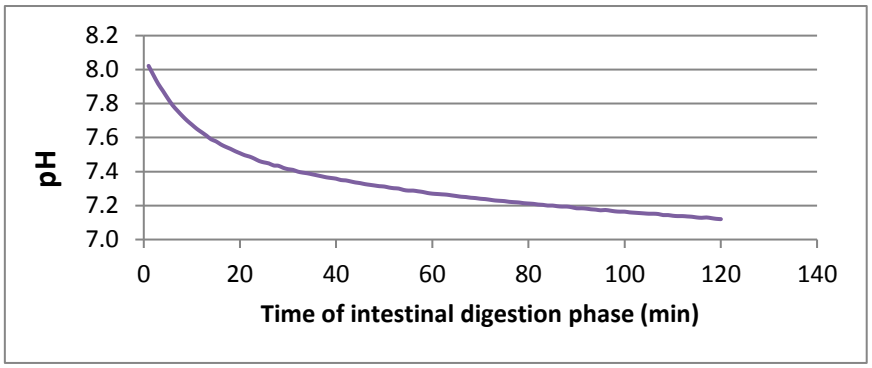


Fig 3.2 pH reducing during the *in vitro* intestinal digestion

3.4. Conclusions

The model digestive unit enables studying key digestion parameters such as pH, particle size, protein digestibility, lipid digestibility, structural and rheological changes in the digesta. The results of the trials with infant formula proved the suitability of the unit for carrying out digestion trials on relatively small quantities of sample formulas. This makes the *in vitro* digestion unit relatively economical, as it requires use of small quantities of enzymes and other chemicals. The instrumentation of the unit made it possible to monitor in real time the pH drop within the reaction vessels and enables studying the effect that different infant formula ingredients have on digestibility over time.

We propose that the bench-top *in vitro* digestion unit has the advantages of easy control and operation and furthermore could be an ideal tool for routine *in vitro* digestion studies.

3.5. References

Hsu, H., Vavak, D., Satterlee, L., & Miller, G. (1977). A multienzyme technique for estimating protein digestibility. *Journal of Food Science*, 42(5), 1269-1273.

Oomen, A. G., Hack, A., Minekus, M., Zeijdner, E., Cornelis, C., Schoeters, G., . . . Rompelberg, C. J. (2002). Comparison of five in vitro digestion models to study the bioaccessibility of soil contaminants. *Environmental Science & Technology*, 36(15), 3326-3334.

Pérez, O. E., David-Birman, T., Kesselman, E., Levi-Tal, S., & Lesmes, U. (2014). Milk protein–vitamin interactions: Formation of beta-lactoglobulin/folic acid nano-complexes and their impact on in vitro gastro-duodenal proteolysis. *Food Hydrocolloids*, 38, 40-47.

CHAPTER 4 GASTROINTESTINAL DIGESTION OF DAIRY AND SOY PROTEINS IN INFANT FORMULAE: AN *IN VITRO* STUDY

4.1. Introduction

Although mother's milk is the best food for infants, infant formula can become the alternative when breastfeeding is not possible or is discontinued for other reasons. Infant formulae supply infants with the nutrients needed for their adequate growth and development (Alles, Scholtens, and Bindels, 2004). Protein and essential amino acid requirement for infants are higher (per unit of body weight) than that for adults (Heird, 2012). Protein in infant formula should contain similar amounts of essential amino acids present in mother's milk (Heird, 2012). The current sources of proteins for infant formula are either cow's milk protein or soy protein, or their derivatives. Due to the difference in protein composition between mother's milk, cow's milk, and soy protein, infant formula based on cow's milk protein and soy protein isolate are modified to resemble mother's milk as much as possible. However, there are limited studies on the digestibility, rheology, and structural changes during digestion of various proteins used in the manufacture of infant formula.

It is well known that digestibility of protein in mother's milk is exceptionally high (Lönnerdal, 2003). Both mother's and cow's milk contain two types of proteins, namely whey and caseins. The whey: caseins ratio in mother's milk varies through the lactation stage with the ratio being 9:1 for colostrum (the first day of lactation), 6:4 for mature milk and 5:5 for late lactation (Kunz and Lönnerdal, 1992). In contrast, whey: caseins ratio in cow's milk is 2:8 which is much lower than that in mother's milk (Thompkinson and Kharb, 2007). This lower proportion of caseins and higher proportion of whey makes the protein in mother's milk easier to digest because caseins clot in the stomach under condition of gastric acidity. This casein precipitation leads to its longer stay time in the infant stomach as compared to whey protein, which is more soluble (Gurr, 1981; Hernell, 2011; Thompkinson and Kharb, 2007). In addition, the difference in the composition of whey protein in mother's and cow's milk could be the cause for difference in digestibility of this protein. While, β-lactoglobulin is not at all present in mothers' milk, it is the dominant whey protein in cow's milk that accounts for approximately 50% of total bovine whey protein (Gurr, 1981). The whey protein dominant in human milk is α-lactalbumin which accounts for 41% of whey and 17-28% of the total protein, while in bovine milk it only accounts for only 3-3.5% of total protein (Gurr, 1981; Heine, Klein, and Reeds, 1991). It has also been reported that β-lactoglobulin and α-lactalbumin resist *in vitro* stomach

digestion at different gastric pH (Astwood, Leach, and Fuchs, 1996; Chatterton, Rasmussen, Heegaard, Sørensen, and Petersen, 2004; Dupont et al., 2010a, Kitabatake and Kinekawa, 1998).

Soy protein based infant formula is used as a breastfeeding substitute for infants allergic to milk protein or for religious, philosophical, or ethical reasons (Agostoni et al., 2006). Although soybean protein quality has been ranked to be as high as cow's milk protein based on the Protein Digestibility-Corrected Amino Acid Scores (PDCAAS) (Schaafsma, 2000; Hughes, Ryan, Mukherjea, and Schasteen, 2011), it has a lower nitrogen conversion factor hence the protein content calculated from the total nitrogen content for soy protein is lower than that for cow's milk protein (Agostoni et al., 2006). Also, soybean protein and cow's milk protein have different amino acid composition profiles. Soy protein contains lower content of methionine, branched-chain amino acids (BCAA) essential for infants growth and development, lysine and proline, and higher amounts of aspartate, glycine, arginine, and cystine than cow's milk protein (Bos et al., 2003; Agostoni et al., 2006). Hence, for normal growth in infants it has been recommended to add methionine to soy infant formula (Fomon, Ziegler, Filer, Nelson, and Edwards, 1979; Agostoni et al., 2006). Digestibility of soy protein has also been reported to be lower than that for cow's milk hence the minimum protein content recommended by the European Union for soy infant formula is 2.25 g/100 kcal as opposed to 1.8 g/100 kcal for cow's milk protein (Agostoni et al., 2006).

An *in vitro* digestion model is a common model which offer many advantages (less expensive, no ethical issues, easy sampling accessibility) over *in vivo* models to understand the digestibility and structural changes of ingested food under simulated physiological conditions in the human gastrointestinal tract (Hur, Lim, Decker, and McClements, 2011). However, there are very few *in vitro* protein digestion studies on human infants with those present in the literature mainly on digestibility of the different proteins such as caseins and β-lactoglobulin (Dupont et al., 2010a; Dupont et al., 2010b). Normally the gastric juice in infants is acidic and contains only pepsin, lipase enzyme, while the intestinal juice is more alkaline with bile salts and more enzymes to digest protein, fat, and carbohydrate (Hamosh, 1996). The composition of infant digestive juices is different compared to that of adult digestive juices. Adult digestive juice has a much lower gastric pH than infant gastric pH and differs in the concentration of enzymes in both gastric and intestinal juices. Recently, Dupont et al. (2010b) set up an *in vitro* protein digestion model for infants with the gastric and intestinal phases using commercial enzymes, bile salts, and surfactants. The concentration of the enzymes, bile salts, and surfactants were based on the available references for infants' gastrointestinal

system. They investigated the effect of heat treatment on purified caseins digestion in infants and the allergic response of formed peptides over 60 minutes in the stomach and 30 minutes in the small intestine. In another study, Dupont et al. (2010a) compared the resistance of purified β-lactoglobulin and β-casein under *in vitro* adult and infant digestion models. They observed β-casein digested quickly after 10 minutes in the stomach of infant model, but β-lactoglobulin remained stable and were only hydrolysed in the small intestine phase. On the other hand, the purified caseins from raw and processed milk (pasteurized) disappeared in the infant gastric phase after 20-40 minutes (Dupont et al., 2010a). In another study, Böttger, Etzel, and Lucey (2013) used the same infant gut models reported by Dupont et al (2010a, 2010b) with some modifications, by extending the intestinal phase to 180 minutes and using pancreatin instead of trypsin and chymotrypsin. They studied the behaviour of whey protein-dextran glycates under simulated infant digestion and observed β-lactoglobulin to be resistant to gastric digestion while native α-lactalbumin rapidly cleaved.

The gastric pH is a very critical consideration while studying infant *in vitro* models and is based on the fasting or fed condition. Hence, different researchers have taken this into account while designing the *in vitro* models. Li-Chan and Nakai (1989) observed the gastric pH in the infant stomach to be between 4 and 5 after two hours of feeding while Nagita et al. (1996) studied the gastric pH during fasting condition and noticed a pH of 3.0-4.0 in neonates and 1.5-3.0 in infants. In 2010, Lönnerdal (2010) used a pH between 3.5 and 5.0 to simulate the infant stomach condition from newborn (pH 5) to 4-6 month-infants (pH 3.5). In a recent study, Lönnerdal (2013) again used a pH 3.5 to mimic *in vitro* stomach digestion in infants. Dupont et al (2010a, 2010b) and Böttger et al. (2013) used a gastric pH of 3.0 for newborns and this possibly could be a study under fasting condition. All of the above studies indicate that the infant gastric pH under the fed condition should be higher than 3.0.

There are no systematic studies in the literature focussed on the digestion of various types of proteins and their physical changes during their passage through the digestive tract. Hence, the main aim of this work was to enhance further understanding on the physical and digestive properties of proteins that have been potentially used in infant formulae. With all the above background information the objectives of the current study were designed:

a) To understand and compare the digestibility of dairy and soy proteins in infant formulae in the absence of lipolytic enzymes.
b) To understand the microstructural changes of infant formulae with an *in vitro* digestive model

4.2. Materials and method

4.2.1. Bench-top *in vitro* digestion unit

A static *in vitro* digestion unit equipped with water bath, overhead stirrer, and pH meter was used for this study. Details and the flow diagram of the bench-top *in vitro* digestion unit was as shown in section 3.2.

4.2.2. Enzymes and chemicals

All enzymes used for the experimental trials were obtained from Sigma-Aldrich, Castle Hill, New South Wales, Australia. Pepsin from porcine gastric mucosa (EC 3.4.23.1, 3840 units/mg protein, one unit will produce a change in A_{280} of 0.001 per min at pH 2.0 at 37°C, measured as TCA-soluble products using hemoglobin as substrate). Trypsin from bovine pancreas (EC 3.4.21.4, 13165 units/mg protein, one unit will produce a change in A_{253} of 0.001 per minute at pH 7.6 at 25°C using Nα-Benzoyl-L-arginine Ethyl Ester (BAEE) as a substrate. Chymotrypsin from bovine pancreas (EC 3.4.21.1, 54.49 units/mg protein, one unit will hydrolyze 1.0 μmol of N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE) per min at pH 7.8 at 25°C as stated by manufacturer). All the above enzymes were stored at -20°C.

Bile salt used contained sodium taurocholate and was obtained from Sigma-Aldrich, Castle Hill, New South Wales, Australia and sodium glycodeoxycholate was obtained from Merck, Kilsyth, Victoria, Australia. Pepstatin and trypsin-chymotrypsin inhibitor obtained from Sigma-Aldrich, Castle Hill, New South Wales, Australia were stored between 2-8°C.

The other ingredients used in the study such as lactose, sodium chloride, hydrochloric acid, sodium hydroxide, and sodium azide were at analytical grade.

4.2.3 Dairy and soybean proteins

Whey protein isolate (WPI 85.1% protein, 1.0% fat, 1.2% carbohydrate) and calcium caseinate (CC 86.7% protein, 1.0% fat, 0.1% carbohydrate) were purchased from Total Foodtec (Australia). Soy

protein isolate (SPI 83.0% protein, 0.5% fat, 3.0% carbohydrate) was purchased from Food Manufacturers Pty (Australia). Sunflower vegetable oil was obtained from a local supermarket.

4.2.4. Preparation of infant milk formulae

100 mL of mother's milk contains 0.9-1.2 g of protein, 3.2-3.6 g of lipid, and 6.7-7.8 g of lactose (Ballard & Morrow, 2013). The quantity of protein, lipid, and lactose used in our formulae was based on the recommendation for infant formula from the European Union (Koletzko et al., 2005) that uses cow and soy proteins. Therefore, 100 mL of liquid formula containing 1.5 g of protein, 4.0 g of lipid and 6.5 g of lactose was chosen. The amount of protein recommended by the European Union is higher than that in mother's milk due to the difference in amino acid profile between mother's milk, cow's milk and soy protein. Preliminary screening of the commercial infant formula available in Australia suggests they are mostly dairy (whey and caseins based in the ratio 6:4, 4:6 and 2:8) or soy based. Hence, the same whey to caseins ratios, and soy protein isolate values were used to make infant formulae in our study. The measured quantity of WPI and CC in the ratio of 6:4, 4:6, and 2:8 were mixed to achieve the final 1.5 g protein/100 mL in cow's milk protein formulas. For soy formula, the same protein content of 1.5 g soy protein isolate/100 mL was used.

The step-by-step preparation of infant formula is as shown in Fig 4.2. The mixtures of WPI and CC were then mixed with deionised water and left overnight for rehydration at room temperature. After rehydration in water, vegetable oil (4.0 g/100 mL) and lactose (6.5 g/100 mL) were mixed uniformly using Silverson at 5000 rpm (Multimix) immediately before transfer to homogenizer at 5/25 MPa (Twin Panda 400, GEA). The liquid formulae was kept at 4 ºC for a maximum two days with the addition of sodium azide (0.02% w/v) (Gallier, Ye, and Singh, 2012).

4.2.5. *In vitro* infant protein digestion

The bench-top *in vitro* digestive unit (as shown in Fig 3.1, chapter 3) was used to carry out the *in vitro* digestion. The two-step digestion procedure of gastric and intestinal phase was performed in the water-jacketed reactors at 37°C by continuous stirring at 250 rpm. The concentration of enzymes and bile salts used were prepared following the method reported by Dupont et al. (2010b). The flow diagram of *in vitro* protein digestion in infants is summarised in Fig 4.2.

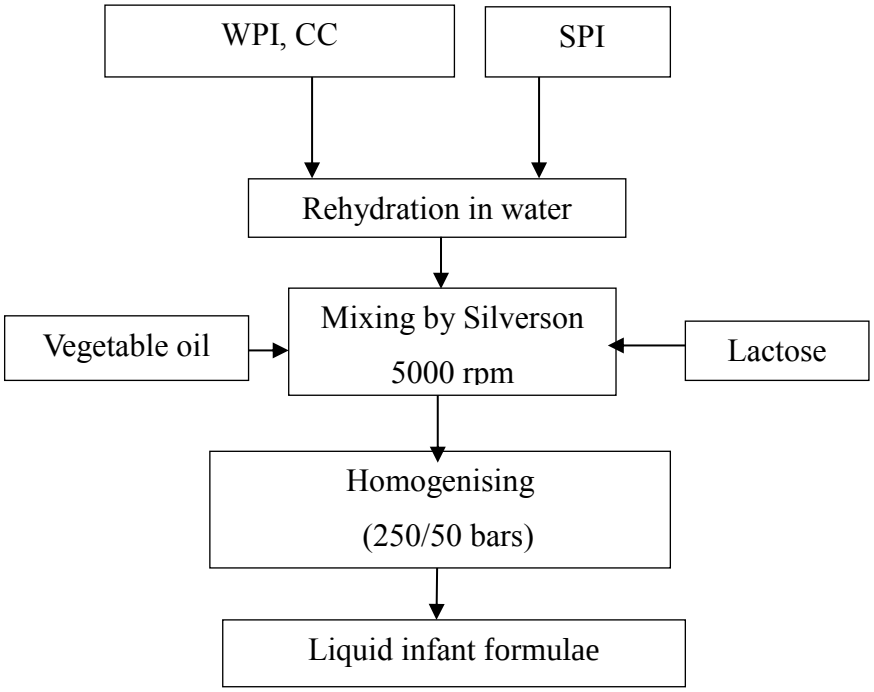


Fig 4.1 Schematic diagram of making infant milk formulae

4.2.5.1. Gastric digestion

Normal gastric pH in infants is between 4 and 5 (Agunod, Yamaguchi, Lopez, Luhby, and Glass, 1969; Lönnerdal and Lien, 2003). In this study, pH 4.0 was chosen to simulate the infant gastric condition. Simulated gastric juice was prepared by using 0.15M NaCl solution with its pH adjusted to 4.0 by adding 0.1M HCl. The liquid infant formula was mixed with this simulated gastric juice in the ratio 2:1 (v/v) and then the pH was readjusted to 4.0. The mix was then loaded to the water-jacketed reactor vessel with continuous stirring until the temperature reached 37 ^{o}C (about 15 min), following which the enzyme pepsin in gastric juice was added to give 22.75 U/mg of total proteins, and gastric digestion commenced. The stomach digestion lasted for 60 min and digesta samples were collected at the start and after 30 and 60 min of digestion for gel electrophoresis, particle size, and structural distribution. Immediately after sample collection, pepsinolysis was stopped by adding 0.85 μM of pepstatin to inhibit the equivalent amount of pepsin in the sample (Rich and Sun, 1980).

4.2.5.2. Intestinal digestion

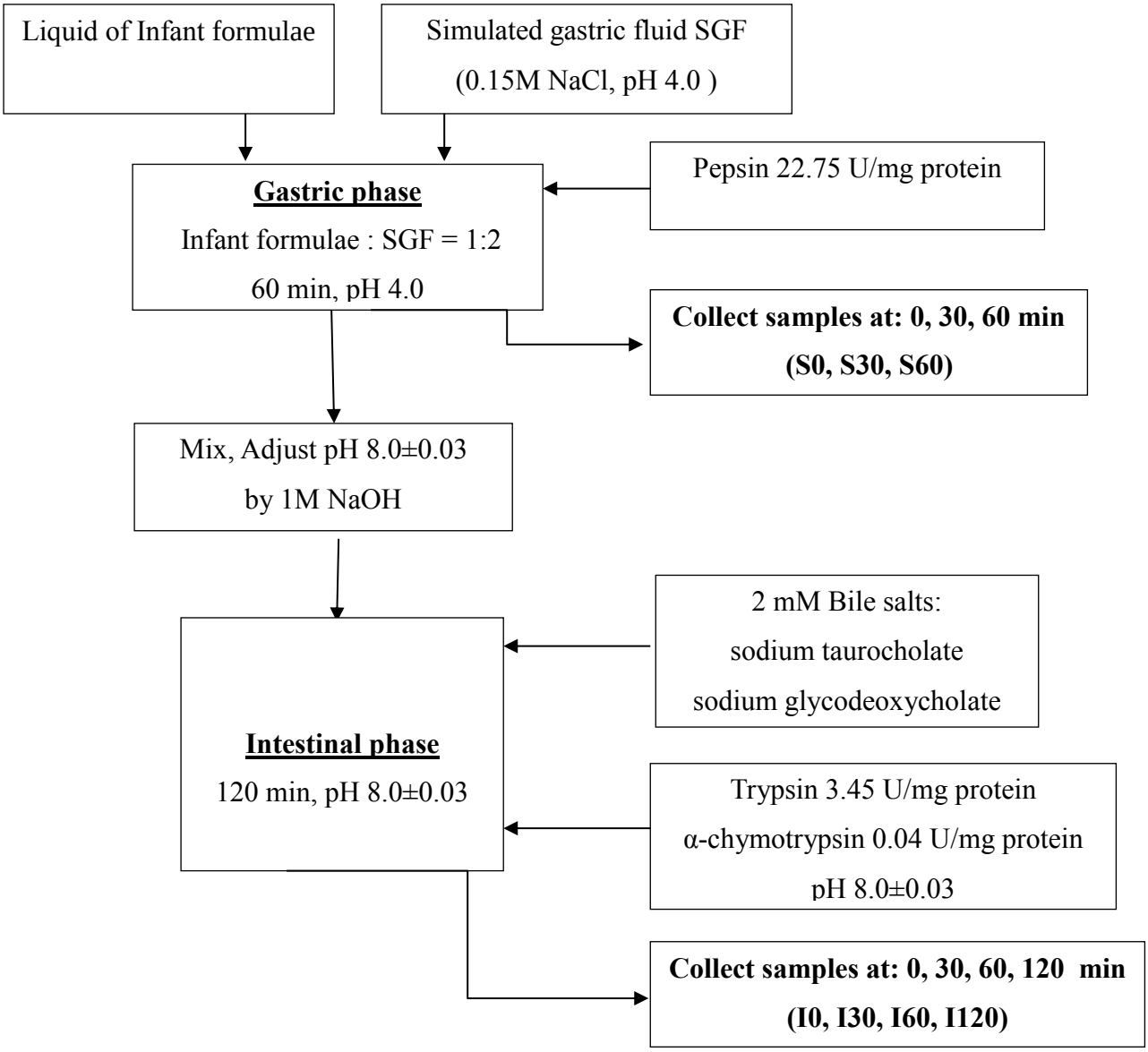


Fig 4.2 Flow diagram of *in vitro* protein digestion in infants

The intestinal digestion phase was carried out with the remaining of the 60 min gastric digesta as the starting material. The pH of the digesta was adjusted to 8.0±0.03 by drop wise addition of 1M NaOH. The bile salt mixture containing equimolar quantities of sodium taurocholate and sodium glycodeoxycholate in the solid form were added to the digesta to give the final concentration of 2 mM, then the pH was readjusted to 8.0±0.03. Following this, trypsin (3.45 U/mg of total protein) and α-chymotrypsin (0.04 U/mg of total protein) were added to the digesta. These enzymes were adjusted to pH 8.0±0.03 by adding simulated intestinal juice (0.15M NaCl, pH 8.0±0.03) at the temperature of digestion (37°C), and the intestinal phase of digestion started immediately after their addition to the digesta.

The digested samples were collected at the start (0 min) and after 30, 60, and 120 min of intestinal digestion for gel electrophoresis, particle size, and microstructural analysis. Trypsin-chymotrypsin inhibitor was added at a concentration (0.82 μM) to inhibit twice the amount of trypsin and chymotrypsin in the sample (Benedé et al., 2014).

4.2.6. Protein digestibility assay - pH drop method

The pH drop method was used to determine the rate of digestibility of the infant formulas with various whey-to-caseins ratios, and soy protein isolate (Nguyen, Gidley, and Sopade (2015) and Bassey, Mcwatters, Edem, and Iwegbue (2013). The pH method adopted in this study as described in Almaas et al. (2006) with a slight modification.

After adding the enzymes at the intestinal phase, the pH decreased rapidly below the adjusted value due to the breakdown of proteins into amino acids and peptides. The pH was measured every minute over a period of two hours. Each infant formula trial was duplicated and three repeated measurements were collected from one formula. The values used for analysis were taken from an average of three repeated measurements from duplication.

Digestibility of each formula was calculated based on the pH after 120 min of digestion (X1) using the equation developed by Hsu, Vavak, Satterlee, and Miller (1977):

$$\text{Digestibility} = 210.46 - 18.10X1 \qquad \text{(Eq. 2.1)}$$

4.2.7. Gel electrophoresis (SDS-PAGE)

Gel electrophoresis is a convenient method that provides an overview of initial stages of protein digestion and the corresponding formation of large peptides with molecular weight > 3.5 kD (Mills et al., 2013). Researchers commonly use this technique to determine the rate of digestion of individual protein components (Dupont et al., 2010a; Gallier, Ye, & Singh, 2012). The protein profile of the digested milk samples at different stages of the gastric and intestinal phase was assayed by reducing SDS-PAGE running on a Mini Protean 3 cell (Bio-Rad) for 37 minute at 200V. The assay was performed according to the protocol described by Laemmli (1970), using 4-20% Tris-HCl precast gel, protein ladder. The gels were run in duplicates for all samples collected during different stages of digestion. Each volume of sample was mixed with four volumes of sample buffer, which contains

0.0625M Tris-HCl buffer pH 6.8, 40% glycerol, 2% SDS, 0.04% bromophenol blue, and β-mercaptoethanol (19:1, v/v). The mixture was heated at 95°C for 5 min then loaded to the wells (10 µL was loaded for both gastric and intestinal phase). Gels scanning was done by densitometry and analysed by Quantity One software.

Hydrolysis of each protein was determined using the equation described by Kim and Barbeau (1991) with slight modification to the time of digestion. In their work, Kim and Barbeau (1991) carried out the digestion phase for 8 hours. However, it is very common to study *in vitro* digestion of milk with 30-60 minutes in gastric phase and 120 minutes in intestinal phase (Chatterton et al., 2004; Almaas et al., 2006; Ohsawa et al., 2008). Also, preliminary works showed the drastic changes happened in the initial stages of digestion. Hence, we carried out digestion study for three hours.

$$\text{Protein degradation \%} = \frac{\text{total peak area of undigested sample} - \text{total peak area of digested sample}}{\text{total peak area of undigested sample}}$$

(Eq 2.2)

4.2.8. Particle size distribution

Particle size distribution of native and digested milk samples were measured before and during *in vitro* gastric and intestinal digestions by Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershine, UK). The refractive index of milk value is 1.35 was used for the dispersed phase and 1.33 for water for the continuous phase. Samples were diluted in deionised water in the measurement cell of the equipment until the obscuration reached 15%. The particle size values were measured as d(0.1), d(0.5), d(0.9) and D[4,3]. The first three values indicate the size of the population of the particles existing below 10, 50, 90% of the total number of particles. D[4,3] is a volume mean of the population which is sensitive to the presence of large particles. Mean particle sizes and distribution were determined as the average of three repeated measurements from duplication.

4.2.9. Confocal Laser Scanning Microscopy (CLSM)

The physical arrangement of protein and fat globules of native and digested sample were observed by Zeiss LSM 700 Confocal Laser Scanning Microscope. Protein were stained with Rhodamine B (1% w/w in MiliQ water) and excited with the laser light at a wavelength 540 nm (Nagano, Tamaki, and Funami, 2008; van de Velde, Weinbreck, Edelman, van der Linden, and Tromp, 2003; van Riemsdijk, Sprakel, van der Goot, and Hamer, 2010). Nile red (0.1% w/w in acetone) was used to

stain triglycerides and excited with the laser light wavelength of 515-530 nm (Gallier, Ye, &Singh, 2012; Ye, Cui, and Singh, 2011).

For slide preparation, 100 µl of infant formula samples was mixed with 25 µl of Rhodamine B or 10 µl of Nile red solution by using vortexer (Ratex VM1) for 5 sec. Samples were stained for at least 10 minutes. 10 µl of stain samples was loaded onto 26x76 mm slides (Sail Brand) and then covered with 18x18 mm cover slip (Menzel Glaser). The edges of the cover slips were coated with a transparent nail polish to fix the sample position and prevent the sample from drying. The observations for fat globules and the breakdown of protein aggregation was done with a magnification lens at 63x and 10x, respectively.

4.2.10. Statistical analysis

The samples for pH drop were measured in triplicate from duplication. Experimental data were assessed by ANOVA tests to determine the significant differences among the means at 95% confident level. The treatment means were considered to be significantly different when $P<0.05$.

4.3. Results and discussion

4.3.1. Protein digestion determined by SDS-PAGE

4.3.1.1. Dairy protein (whey protein and caseins)

Fig 4.3 (A-C) presents the PAGE patterns of the three different dairy milk formulae (WPI and CC in the ratio 6:4, 4:6 and 2:8) at 0, 30 and 60 min of stomach digestion and at 0, 30, 60, 120 min of intestinal digestion. After one hour of gastric digestion with pepsin, less than 20% of caseins was hydrolysed (calculated using equation 2). This is also indicated by the intensity of the bands at a molecular weight of approximately 23 and 24 kDa for α- and β-casein, respectively, that show a slight decrease in intensity towards the end of one hour (Fig 4.3, S60). Similar observations were reported by Sakai et al. (2000). In the intestinal phase, the enzymes trypsin and chymotrypsin completely digested α–casein and β–casein. The bands markedly became faint at point I0 and completely disappeared soon after, between I30- I120, Fig 4.3 (A-C).

The bands of whey proteins, α–lactalbumin and β–lactoglobulin observed at molecular weights of approximately 14.4 kDa and 18 kDa completely resisted proteolysis by pepsin during the duration of digestion in the stomach (Fig 4.3, S60). However, in the intestinal phase, while α–lactalbumin was partly hydrolysed (less than 8% hydrolysed), β–lactoglobulin was completely digested after only 30 min of digestion for the three different formulae [Fig 4.3 (A-C)]. This indicates that the β–lactoglobulin was completely hydrolysed by trypsin and chymotrypsin, as observed in an earlier study by Kitabatake & Kinekawa, (1998). The negligible digestion of β-lactoglobulin during one hour in stomach at pH 1.5-7.0 has also been reported in earlier studies (Li, Zhu, Zhou, Peng, and Guo, 2013; Inglingstad et al., 2010; Chatterton et al., 2004; Sakai et al., 2000, Kitabatake & Kinekawa, 1998; Astwood et al., 1996).

The limited digestion of α-lactalbumin under simulated gastric digestion as observed in this study has also been observed earlier by researchers. Jakobsson, Lindberg, & Benediktsson (1982) reported that only 1 mg of α-lactalbumin was digested as opposed to 30 mg of caseins under the same condition: at pH 4.5-5.0 (normal gastric pH of infants) or at pH 1.5-2.0 which is optimal for pepsin.

Sakai et al. (2000) studied the *in vitro* digestibility of α-lactalbumin of commercial infant formula in the stomach at pH 1.5-4.0 and observed that α-lactalbumin hydrolysed at pH 1.5-2.5 but it was resistant to proteolysis at pH above 3.0. Similar results were obtained during a human newborn *in vivo* digestion study by Chatterton et al. (2004). It can be seen that α-lactalbumin significantly resists *in vitro* digestion and it is likely that α-lactalbumin in both human and cow's milk have the same *in vitro* digestibility pattern. Even during the intestinal digestion, α–lactalbumin is only partially hydrolysed as the bands for α–lactalbumin are still visible. Similar results at pH > 3 have been reported by Chatterton et al. (2004) and Sakai et al. (2000) and are attributed to the absence of peptidases enzymes in the duodenum that is responsible for complete hydrolysis of α–lactalbumin (Lönnerdal, 2013).

In disparity to *in vitro*, *in vivo* studies on digestibility of α-lactalbumin suggest complete digestion in the upper part of the gastrointestinal tract such as the stomach and duodenum (Davidson and Lönnerdal, 1987 and Donovan, Atkinson, Whyte, and Lönnerdal, 1989) with no intact α-lactalbumin detected in the stool sample of preterm and term infants fed on mother's milk. Heine, Radke, Wutzke, Peters, and Kundt (1996) also observed similar levels of plasma tryptophan (α-lactalbumin has high proportion of tryptophan) in infants fed on mother's and formula enriched with α-lactalbumin. In

addition, Lien *et al*., (2004) reported comparable growth rates and serum albumin content between the infant groups feeding on standard formula and enriched α-lactalbumin formula. All these above studies indicate complete hydrolysis of α-lactalbumin during *in vivo* gastrointestinal digestion study. However comparison of *in vitro* and *in vivo* studies should be treated with caution as there is a constant influx of enzymes with digestion and adsorption taking place simultaneously in the *in vivo* system as opposed to *in vitro* studies.

4.3.1.2 Soy protein

The sequential PAGE patterns of soy based infant formulae after 1 h of gastric digestion with pepsin and 2 h of intestinal digestion with trypsin, chymotrypsin and bile salts are as shown in Fig 4.3 (D). Soy protein contains β-conglycinin with three subunits (α: 76 kDa, α':72 kDa, β: 53 kDa) and glycinin with acidic polypeptide (31- 45 kDa) and basic polypeptide (18-20 kDa). This was also reported in earlier studies (Brooks and Morr, 1985; Shuttuck-Eidens and Beachy, 1985; Thanh and Shibasaki, 1977). The intensity of the band for β-conglycinin, acidic polypeptide, and basic polypeptide decreased with increasing incubation time in the stomach [Fig 4.3(D)] indicating partial hydrolysis of these proteins by pepsin. The degradation of these polypeptides were at 63%, 78%, and 60% respectively after 1 hour in gastric phase. The hydrolysis of β-conglycinin, acidic polypeptide, and basic polypeptide progressed in the simulated intestinal phase, these proteins indicated by lighter bands from I30 to I120. As hydrolysis progressed, a large amount of small peptides were formed at approximately 20 kDa.

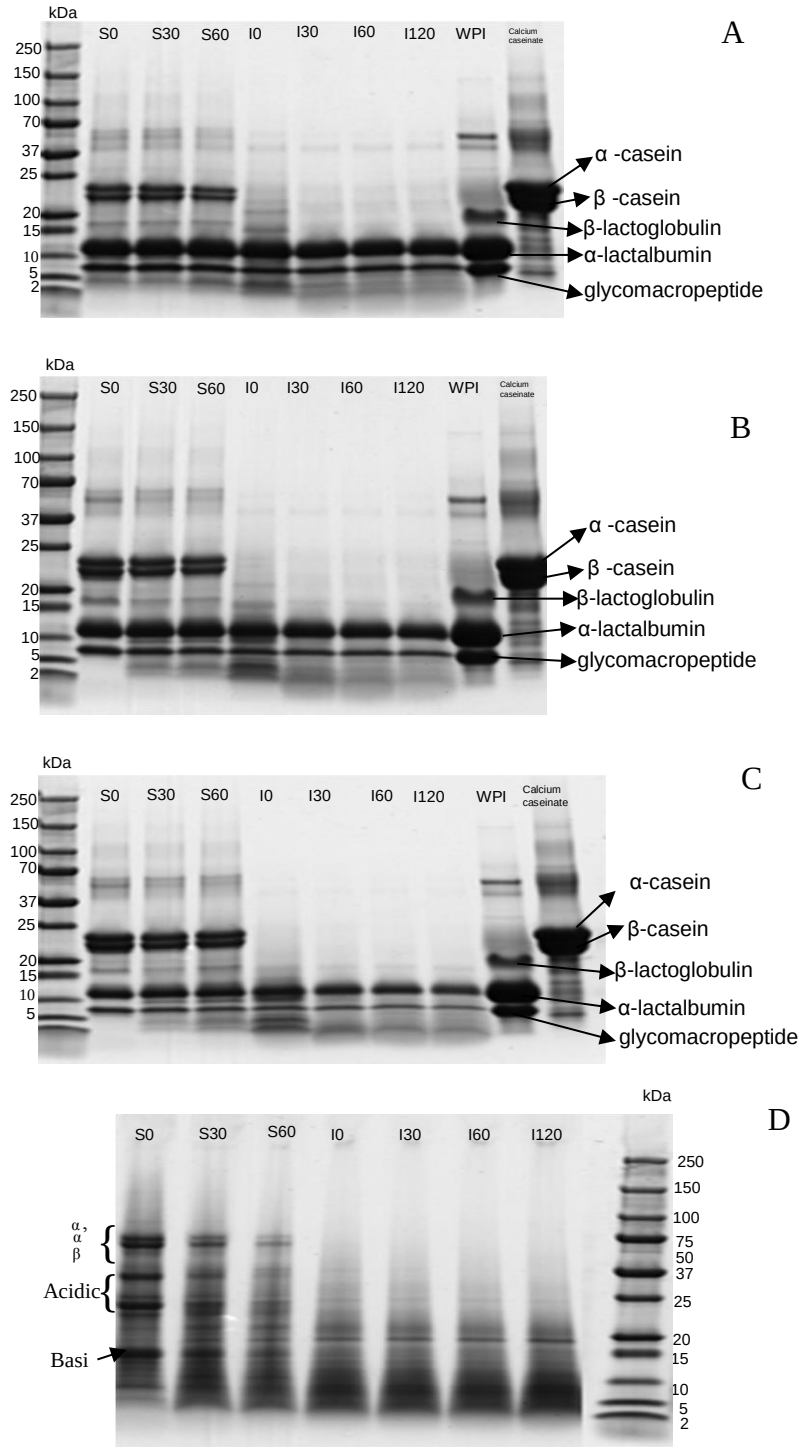


Fig 4.3 Reducing SDS-PAGE analysis of *in vitro* digested samples of the four infant milk formulae: WPI:CC=6:4 (A), WPI:CC=4:6 (B), WPI:CC=2:8 (C), and 100% SPI (D) during gastric phase from 0 min (S0) to 60 min (S60) and intestinal phase from 0 min (I0) to 120 min (I120).

4.3.2. Digestibility assay - pH drop method

Table 4.1 illustrates the *in vitro* digestibility rate of the four infant formulae calculated using equation 1. It was found that the digestibility rate is highest for formulae with a higher proportion of caseins (formulae with whey to caseins ratio of 2:8) and least for soy protein formulae.

Table 4.1 *In vitro* digestibility of the four infant formulae WPI:CC 6:4, WPI:CC 4:6, WPI:CC 2:8, and SPI.

Sample	***In vitro digestibility***
100% SPI	76.4 ± 0.1^{d}
WPI:CC = 6:4	81.5 ± 0.1^{c}
WPI:CC = 4:6	84.3 ± 0.2^{b}
WPI:CC = 2:8	86.4 ± 0.1^{a}

Mean values of digestibility that do not share the same letter are significantly different at $P<0.05$. Triplicate samples were measured from duplication.

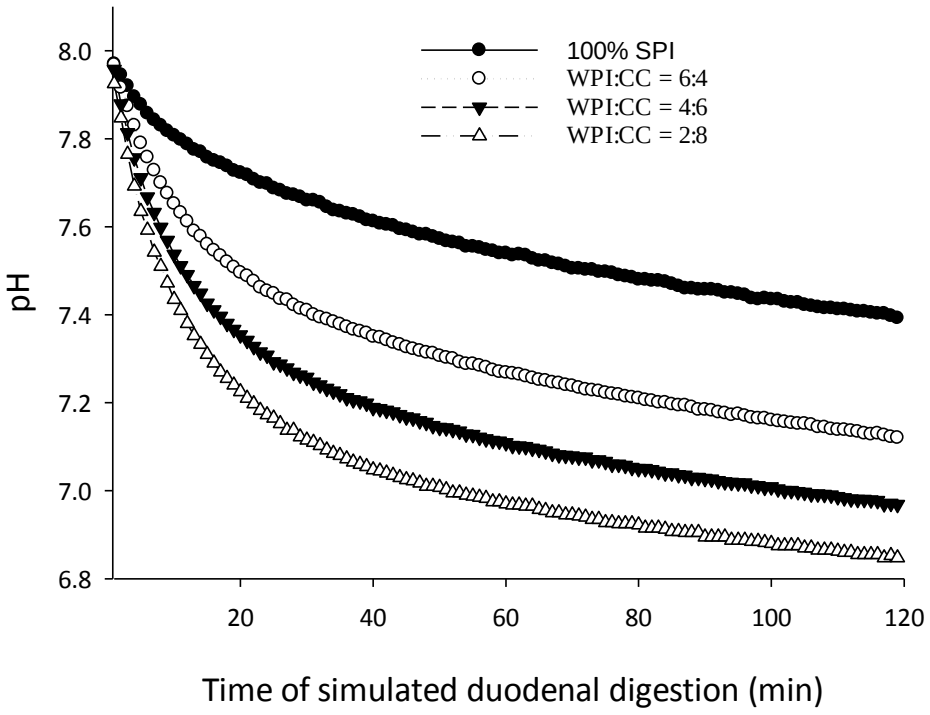


Fig 4.4 Reduction in pH during *in vitro* intestinal digestion of the four infant formulae: WPI:CC 6:4, WPI:CC 4:6, WPI:CC 2:8, and 100% SPI.

The rate of digestibility is characterized by the extent of the pH drop at 2 hours after enzyme addition in the intestinal phase. Fig 4.4 demonstrates the difference in digestion of the three dairy infant milk formulae and the soy protein formula. Formulae with a WPI to CC ratio of 2:8 show a maximum pH drop, while soy formula created the least drop. The pH drop method suggests rapid digestion of the formula with a higher proportion of caseins which is in agreement with the digestibility rate calculated using equation 1 (Table 4.1) and the PAGE patterns (Fig 4.3c). PAGE patterns for formulae with whey to casein ratios of 6:4 (Fig 4.3a) and 4:6 (Fig 4.3b) show faint bands at the start of the intestinal phase while this is not observed in formulae with whey to casein ratio of 2:8. This suggests that in the small intestine proteases hydrolyse caseins quicker than whey proteins. This difference in digestibility can be related to the difference in the structure and composition of amino acids in caseins and whey. Due to the high degree of phosphorylation, caseins have an open tertiary structure (Holt, Carver, Ecroyd, and Thorn, 2013; Swaisgood, 1993) and are sensitive to proteolysis. In contrast, whey contains a high amount of sulfur-containing amino acids (methionine, cystein, lysine, threoin and tryptophan) that creates disulfide bonds making whey proteins a compact structure that restricts the action of digestive proteases (Lacroix et al., 2006). Hsu et al. (1977), who pioneered the pH drop method using multi-enzymes, also found the pH drop for caseins to be more rapid than that for whey - the pH for caseins dropped from 8.0 to 6.7, while for whey the pH dropped from 8.0 to 7.4 after 10 min of digestion.

From Fig 4.4 and Table 4.1, it is clear that soy-based formula has the least digestibility. One would associate the low digestibility to the proteases inhibitors, tannins or phytates found in less refined soy grains. However, the concentration of these elements is very low in soy products and could not possibly affect digestibility. Hence, the low digestibility is due to the structural aspects of soy proteins and product processing (Carbonaro et al, 2012; Carbonaro, Maselli, & Nucara, 2014). The secondary structure of soy proteins is dominated by β-sheets as compared to milk proteins that are rich in α-helix. The β-sheet structures of soy protein are highly hydrophobic and encourage protein aggregation making it less soluble and resulting in low digestibility of soy proteins. Also heat treatment during processing causes β-sheet aggregation among molecules that have adverse effect on the resistance to digestion of soy proteins (Carbonaro et al, 2012; Carbonaro, Maselli, & Nucara, 2014). Therefore, precaution should be taken when comparing the protein digestibility of soy products because its properties such as denaturation and aggregation can vary considerably between products and also between manufacturers. Based on the low digestibility of soy proteins, the European Society for Paediatric Gastroenterology Hepatology and Nutrition Committee (ESPGHAN) recommended

employing a higher proportion of protein in soy based infant formula (2.25 g of protein/100 kcal) than the one based on cow's milk proteins (1.8 g of protein/100 kcal) (Agostoni et al., 2006).

The amount of amino acids and peptides formed during *in vitro* digestion will provide valuable information as to where and to what extent the protein breaks down. However, this information is still limited in the literature and requires further research to quantify and compare the amount of amino acids and peptides obtained in the gastric and intestinal digestion phases.

4.3.3 Particle size distribution

Particle size can influence the viscosity and dissolvability of infant formulas. The particle size distribution of infant formula affects rheological behavior during *in vitro* infant formula digestion (Prakash, Ma, and Bhandari, 2014) and provides useful information for design of infant formula. In this study, particle size distribution of infant formula was reported during infant gastro intestinal digestion.

The particle size distribution of the four infant formulae in their native state and during gastric and intestinal digestion were studied [Fig 4.5 (a-d)]. The figures clearly suggest a bimodal distribution for all the four formulae in their native state with a size range from 0.1 to 4 μm. However, the addition of simulated gastric fluid to the native milk, remarkably increases the particle size distribution due to caseins precipitation. The particle populations that exist below 10, 50, 90% of the total number of particles, are represented as d(0.1), d(0.5), d(0.9) in Table 4.2, which shows an increase in particle size immediately after addition of simulated gastric fluid to the four native formulae.

Table 4.2 Particle size distribution of native and gastric digested samples of the four formulae: WPI:CC 6:4, WPI:CC 4:6, WPI:CC 2:8, and 100% SPI

Name of formulae	Samples	d(0.1) µm	d(0.5) µm	d(0.9) µm
WPI:CC=6:4	Native	0.2±0.2	0.3±0.1	0.9±0.1
	S0	70.9±15.6	237.3±49.2	521.5±91.7
	S30	0.7±0.1	101.5±9.7	265.7±16.7
	S60	0.6±0.1	42.1±4.4	234.2±20.7
WPI:CC=4:6	Native	0.2±0.1	0.3±0.1	0.9±0.1
	S0	138.6±7.5	341.0±16.2	660.1±37.9
	S30	0.7±0.1	243.7±16.3	570.9±41.6
	S60	0.7±0.1	187.3±26.6	491.5±73.8
WPI:CC=2:8	Native	0.2±0.1	0.3±0.1	0.9±0.1
	S0	179.3±25.1	566.2±50.3	1168.5±82.4
	S30	0.9±0.1	552.2±55.7	1188.0±79.7
	S60	0.7±0.1	272.1±72.6	511.3±50.0
100% SPI	Native	0.2±0.1	0.3±0.1	0.7±0.1
	S0	0.6±0.1	31.6±1.2	74.8±4.9
	S30	0.6±0.1	18.5±2.9	61.3±5.7
	S60	0.6±0.1	14.6±1.2	55.9±2.4

With formula WPI:CC= 6:4, d(0.9) remarkably increased from 0.92 µm for native milk to over 520 µm for S0. A similar pattern was also observed for other formulae (Table 4.2). Over the 60 minutes of gastric digestion (S0-S60), small and medium particles appeared as a result of the breakdown of the aggregation by enzyme pepsin. After 1 hour of pepsinolysis, the small and medium particles were in the size range 0.5-4 µm and 4-100 µm, respectively. The largest particle size of the digesta is extremely large >100 µm. Since the size of fat is only around 2 µm, it is not possible for it to contribute towards the particle size of the digesta and the large particle size is due to aggregation of proteins.

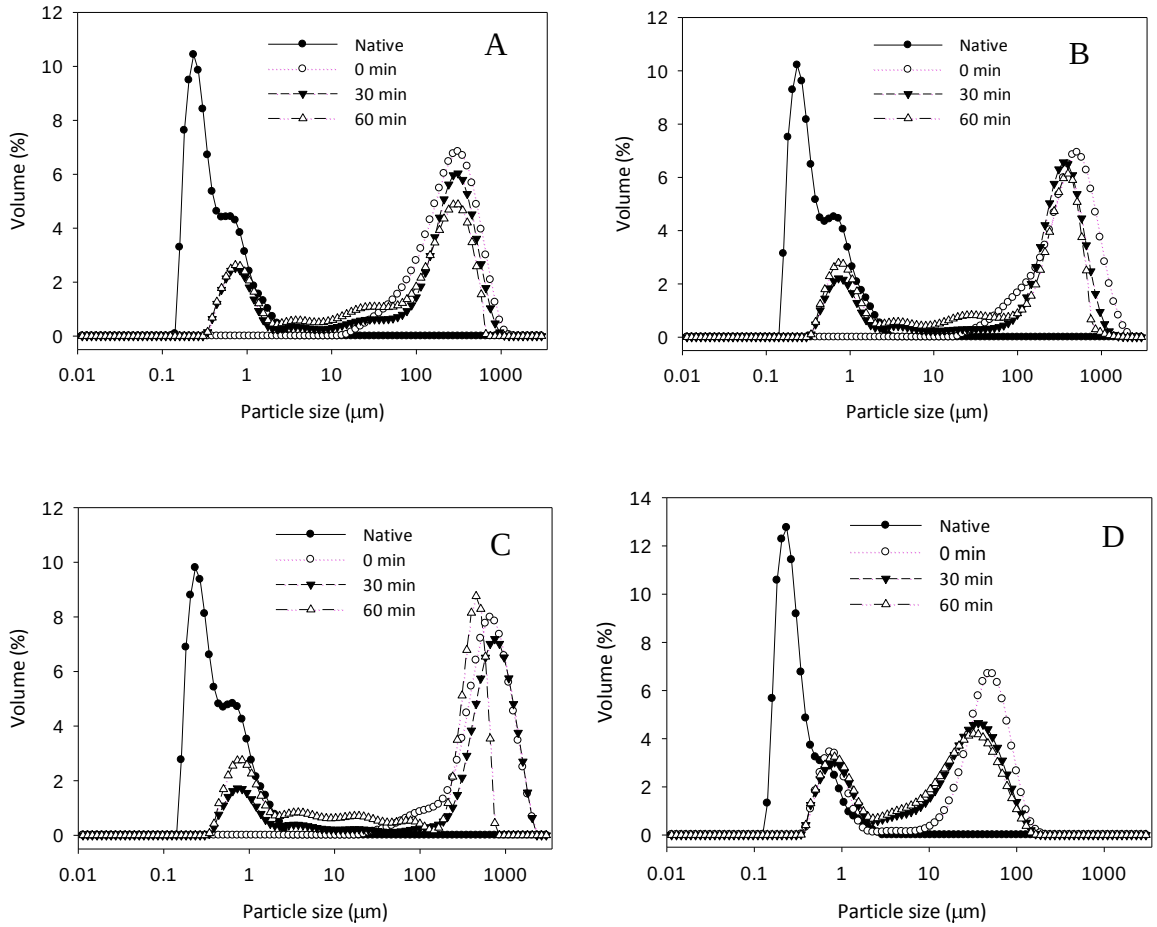


Fig 4.5 Size distribution of native and digested samples under *in vitro* gastric digestion of the four infant formulae: WPI:CC 6:4 (A), WPI:CC 4:6 (B), WPI:CC 2:8 (C), and 100% SPI (D)

The breakdown of the aggregates by pepsin also led to a decrease of the volume mean D[4,3] diameter over 60 min of gastric digestion at the time of mixing with SGF. A similar result was observed by Prakash, Ma, & Bhandari (2014). However, D[4,3] increased remarkably as compared to that of native milk for all formulae (Fig 4.7). The higher the amount of caseins (formula with whey to caseins ratio of 2:8), the larger of D[4,3] was observed due to the agglomeration of caseins in the samples. While D[4,3] for the soy based formula was the smallest. The changes in the particle size distribution of soy protein formula during the gastric and intestinal digestion was very similar to dairy formulae as observed in Fig 4.6.

In the intestinal phase, at pH 6.5, all the protein agglomerates in the digesta dissolved and the particle size distribution is similar to native proteins and has not been reported in Fig 4.6.

4.3.4. Microstructural changes

The gastric and intestinal digestion of the four infant formulae were followed with CLSM (Fig 4.7-4.9) that compares the micrographs at the start and end of digestion (the particle size of native samples were very small and could not be captured through CLSM and therefore has not been presented). At the start of the gastric digestion (Fig 4.7, S0) the dairy protein (caseins and whey proteins) and soy proteins are in large aggregates as confirmed by Fig 4.5 and Fig 4.6. After one hour of proteolysis in the stomach (S60), the large aggregates of milk protein (Fig 4.7 A-C) and soy protein (Fig 4.8D) become smaller as compared to that in S0 (Fig 4.7 A-D). However, the confocal micrographs of fat suggested no change in the size of fat globules during the one-hour gastric digestion and two hour of intestinal digestion. This is due to the absence of gastric and pancreatic lipases (Fig 4.8 and 4.9 A-D). In this study while preparing the infant formula samples, the fat is homogenized during which the surface-active proteins will be adsorbed at the interface of fat particles, forming fat globule membrane. One would expect the protein in the fat globules will undergo digestion that can cause destabilization and coalescence of fat droplets and this would have appeared in confocal micrographs. However, no fat coalescence or free fat smear was noticed in the CLSM images for both simulated gastric and intestinal digestion. This may be explained by the immediate re-adsorption of the surface active proteolytic products at the interface of fat particles in stomach phase. The lower chain polypeptides and peptides formed during the digestion process will still be surface-active and are adsorbed at the interface of fat particles in the absence of lipase that would have affected the behavior of fat particles. Similar results were also reported by Li, Ye, Lee, and Singh (2013), Gallier, Ye, & Singh (2012) and Ye, Cui, & Singh (2011) who showed that the fat globule membrane was stable during proteolysis in the stomach. They also postulated that peptides generated by any proteolysis of membrane proteins will be adsorbed into the fat globule membrane, preventing the coalescence of fat globules. However, in the intestinal phase, the stabilization of fat globules is due to the replacement of peptides or remaining proteins by bile acids at the fat globule membrane (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011).

In the intestinal phase, all the protein agglomerates in the digesta dissolved under the intestinal pH. Hence, confocal images could not be obtained for the small particles.

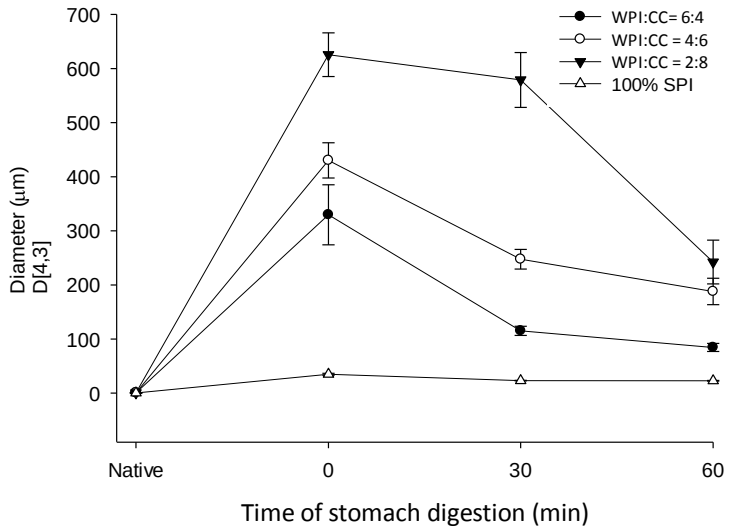


Fig 4.6 Volume mean D[4,3] diameter of native and gastric digested samples under *in vitro* gastric digestion of the four infant formulae: WPI:CC 6:4, WPI:CC 4:6, WPI:CC 2:8, and 100% SPI.

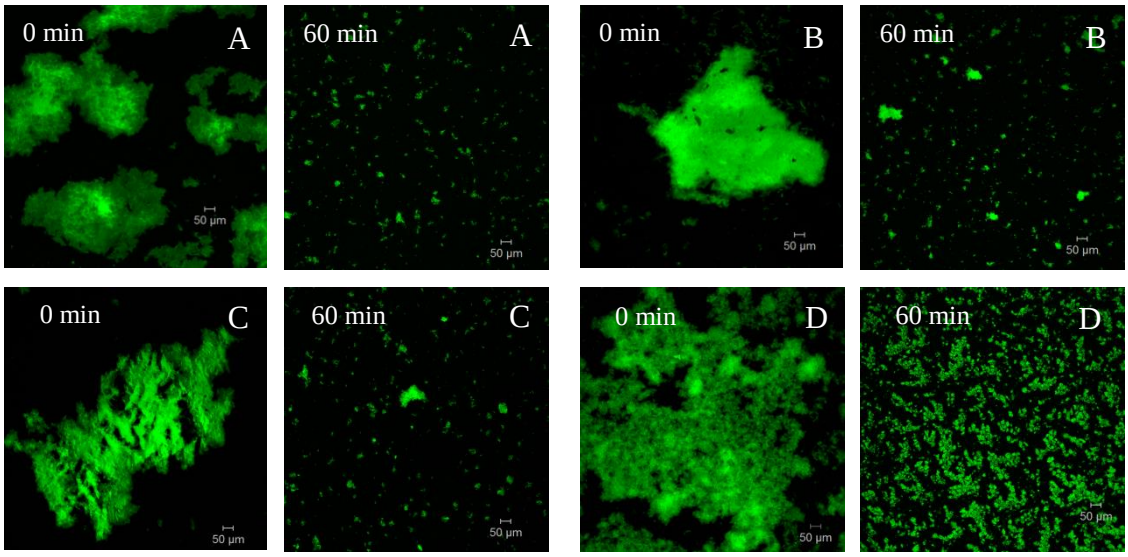


Fig 4.7 CLSM of protein agglomerates in gastric digested samples at 0 min and 60 min of the four infant formulae: WPI:CC 6:4 (A), WPI:CC 4:6 (B), WPI:CC 2:8 (C), and 100% SPI (D).

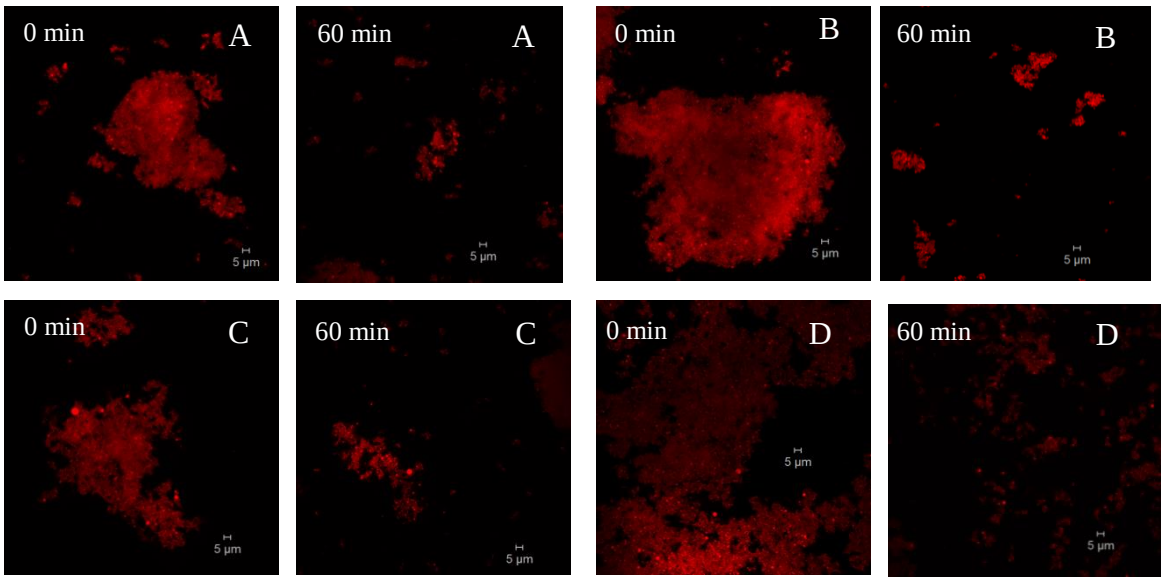


Fig 4.8 CLSM of fat globules in gastric digested samples at 0 min and 60 min of the four infant formulae: WPI:CC 6:4 (A), WPI:CC 4:6 (B), WPI:CC 2:8 (C), and 100% SPI (D).

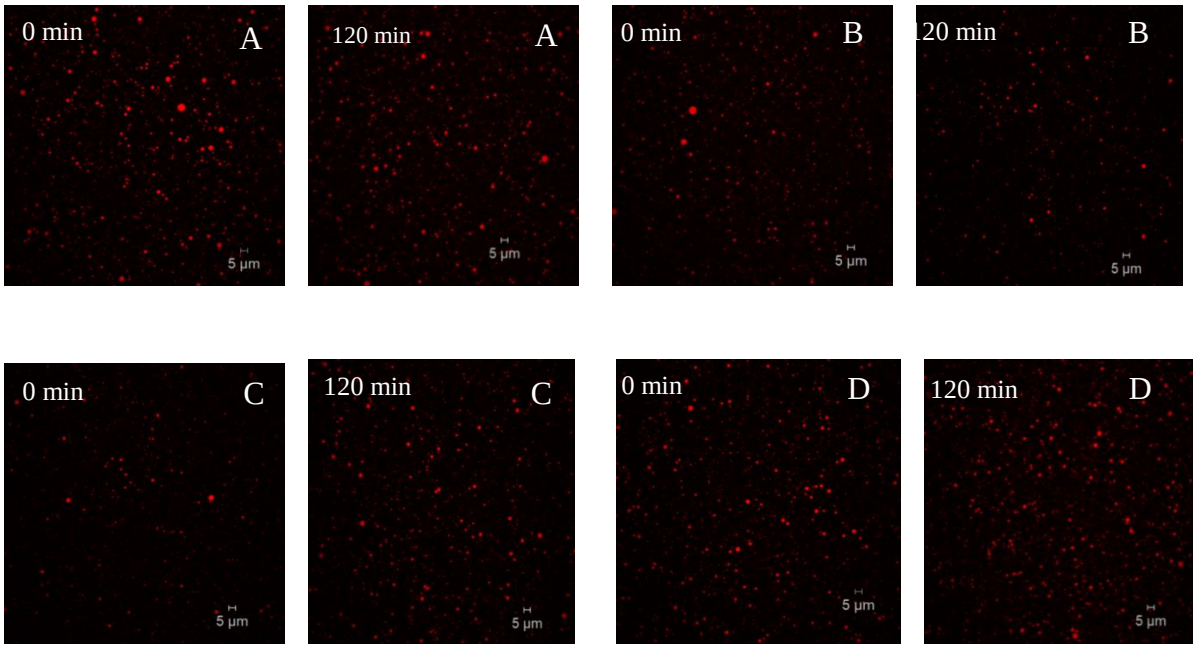


Fig 4.9 CLSM of fat globules in intestinal digested samples at 0 min and 120 min of the four infant formulae WPI:CC 6:4 (A), WPI:CC 4:6 (B), WPI:CC 2:8 (C), and 100% SPI (D).

4.4. Conclusions

The above results from the static *in vitro* digestion, simulating infant gastrointestinal tract suggests dairy proteins to be first partially hydrolysed by pepsin following which they are further digested by proteases. A higher percentage of caseins in dairy infant formulae resulted in an increase in protein

degradation due to the ease of digestion of caseins in the simulated intestinal phase. No coalescence of fat globules was observed through simulated gastric and intestinal digestion in the absence of lipase. Further work is being pursued to understand *in vitro* lipolysis with and without proteases.

Soy-based infant formulae showed the least *in vitro* protein hydrolysis compared to dairy formulae. This is due to the hydrophobic β-sheet structures of soy protein that encourage protein aggregation and the possible effect of heat treatment on soy protein structure during processing. However, it is worth noting that digestibility of soy proteins considerably varies between products and manufacturers.

Digestion of ingredients in infant formula is a complex issue. A range of systematic studies on dairy and soy proteins digestion by evaluation of the released amino acids will help understand the digestibility of these ingredients better and to some extent help determine the bioaccessibility of nutrients.

4.6. References

Agostoni, C., Axelsson, I., Goulet, O., Koletzko, B., Michaelsen, K. F., Puntis, J., Rieu, D., Rigo, J., Shamir, R., Szajewska, H., & Turck, D. (2006). Soy protein infant formulae and follow-on formulae: a commentary by the ESPGHAN Committee on Nutrition. *Journal of Pediatric Gastroenterology and Nutrition*, *42*(4), 352-361.

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

Alles, M. S., Scholtens, P. A., & Bindels, J. G. (2004). Current trends in the composition of infant milk formulas. *Current Paediatrics*, *14*(1), 51-63.

Almaas, H., Cases, A.-L., Devold, T. G., Holm, H., Langsrud, T., Aabakken, L., Aadnoey, T., & Vegarud, G. E. (2006). In vitro digestion of bovine and caprine milk by human gastric and duodenal enzymes. *International Dairy Journal, 16*(9), 961-968.

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

Ballard, O., & Morrow, A. L. (2013). Human Milk Composition. *Pediatric Clinics North America*, *60*(1), 49-74.

Bassey, F. I., Mcwatters, K. H., Edem, C. A., & Iwegbue, C. (2013). Formulation and nutritional evaluation of weaning food processed from cooking banana, supplemented with cowpea and peanut. *Food Science & Nutrition*, 1(*5*), 384-391.

Benedé, S., López-Expósito, I., Giménez, G., Grishina, G., Bardina, L., Sampson, H. A., Molina, E., & López-Fandiño, R. (2014). In vitro digestibility of bovine β-casein with simulated and human oral and gastrointestinal fluids. Identification and IgE-reactivity of the resultant peptides. *Food Chemistry, 143*, 514-521.

Bos, C., Metges, C. C., Gaudichon, C., Petzke, K. J., Pueyo, M. E., Morens, C., & Tomé, D. (2003). Postprandial kinetics of dietary amino acids are the main determinant of their metabolism after soy or milk protein ingestion in humans. *The Journal of Nutrition, 133*(5), 1308-1315.

Böttger, F. H., Etzel, M. R., & Lucey, J. A. (2013). In Vitro Infant Digestion of Whey Protein–Dextran Glycates. *Food Digestion, 4*(2-3), 1-9.

Brooks, J., & Morr, C. (1985). Current aspects of soy protein fractionation and nomenclature. *Journal of the American Oil Chemists' Society, 62*(9), 1347-1354.

Carbonaro, M., Maselli, P., & Nucara, A. (2012). Relationship between digestibility and secondary structure of raw and thermally treated legume proteins: a Fourier transform infrared (FT-IR) spectroscopic study. *Amino Acids, 43*(2), 911-921.

Carbonaro, M., Maselli, P., & Nucara, A. (2014). Structural aspects of legume proteins and nutraceutical properties. *Food Research International*. In Press.

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

Davidson, L. A., & Lönnerdal, B. (1987). Persistence of human milk proteins in the breast-fed infant. *Acta Paediatrica, 76*(5), 733-740.

Donovan, S. M., Atkinson, S. A., Whyte, R. K., & Lönnerdal, B. (1989). Partition of nitrogen intake and excretion in low-birth-weight infants. *Archives of Pediatrics & Adolescent Medicine, 143*(12), 1485.

Dupont, D., Mandalari, G., Molle, D., Jardin, J., Léonil, J., Faulks, R. M., Wickham, M.S.J., Mills E.N.C., & Mackie, A. R. (2010a). Comparative resistance of food proteins to adult and infant in vitro digestion models. *Molecular Nutrition & Food Research, 54*(6), 767-780.

Dupont, D., Mandalari, G., Mollé, D., Jardin, J., Rolet-Répécaud, O., Duboz, G., Léonil, J., Mills E.N.C., & Mackie, A. R. (2010b). Food processing increases casein resistance to simulated infant digestion. *Molecular Nutrition & Food Research, 54*(11), 1677-1689.

Mills, E.N. C. , Marsh, J. T., Boyle, R., Hoffmann-Sommergruber K., DuPont, D., Bartra, J., Bakalis, S.,, McLaughlin, J. , Shewry, P. R. (2013). *Literature review: 'in vitro digestibility tests for allergenicity assessment'*. Retrieved from http://www.efsa.europa.eu/en/supporting/pub/529e.htm.

Fomon, S. J., Ziegler, E. E., Filer, L., Nelson, S. E., & Edwards, B. B. (1979). Methionine fortification of a soy protein formula fed to infants. *The American Journal of Clinical Nutrition, 32*(12), 2460-2471.

Gallier, S., Ye, A., & Singh, H. (2012). Structural changes of bovine milk fat globules during in vitro digestion. *Journal of Dairy Science, 95*(7), 3579-3592.

Gurr, M. I. (1981). Review of the progress of dairy science: human and artificial milks for infant feeding. *Journal of Dairy Research, 48*(3), 519-554.

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

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

Heird, W. C. (2012). Infant nutrition. In J. W. Erdman, I. A. Macdonald & S. H. Zeisel (Eds), *Present Knowledge in Nutrition* (pp.624-636). Wiley-Blackwell, Oxford, UK.

Hernell, O. (2011). Human milk vs. cow's milk and the evolution of infant formulas. In R. A. Clemens, O. Hernell & K. M. Michaelsen (Eds.), *Milk and milk products in human nutrition*, (pp.17-28). Karger.

Holt, C., Carver, J. A., Ecroyd, H., & Thorn, D. C. (2013). Invited review: Caseins and the casein micelle: Their biological functions, structures, and behavior in foods. *Journal of Dairy Science, 96*(10), 6127-6146.

Hsu, H., Vavak, D., Satterlee, L., & Miller, G. (1977). A multienzyme technique for estimating protein digestibility. *Journal of Food Science, 42*(5), 1269-1273.

Hughes, G. J., Ryan, D. J., Mukherjea, R., & Schasteen, C. S. (2011). Protein digestibility-corrected amino acid scores (PDCAAS) for soy protein isolates and concentrate: criteria for evaluation. *Journal of Agricultural and Food Chemistry, 59*(23), 12707-12712.

Hur, S. J., Lim, B. O., Decker, E. A., & McClements, D. J. (2011). In vitro human digestion models for food applications. *Food Chemistry, 125*(1), 1-12.

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

Jakobsson, I., Lindberg, T., & Benediktsson, B. (1982). In vitro digestion of cow's milk proteins by duodenal juice from infants with various gastrointestinal disorders. *Journal of Pediatric Gastroenterology and Nutrition, 1*(2), 183-192.

Kim, Y. A., & Barbeau, W. E. (1991). Evaluation of SDS-PAGE method for estimating protein digestibility. *Journal of Food Science, 56*(4), 1082-1086.

Kitabatake, N., & Kinekawa, Y.-I. (1998). Digestibility of bovine milk whey protein and β-lactoglobulin in vitro and in vivo. *Journal of Agricultural and Food Chemistry, 46*(12), 4917-4923.

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

Lacroix, M., Bos, C., Léonil, J., Airinei, G., Luengo, C., Daré, S., Benamouzig, R., Fouillet, H., Tomé, D. (2006). Compared with casein or total milk protein, digestion of milk soluble proteins is too rapid to sustain the anabolic postprandial amino acid requirement. *The American Journal of Clinical Nutrition, 84*(5), 1070-1079.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680-685.

Li-Chan, E., & Nakai, S. (1989). Enzymic dephosphorylation of bovine casein to improve acid clotting properties and digestibility for infant formula. *Journal of Dairy Research, 56*(3), 381-390.

Li, H. J., Zhu, K. X., Zhou, H. M., Peng, W., & Guo, X. N. (2013). Comparative study about some physical properties, in vitro digestibility and immunoreactivity of soybean protein isolate for infant formula. *Plant Foods for Human Nutrition, 68*(2), 124-130.

Li, J., Ye, A., Lee, S. J., & Singh, H. (2013). Physicochemical behaviour of WPI-stabilized emulsions in in vitro gastric and intestinal conditions. *Colloids and Surfaces B: Biointerfaces, 111*, 80-87.

Lien, E. L., Davis, A. M., Euler, A. R., & Group, M. S. (2004). Growth and safety in term infants fed reduced-protein formula with added bovine alpha-lactalbumin. *Journal of Pediatric Gastroenterology and Nutrition, 38*(2), 170-176.

Liener, I. E. (1994). Implications of antinutritional components in soybean foods. *Critical Reviews in Food Science and Nutrition, 34*(1), 31-67.

Lönnerdal, B. (2010). Bioactive proteins in human milk: mechanisms of action. *Journal of Pediatrics, 156*(2), 017.

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

Lönnerdal, B. (2013). Bioactive proteins in breast milk. *Journal of Paediatrics and Child Health, 49*(S1), 1-7.

Lönnerdal, B., & Lien, E. L. (2003). Nutritional and physiologic significance of alpha-lactalbumin in infants. *Nutrition Reviews, 61*(9), 295-305.

Maldonado-Valderrama, J., Wilde, P., Macierzanka, A., & Mackie, A. (2011). The role of bile salts in digestion. Advances in Colloid and Interface Science, 165(*1*), 36-46.

Nagano, T., Tamaki, E., & Funami, T. (2008). Influence of guar gum on granule morphologies and rheological properties of maize starch. *Carbohydrate Polymers, 72*(1), 95-101.

Nagita, A., Amemoto, K., Yoden, A., Aoki, S., Sakaguchi, M., Ashida, K., & Mino, M. (1996). Diurnal variation in intragastric pH in children with and without peptic ulcers. *Pediatric Research, 40*(4), 528-532.

Nguyen, G. T., Gidley, M. J., & Sopade, P. A. (2015). Dependence of in-vitro starch and protein digestions on particle size of field peas (*Pisum sativum L.*). *LWT-Food Science and Technology*. In Press.

Ohsawa, K., Satsu, H., Ohki, K., Enjoh, M., Takano, T., & Shimizu, M. (2008). Producibility and digestibility of antihypertensive β-casein tripeptides, Val-Pro-Pro and Ile-Pro-Pro, in the gastrointestinal tract: analyses using an in vitro model of mammalian gastrointestinal digestion. *Journal of Agricultural and Food Chemistry*, 56(*3*), 854-858.

Peace, R., Sarwar, G., & Touchburn, S. (1992). Trypsin inhibitor levels in soy-based infant formulas and commercial soy protein isolates and concentrates. *Food Research International, 25*(2), 137-141.

Pérez, O. E., David-Birman, T., Kesselman, E., Levi-Tal, S., & Lesmes, U. (2014). Milk protein-vitamin interactions: Formation of beta-lactoglobulin/folic acid nano-complexes and their impact on in vitro gastro-duodenal proteolysis. *Food Hydrocolloids, 38*, 40-47.

Prakash, S., Ma, Q., & Bhandari, B. (2014). Rheological behaviour of selected commercially available baby formulas in simulated human digestive system. *Food Research International, 64*, 889-895.

Rich, D. H., & Sun, E. T. (1980). Mechanism of inhibition of pepsin by pepstatin: Effect of inhibitor structure on dissociation constant and time-dependent inhibition. *Biochemical Pharmacology, 29*(16), 2205-2212.

Sakai, K., Yoshino, K., Satter, M. A., Ota, F., Nil, Y., Fututa, K., Ueda, N., Shimizu, Y., & Yamamoto, S. (2000). Effects of pH variation and NaCl on in vitro digestibility of cow's milk

proteins in commercially available infant formulas. *Journal of Nutritional Science and Vitaminology, 46*(6), 325-328.

Sarwar Gilani, G., Wu Xiao, C., & Cockell, K. A. (2012). Impact of antinutritional factors in food proteins on the digestibility of protein and the bioavailability of amino acids and on protein quality. *British Journal of Nutrition, 108*(S2), S315-S332.

Schaafsma, G. (2000). The protein digestibility-corrected amino acid score. *The Journal of Nutrition, 130*(7), 1865S-1867S.

Shuttuck-Eidens, D. M., & Beachy, R. N. (1985). Degradation of β-conglycinin in early stages of soybean embryogenesis. *Plant Physiology, 78*(4), 895-898. Swaisgood, H. E. (1993). Review and Update of Casein Chemistry 1, 2. *Journal of Dairy Science, 76*(10), 3054-3061.

Thanh, V. H., & Shibasaki, K. (1977). Beta-conglycinin from soybean proteins. Isolation and immunological and physicochemical properties of the monomeric forms. *Biochimica et Biophysica Acta (BBA)-Protein Structure, 490*(2), 370-384.

Thompkinson, D., & Kharb, S. (2007). Aspects of infant food formulation. *Comprehensive Reviews in Food Science and Food Safety, 6*(4), 79-102.

van de Velde, F., Weinbreck, F., Edelman, M. W., van der Linden, E., & Tromp, R. H. (2003). Visualisation of biopolymer mixtures using confocal scanning laser microscopy (CSLM) and covalent labelling techniques. *Colloids and Surfaces B: Biointerfaces, 31*(1), 159-168.

van Riemsdijk, L. E., Sprakel, J. H., van der Goot, A. J., & Hamer, R. J. (2010). Elastic networks of protein particles. *Food Biophysics, 5*(1), 41-48.

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

CHAPTER 5 *IN VITRO* DIGESTION OF INFANT MILK FORMULAE WITH HYDROLYSED AND NON-HYDROLYSED PROTEINS FROM DAIRY AND SOYBEAN

5.1. Introduction

Infant formula is a milk product that is intended to replace mother's milk when breastfeeding is not possible or is discontinued for some reason. Most of the infant formulas are based on cow's milk and a minority use soy protein isolate as a protein source. The differences in composition between human milk, bovine milk, and soy milk led to the modification of the infant formula contents to be closer to human milk as much as possible (Goedhart & Bindels, 1994). At present, due to the advances in food technology and engineering, the main targets of current infant formula have been supposedly met from the point of view of safety for infants and the composition in macro-nutrients (protein, fat, carbohydrates) and micro-nutrients (vitamins, minerals) comparable to mothers' milk (Hernell, 2011). Healthy infants can be fed on regular infant formula, but premature infants with digestive concerns or infants with allergy regularly that require formula based on hydrolysed proteins.

Extensively hydrolysed formula (eHF) contains milk proteins that are treated with enzymes to break down the native protein structure into smaller fragments thereby reducing allergenicity (C. Dupont, Hol, & Nieuwenhuis, 2015; Hays & Wood, 2005). According to the American Academy of Pediatrics, eHF is defined as a formula containing only peptides that have molecular weight less than 3 kDa (Hays & Wood, 2005; S. Koletzko et al., 2012). Infants with cow's milk protein allergy (CMPA) or those intolerant to intact proteins, are advised to be fed on extensively hydrolysed formula (Agostoni, Terracciano, Varin, & Fiocchi, 2014; Alles, Scholtens, & Bindels, 2004; C. Dupont et al., 2015; Hernell & Lönnerdal, 2003). There is an increasing evidence that eHF is effective in treatment and prevention of allergy in infants (C. Dupont et al., 2015; O'Connor, 2009) and also in the treatment of infantile colic (Hall, Chesters, & Robinson, 2012; Ventura, San Gabriel, Hirota, & Mennella, 2012).

Partially hydrolysed formula (pHF) containing a combination of intact and partially digested proteins (Ventura et al., 2012) are promoted for infants with mild digestive issues (More, 2013). In Australia and New Zealand, pHF is recommended for non-breast-fed infants with a high risk of allergic diseases whose parents or siblings have history of allergy (Allergy, 2010; Australia, 2014).

Soy infant formula is used as a breastfeeding substitute for infants allergic to cow's milk proteins or for religious, philosophical, or ethical reasons (Agostoni et al., 2006). Due to the different amino acid composition profiles in soy proteins as compared to cow's milk proteins (lower content of methionine, higher amounts of aspartate, glycine, arginine, and cystine) (Agostoni et al., 2006; Bos et al., 2003) and some extent of phytate (may lessen the absorption of iron and zinc), soy formula is not recommended for healthy infants during the first 6 months of life (B. Koletzko et al., 2003). Soy formula could be an option for infants with CMPA who are over six months of age and not accept or tolerate eHF (S. Koletzko et al., 2012).

The formation of peptides during enzymatic processing of hydrolysed proteins, introduces the bitter taste that may reduce their acceptability among infants. To hide the bitter taste of hydrolysed proteins, saccharose or fructose might be added (B. Koletzko et al., 2003). The Coordinated International Expert Group of European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) recommend the protein content in infant formulas based on cow's milk protein hydrolysates should be between 1.8-3.0 g/ 100 kcal (B. Koletzko et al., 2005).

Despite all the advantages of eHF and pHF in improved digestion and treatment of colic in infants, there is limited information on the structural and physical changes encountered by the hydrolysed proteins during their passage through the digestive tract. Hence, the objective of this research is to compare the digestibility and the microstructural changes that infant formulae (based on hydrolysed and non-hydrolysed dairy and soy proteins) undergo in an *in vitro* GI tract. This was achieved by determining the digestibility rate (pH-drop method), gel electrophoresis (SDS-PAGE), the released amount of ninhydrin reactive amino nitrogen, particle size distribution, and micro-structural changes during enzymatic hydrolysis.

5.2. Materials and method

5.2.1. Materials

5.2.1.1. Enzymes and chemicals

The following enzymes and chemicals were used for the experiment: Pepsin from porcine gastric mucosa (EC 3.4.23.1, 3840 units/mg protein, one unit will produce a change in A280 of 0.001 per

min at pH 2.0 at 37°C, measured as TCA-soluble products using haemoglobin as substrate). Trypsin from bovine pancreas (EC 3.4.21.4, 13165 units/mg protein, one unit will produce a change in A253 of 0.001 per minute at pH 7.6 at 25°C using Nα-Benzoyl-L-arginine Ethyl Ester (BAEE) as a substrate. Chymotrypsin from bovine pancreas (EC 3.4.21.1, 54.49 units/mg protein, one unit will hydrolyze 1.0 μmol of N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE) per min at pH 7.8 at 25°C as stated by manufacturer).

Sodium taurocholate, pepstatin, trypsin-chymotrypsin inhibitor, and ninhydrin reagent were obtained from Sigma-Aldrich, Castle Hill, New South Wales, Australia. Sodium glycodeoxycholate was obtained from Merck, Kilsyth, Victoria, Australia. They were stored between 2-8°C. All enzymes used for the experimental trials were obtained from Sigma-Aldrich, Castle Hill, New South Wales, Australia and stored at the recommended temperature of -20°C.

The other ingredients used in the study such as lactose, sodium chloride, hydrochloric acid, and sodium hydroxide were of analytical grade.

5.2.1.2. Dairy and soy proteins

Whey protein isolate (WPI 85.1% protein, 1.0% fat, 1.2% carbohydrate) and calcium caseinate (CC, 86.7% protein, 1.1% fat, 0.1% carbohydrate) were purchased from Total Foodtec Pty Ltd (Australia).

Hydrolysed whey protein (HWP, 81.9% protein, 0.5% fat, 2.6% carbohydrate, degree of hydrolysis 18.5%) and hydrolysed casein protein (HCP, 86.7% protein, 0.9% fat, 0.2% carbohydrate, degree of hydrolysis 6%) were purchased from Total Foodtec Pty Ltd. and Myopure Pty, Australia respectively.

Soy protein isolate (SPI, 82.4% protein, 4.5% fat, less than 1.0% carbohydrate) purchased from Food Manufacturers Pty., Australia. Hydrolysed SPI (HSPI) was made from SPI by pepsin at a concentration of 22.75 U/mg of total protein, at 37°C for 1 hour. The hydrolysed mixture was alkalised to pH 8.0-8.5 by 0.1N NaOH in order to inactivate the enzyme pepsin. The residue of NaOH was then neutralised by 0.01N HCl followed by freeze-drying for 72 hours (Pasupuleti & Braun, 2008).

Sunflower vegetable oil was obtained from a local supermarket.

5.2.2. Method

Preparation of infant formulae and procedure of *in vitro* infant protein digestion are as described at section 4.2.5, chapter 4. Infant milk formula used in this study were 100% HCP, 100% CC, 100% HWP, 100% WPI, HWP: HCP =1:1, WPI:CC=1:1, 100% SPI, 100% HSPI. The ratio of WPI and CC was chosen based on the the ratio of available commercial infant formulae. For hydrolysed infant formulae a single protein source is commonly used. This research study aimed to compare the digestibility of proteins in infant formulae which is based on sole hydrolysed whey protein isolate, sole hydrolysed casein protein, and a mixture of both. Therefore, a ratio 1:1 of HWP:HCP was chosen.

5.2.2.1. pH-drop method

The pH-drop method was used to determine the rate of digestibility of the infant formulae with hydrolysed and non-hydrolysed proteins from dairy and soybean as described by Hsu et al. (1977). This method was modified with a lower level of enzymes and in the absence of peptidase to adapt to infant digestion as recommended by Dupont et al (2010). Although the enzymes used were less and without peptidase, they were capable of hydrolysing proteins and releasing protons that can drop the pH, which was adjusted to 8.0±0.03. Due to the breakdown of proteins into amino acids and peptides, the decreasing pH was measured every minute by pH meter for duration of two hours. The values used for analysis were taken from an average of duplicate measurements.

Digestibility of each formula was calculated based on the pH after 120 min of digestion (X1) using the equation developed by Hsu et al. (1977):

$$\text{Digestibility} = 210.46 - 18.10X1 \qquad \text{Equation (1)}$$

5.2.2.2. SDS-PAGE

Degradation of proteins during *in vitro* digestion based on molecular weight of proteins and polypeptides was assessed by SDS-PAGE (Nguyen et al., 2015b). The protein breakdown of the digesta over the gastric and intestinal phase was analysed by reducing SDS-PAGE running on a Mini Protean 3 cell (Bio-Rad) for 37 min at 200V. The assay was performed according to the protocol

described by Laemmli (1970), using 4-20% Tris-HCl precast gel, protein ladder. Each volume of sample was mixed with four volume of sample buffer (0.0625 M Tris-HCl buffer pH 6.8), 40% glycerol, 2% SDS, 0.04% bromophenol blue, and β-mercaptoethanol (19:1, v/v). The mixture was heated at 95^0C for 5 min then loaded to the wells (10 µL was loaded for both gastric and intestinal phase).

5.2.2.3. Ninhydrin-reactive amino nitrogen

Small peptides and amino acids were assayed by spectrophotometer at wavelength 570nm with ninhydrin reagent. Ninhydrin hydrates with amino groups producing the colored ninhydrin chromophore called Ruhemann's purple (λmax=570 nm, ε=22 000). Frozen digested samples were thawed on ice, followed by centrifugation at 4000 rpm, 4°C for 20 minutes. The supernatant after fat removal was mixed with sulfosalicylic acid 6% (2:1 v/v) and protein precipitate was removed immediately by centrifugation at 4000 rpm, 4°C for 20 minutes. Collected supernatant phase was then purified with a 0.45 µm syringe filter to remove molecules >10kDa (Ventura et al., 2012). The clear supernatant was used to analyse ninhydrin-reactive amino nitrogen using ninhydrin reagent. The amount of ninhydrin-reactive amino nitrogen generated from gastric and intestinal digestion was calculated based on L-Leucine as the amino acid standard. Standard curve was built from absorbance at 570 nm of 12.5, 25.0, 37.5, 50.0 µmol/ mL of L-Leucine solution. Stock solution was prepared from 50 µmol/ mL of L-Leucine in 0.05% glacial acetic acid. This method has previously been used (Kaur, Maudens, Haisman, Boland, & Singh, 2014; Moore, 1968).

5.2.2.4. Particle size distribution

Particle size distribution of native and digested milk samples were measured before and during *in vitro* gastric and intestinal digestions by Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershine, UK). The refractive index value for dispersed phase was 1.35 (milk), and for continuous phase was 1.33 (water). Values of d(0.1), d(0.5), d(0.9), and D[4,3] were recorded. The first three values indicate the size of the population of the particles existing below 10, 50, 90% of the total number of particles. D[4,3] is a volume mean of the population which is sensitive to the presence of large particles. Mean particle sizes and distribution were determined as the average of three repeated measurements from duplication.

5.2.2.5. Confocal Laser Scanning Microscopy (CLSM)

The physical arrangement of protein and fat globules of native and digested sample were observed by Zeiss LSM 700 Confocal Laser Scanning Microscope. Protein were stained with Rhodamine B (0.01% w/w in ethanol) and excited with the laser light at a wavelength 540 nm (Nagano, Tamaki, & Funami, 2008; van de Velde, Weinbreck, Edelman, van der Linden, & Tromp, 2003; van Riemsdijk, Sprakel, van der Goot, & Hamer, 2010). Nile red (0.01% w/w in ethanol) was used to stain triglycerides and excited with the laser light wavelength of 515-530 nm (S. Gallier, Ye, & Singh, 2012; Ye, Cui, & Singh, 2011).

For slide preparation, 500 μL of infant formula samples were mixed with 20 μL of Rhodamine B and 20 μL of Nile red solution by using vortexer (Ratex VM1) for 5 sec. Samples were stained for at least 10 minutes. 2μL of stain samples were loaded onto 26x76 mm slides (Sail Brand) and then covered with 18x18 mm cover slip (Menzel Glaser). The edges of the cover slips were coated with a transparent nail polish to fix the sample position and prevent the sample from drying. The observations of fat globules and the protein aggregates were done with a magnification lens at 63x and 10x, respectively.

5.2.2.6. Data analysis

Experimental data were assessed by one-way ANOVA with Minitab® 16 and Tukey test for pairwise comparison to determine the significant differences among the means at 95% confidence level. The treatment means were considered to be significantly different when $P<0.05$.

5.3. Results and discussion

5.3.1. Digestibility – pH-drop method

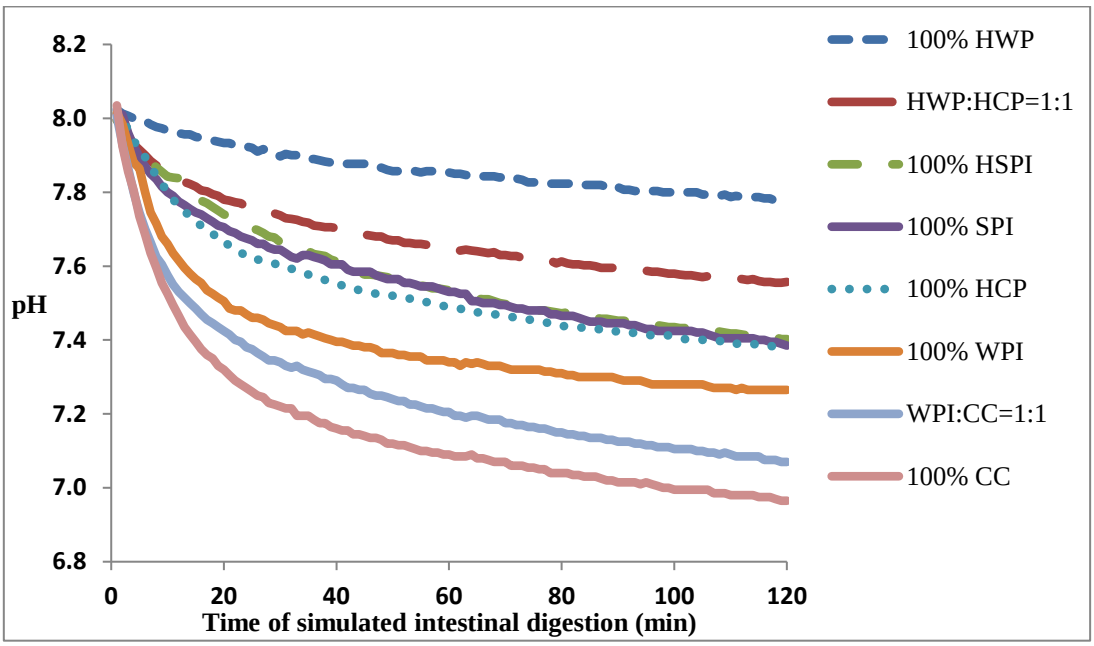
The rate of *in vitro* digestibility is characterized by the extent of pH-drop at 2 hours after enzyme addition in the intestinal phase. Fig 5.1 demonstrates the difference in digestion of infant formulae using hydrolysed and non-hydrolysed proteins. Overall, formulae with non-hydrolysed proteins have the pH dropped to a lower pH compared to formulae with hydrolysed proteins. This could be associated with the high availability of intact proteins to protease enzymes in non-hydrolysed proteins

formulae that breaks down intact proteins into peptides and amino acids resulting in lowering pH more easily. The pH-drop patterns for infant formulae with non-hydrolysed proteins are similar to the results obtained by Nguyen et al (2015b). The results also confirm that among the proteins, formulae containing 100% and 50% caseins showed the maximum pH-drop. That is, in the small intestine proteases can hydrolyse caseins quicker than whey proteins or soy protein. This difference in digestibility between caseins and whey can be related to the difference in the structure and composition of amino acids in these proteins. Due to the high degree of phosphorylation, caseins have an open tertiary structure (Holt, Carver, Ecroyd, & Thorn, 2013; Swaisgood, 1993) and are more susceptible to proteolysis. Similar results have also been reported by Nguyen et al (2015b). In contrast, whey proteins contain a high amount of sulfur-containing amino acids (methionine, cystein, lysine, threoin and tryptophan) that create disulfide bonds making them a compact structure thereby restricting the action of digestive proteases (Lacroix et al., 2006). Hsu et al.(1977) who pioneered the pH-drop method using multi-enzymes, also found the pH-drop for caseins to be more rapid than that for whey - the pH for caseins dropped from 8.0 to 6.7, while for whey the pH-dropped from 8.0 to 7.4 after only 10 min of digestion.

A comparison between the structures also reveals the difference in digestibility of soy and dairy proteins. The β-sheet conformation is found predominantly in soy proteins, but less in dairy proteins (Carbonaro, Maselli, & Nucara, 2012). The highly hydrophobic nature of β-sheet structures primarily contributes to the less solubility of soy proteins and support protein self-aggregation. Thermal processing also promotes hydrophobic interactions among molecules that diminishes soy proteins susceptibility to proteolysis (Carbonaro, Maselli, & Nucara, 2015).

The *in vitro* digestibility rate of infant formulae with hydrolysed and non-hydrolysed proteins in the intestinal phase, calculated using equation (1) are presented in Table 5.1. Please note the digestibility values for hydrolysed proteins in Table 1 do not represent the total digestibility as the calculated values did not take into account the partially hydrolysed portions during the manufacturing process. It was found that highest digestibility rate is for formulae with 100% CC (84.32%) and with WPI:CC=1:1 (82.51%), least digestibility rate is for 100% HWP (69.75%). Fig 5.1 also demonstrates the difference in digestion of these formulae: 100% CC formula shows a maximum pH-drop, followed by WPI:CC=1:1 and 100% SPI, while 100% HWP formula showed the least drop in pH. The pH-drop method suggests rapid digestion of the formula with a higher proportion of CC, which is in agreement with the calculated digestibility rate (Table 1). Among formulae with hydrolysed proteins,

100% HCP and 100% HSPI obtain similar digestibility (76.95%, 76.81%, respectively), followed by HWP: HCP =1:1 (73.69%), 100% HWP with lowest rate (69.75%).



Mean values of pH (no of replicates = 4); the error bar has not be added for clarity of images

Fig 5.1 Reduction in pH during *in vitro* intestinal digestion of infant formulae with hydrolysed and non-hydrolysed proteins.

Table 5.1 *In vitro* digestibility of infant formulae with hydrolysed and non-hydrolysed proteins in the intestine

Infant milk formulae	*In vitro* digestibility	Infant milk formulae	*In vitro* digestibility
100% HCP	76.9±0.9^{c}	**100% CC**	84.3±0.7^{a}
HWP:HCP =1:1	73.7±1.3^{d}	**WPI:CC=1:1**	82.5±2.1^{ab}
100% HWP	69.7±0.8^{e}	**100% WPI**	78.9±2.1^{bc}
100% HSPI	76.5±1.1^{cd}	**100% SPI**	76.8±0.6^{cd}

Mean values of digestibility of proteins (intestinal phase only) that do not share the same letter are significantly different at $P < 0.05$. Please note the digestibility values for hydrolysed proteins do not represent the total digestibility as the calculated values do not take into account the partially hydrolysed portions during the manufacturing process.

One would expect that hydrolysed proteins could be hydrolysed faster than non-hydrolysed proteins. However, the digestibility rate of the formulae with hydrolysed and non-hydrolysed proteins shown in this study actually expresses the digestion of intact proteins and polypeptides available in those proteins. Hydrolysed proteins already contain amino acids and peptides, which did not contribute to the pH drop as pH was adjusted to 8.0±0.03 earlier. The exact reason why there is a difference in

digestibility between hydrolysed whey, hydrolysed caseins, and hydrolysed soy proteins is still unclear and requires further research.

5.3.2. Protein digestion determined by SDS-PAGE

Fig 5.2 (a-d) and Fig 5.2 (A-D) compares the PAGE patterns of formulae containing hydrolysed and non-hydrolysed dairy and soy proteins over gastric digestion (60 min) and intestinal digestion (120 min). After 120 min of intestinal digestion, no bands of intact proteins are visible with formulae containing hydrolysed proteins. However, for formulae made of non-hydrolysed proteins, intact proteins were detected such as caseins, β-lactoglobulin, α-lactalbumin, basic and acidic polypeptides of glycinin were observed.

The gel images for formulae 100% HCP and 100% CC is as shown in Fig 5.2 (a & A). As can be seen α-casein (23 kDa) and β-casein (24 kDa) are not present in lane for HCP which was present in CC. The only detectable bands are peptides with molecular weight between 10-2 kDa, which are faint after 1 hour of gastric digestion (S0-S60) and completely disappear after 30 minutes of the intestinal phase (D30). Meanwhile, with formula containing 100% CC (Fig 5.2A), most of the caseins remained after 1h of digestion in the stomach, but are completely digested by proteases in the intestine (Fig 5.2 [A, C]). This is because the gastric pH is 4.0 close to the isoelectric point of casein (pHi 4.6 leading to casein agglomeration that prevents pepsinolysis susceptibility of caseins.

For HWP (Fig 5.2b), only a faint band for α-lactalbumin was observed at approximately 14.4 kDa with no visible bands for β-lactoglobulin and glycomacropeptide, while all of them are visible in lanes for WPI formulae (Fig 5.2[B,C]). As *in vitro* digestion progressed, the formulae with 100% and 50% of HWP shows α-lactalbumin along with peptides (10-2 kDa) that have been significantly degraded by proteases under gastrointestinal digestion (Fig 5.2[b,c]). In contrast, for 100% WPI formula, over 70% of β-lactoglobulin, 80% of α-lactalbumin, and 40% of glycomacropeptide resisted pepsin digestion. At the end of the intestinal phase, only β-lactoglobulin is completely digested, and bands for α-lactalbumin and glycomacropeptide are still visible (Fig 5.2B).

Fig 5.2D shows 100% SPI formula that contains glycinin with basic polypeptides (18-20 kDa) and acidic polypeptides (31-45 kDa), and some extent of α, α', β conglycinin (76, 72, 53 kDa). These bands gradually become faint during gastric and intestinal digestion. HSPI contains a small portion

of glycinin basic polypeptides with slight bands detected for basic polypeptides, and no bands for glycinin acidic peptides (31-45 kDa) and α, α', β conglycinin (76, 72, 53 kDa). The intensity of the bands for glycinin basic polypeptides decreased with increasing incubation time for the gastric phase and completely disappeared in the early stages of the intestinal phase (Fig 5.2d). This pattern is quite the same for the digestion of formula with native SPI, albeit a higher intensity of the protein bands is observed after the stomach and intestinal phase (Fig 5.2D).

The results from SDS-PAGE again confirm the different digestibility of proteins from different sources, which reinstates the observations by pH drop method. Although dairy proteins are more susceptible to proteolysis than soy proteins, there are still differences in digestibility among the dairy proteins i.e. between caseins and whey proteins, even between α-lactalbumin, β-lactoglobulin, and glycomacropeptide as observed in our study. A difference in digestibility between α-lactalbumin and β-lactoglobulin (Jakobsson, Borulf, Lindberg, & Benediktsson, 1983) has also been reported, possibly due to the differences in the conformation of α-lactalbumin and β-lactoglobulin. The structure of α-lactalbumin with more disulphide bonds and calcium binding make the compact globular molecule of α-lactalbumin more stable than β-lactoglobulin (Pike, Brew, & Acharya, 1996).

With glycomacropeptide, the monomer molecules of glycomacropeptide are able to self-assemble at gastric pH (pH 4) because of hydrophobic interaction (Farías, Martinez, & Pilosof, 2010) that prohibits its accessibility to pepsin in stomach phase. However, at intestinal phase (pH 7), most of the hydrophobic domains of glycomacropeptide become strong negative charge density due to deprotonation of amino acids (Glu and Asp) that makes glycomacropeptide molecules turn into strong negative shields preventing then self-association (Sharma, Rajput, & Mann, 2013). This possibly makes the glycomacropeptide more susceptible to proteases in intestine than in stomach during digestion.

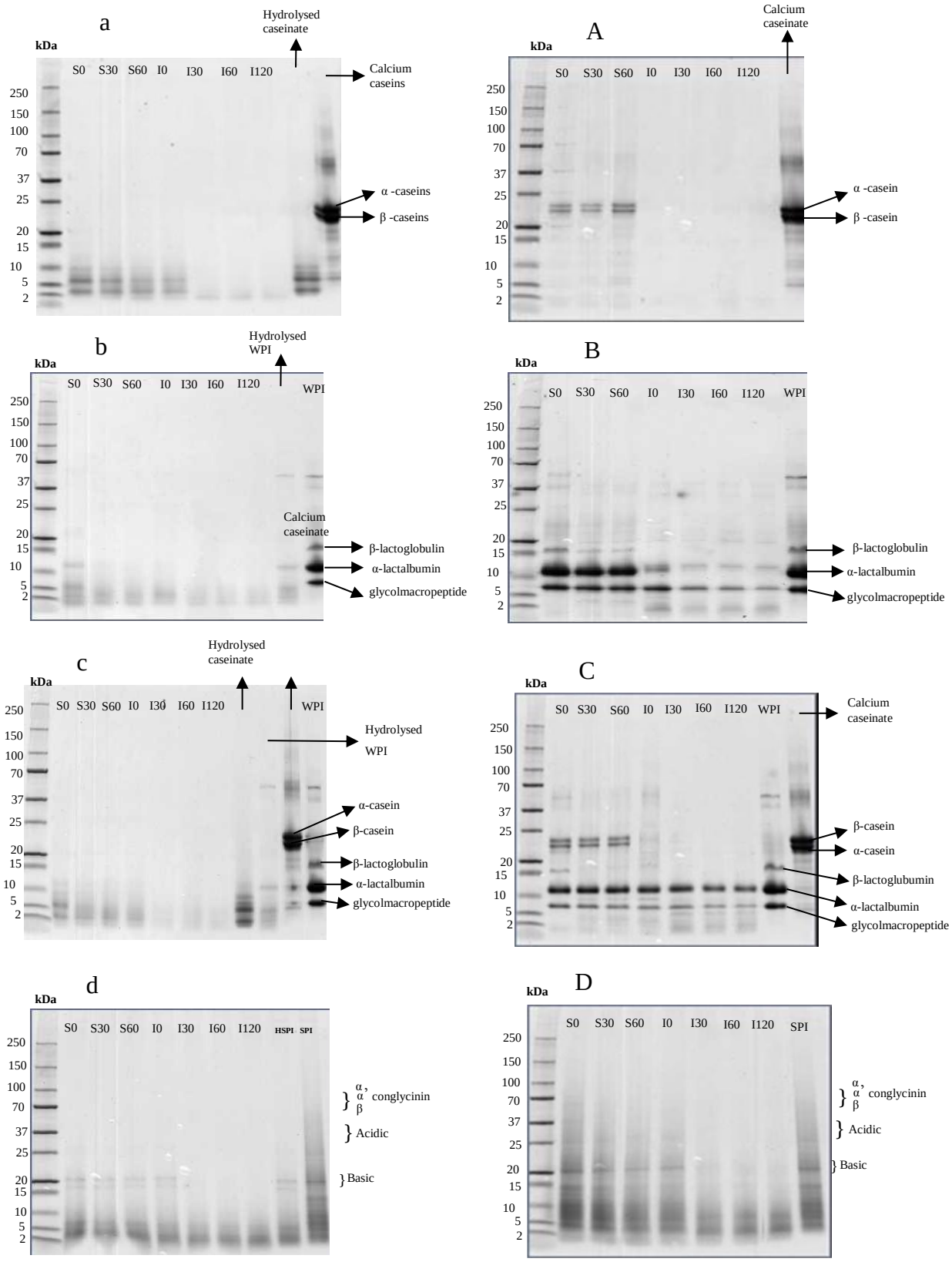


Fig 5.2 Reducing SDS-PAGE analysis of *in vitro* digested samples of infant formulae with 100% HCP (a), 100% CC (A), 100% HWP (b), 100% WPI (B), HWP:HCP=1:1(c), WPI:CC=1:1 (C), 100% HSPI (d) ,100% SPI (D).

At the end of *in vitro* gastrointestinal digestion, no bands of intact proteins were detected for all formulae using hydrolysed proteins suggesting an improved digestibility of hydrolysates compared to their native counterparts. This improvement in digestibility of hydrolysed proteins could be due to changes to their molecular structure and increased solubility making them more accessible to proteases as suggested by Sindayikengera (2006). Sindayikengera (2006) found similar results with hydrolysed caseinate and hydrolysed whey with 5-20% degree of hydrolysis. Moure et al (2005) and Moughan et al (1990) also reported an *in vitro* digestibility enhancement soy hydrolysate as compared to native soy proteins.

Although SDS-PAGE provides valuable information on the proteolytic susceptibility of intact proteins (Nguyen et al., 2015), it is unable to evaluate small peptides (2-3 kDa) and amino acids (Kaur et al., 2014) which is useful for comparing digestibility of infant formulae containing hydrolysed and non-hydrolysed proteins. Hence, ninhydrin-reactive amino nitrogen in the digesta samples of various formulae before and after gastric and intestinal was determined.

5.3.3. Ninhydrin-reactive amino nitrogen

As can be seen in (Fig 5.3 [A-D]) there is a significantly higher amount of ninhydrin-reactive amino nitrogen in digesta samples of all infant formulae using hydrolysed proteins compared to non-hydrolysed proteins at the beginning (S0) and over digestion (S60, I120) except for the formulae made of HSPI and SPI. The results indicate that at the end of gastric (S60) and intestinal digestion (I120), the availability of small peptides and amino acids in formulae made of hydrolysed dairy proteins is certainly higher than in non-hydrolysed ones as hydrolysates already contain free amino acids released during the enzymatic manufacturing process (Pasupuleti & Braun, 2008).

Also, there is an increase in amount of ninhydrin-reactive amino nitrogen generated by proteases during gastrointestinal digestion for formulae using 100%, 100% HCP, 100% WPI, and 100% CC. However, no difference was observed over digestion for formulae with a mixture of HCP-HWP, or a mixture of WPI-CC, or hydrolysed SPI, or HSPI. This anomalous behaviour and the large variations between duplicates is unclear and will need further investigation.

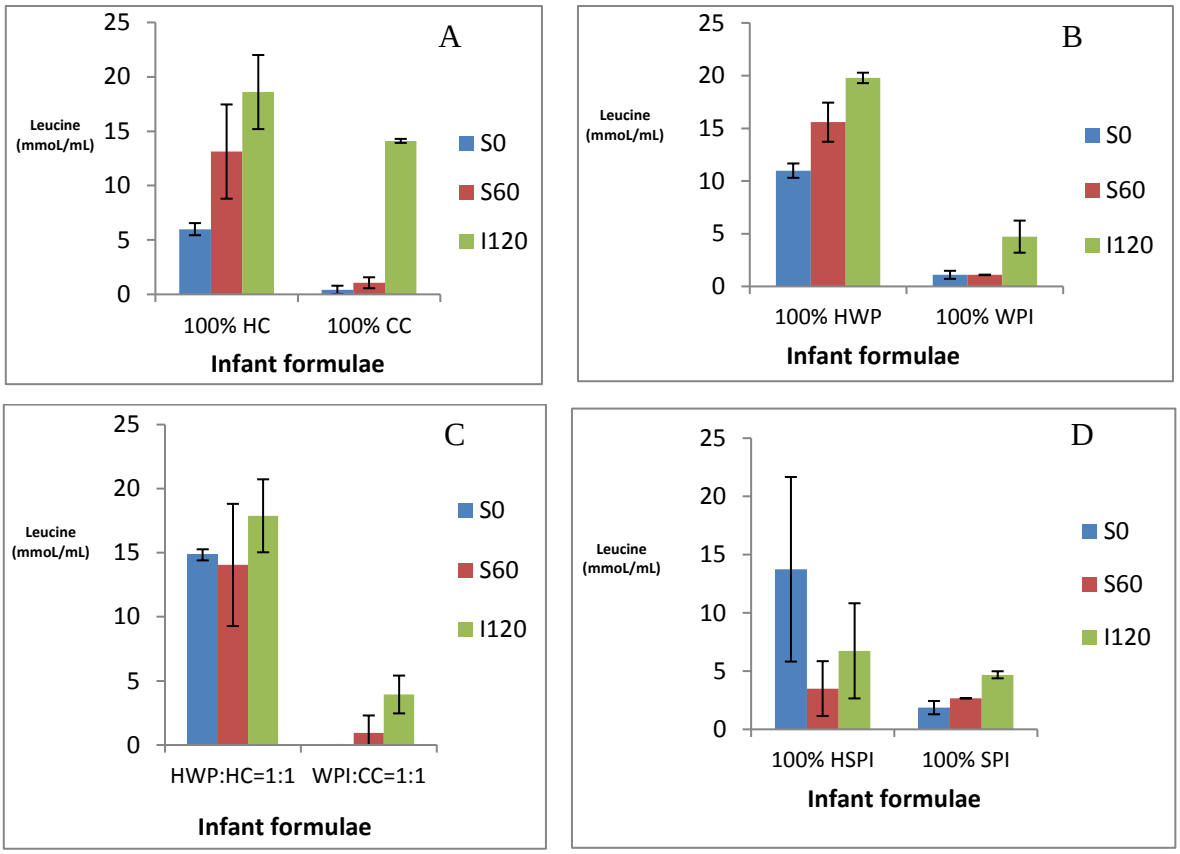


Fig 5.3 Comparison of amino nitrogen released (L-Leucine, mmoL/mL) using ninhydrin reagent during *in vitro* digestion after 0 and 60 min in stomach (S0, S60) and after 120 min in intestinal phase (D120) of infant formulae using 100% HCP – 100% CC (A), HWP – WPI (B), HWP:HCP = 1:1 (C), 100% HSPI – 100% SPI (D).

The pH-drop results are in agreement with SDS-PAGE but not with the ninhydrin-reactive amino nitrogen results. It is a known fact that non-hydrolysed proteins containing higher amount of intact proteins should give higher amount of amino acids and small peptides released during digestion. This is confirmed by the pH-drop method that resulted in more drop in pH with non-hydrolysed proteins compared to hydrolysed proteins during intestinal digestion (Fig 5.1). However, the amount of amino acids and peptides determined using ninhydrin test (Fig 5.3) is higher for hydrolysed proteins that seems to contradict from the pH-drop method results. It is worth noting that the hydrolysed proteins have already gone through hydrolysis during their production that results in a higher amount of amino acids and peptides right from the start. Hence, the hydrolysis step should be taken into account for the total digestibility of hydrolysed proteins.

5.3.4. Particle size distribution

Particle size of the digesta in the gastrointestinal tract can influence its viscosity and dissolvability during digestion. The particle size distribution of infant formula affects the rheological behaviour during *in vitro* infant formula digestion (Prakash, Ma, & Bhandari, 2014) and can provide useful information for design of infant formula. Hence, in this study the particle size distribution of infant formula was measured during infant gastrointestinal digestion. The particle size distribution of the infant formulae in their native state and during gastric and intestinal digestions are as seen in Fig 5.4 (a-d) and Fig 5.4 (A-D). The figures show a bimodal distribution for the formulae with dairy proteins in their native state with a size range from 0.02 to 3 μm. However, the addition of simulated gastric fluid to the native milk make the particle size distribution remarkably increased up to 700 μm due to proteins agglomerated at acidic pH in the gastric phase and then a decrease over the gastric digestion period. The largest protein agglomerates were observed at 100% CC formula due to the highest content of caseinate, then a reduction at formulae containing less caseinate (WPI:CC=1:1, 100% HCP, HWP:HCP=1:1). The particle size distribution of formulae using 100% hydrolysed and 100% non-hydrolysed almost remained unchanged during 60 min in stomach.

The particle size of native milk was similar between formulae made of hydrolysed and non-hydrolysed dairy proteins as per Fig 5.4 and Table 5.2. However, with formulae using soy proteins, particles size of the native samples in HSPI was larger than in SPI. The bigger particle size of soy hydrolysate emulsion in native samples may be due to more exposed hydrophobic residues in soy hydrolysates than in SPI that promotes the hydrophobic interactions among soy hydrolysates resulting in aggregates (Tsumura et al., 2005).

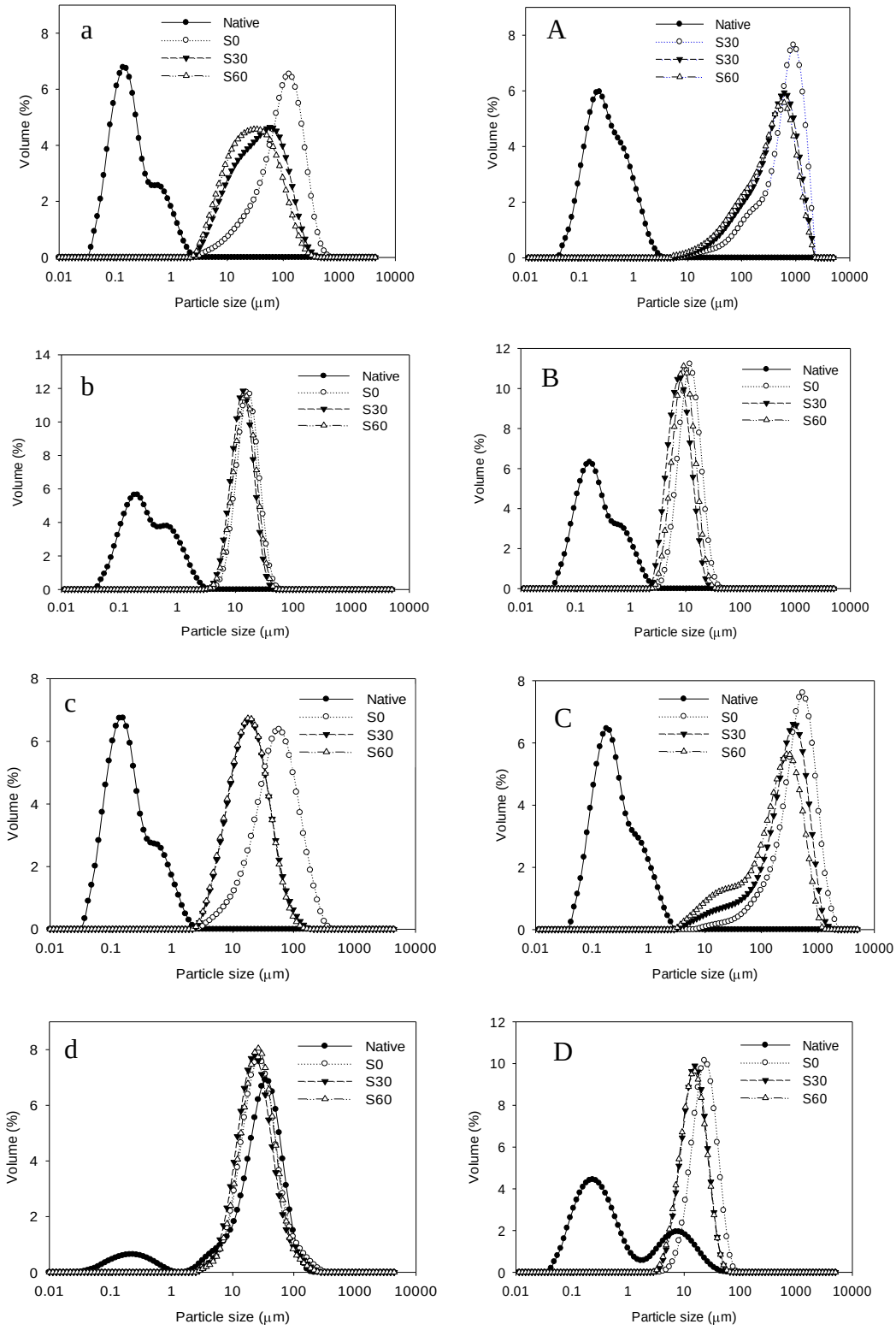


Fig 5.4 Size distribution of native and digested samples under the *in vitro* stomach digestion of the four infant milk formulae from hydrolysed and non-hydrolysed proteins: 100% HCP (a) - 100% CC (B), 100% HWP (b) - 100% WPI (B), HWP:HCP=1:1 (c) WPI:CC=1:1(C), 100% HSPI (d) - 100% SPI (D).

Table 5.2 Particle size distribution of native and *in vitro* gastric digested samples of infant milk formulae

Name of the formulae		d (0.1)	d (0.5)	d (0.9)
	Native	0.1±0.0	0.2±0.0	0.8±0.0
	S0	24.4±3.5	117.6±17.0	301.3±42.0
	S30	9.9±0.3	49.2±3.1	162.0±11.6
100% HCP	S60	7.9±0.1	35.0±1.4	134.1±6.9
	Native	0.1±0.0	0.3±0.0	0.9±0.0
	S0	123.6±12.0	646.0±46.1	1338.1±36.4
	S30	67.2±3.1	401.0±22.5	1055.8±40.7
100% CC	S60	54.8±1.2	345.1±14.9	958.9±66.0
	Native	0.1±0.0	0.2±0.0	1.0±0.0
	S0	9.2±0.6	16.4±1.5	27.8±3.2
	S30	6.5±0.6	11.3±0.2	19.2±0.3
100% HWP	S60	7.1±0.1	13.1±0.1	23.1±0.9
	Native	0.1±0.0	0.2±0.0	0.8±0.0
	S0	6.0±0.1	10.8±0.6	19.0±1.8
	S30	3.7±0.0	6.7±0.1	12.0±0.3
100% WPI	S60	4.7±0.1	8.4±0.4	15.0±0.8
	Native	0.1±0.0	0.2±0.0	0.7±0.0
	S0	15.0±1.5	42.4±4.7	89.3±10.4
	S30	7.0±1.2	17.1±0.4	37.8±1.2
HWP:HCP=1:1	S60	6.8±0.1	16.7±0.6	37.6±1.7
	Native	0.08±0.01	0.21±0.01	0.85±0.01
	S0	117.10±25.4	431.72±92.3	918.54±210.8
	S30	40.45±8.2	270.31±41.4	640.37±100.2
WPI:CC =1:1	S60	19.49±3.6	271.54±30.3	499.34±43.5
	Native	15.49±0.6	33.91±1.6	64.62±3.4
	S0	9.91±0.2	22.50±0.6	45.75±1.4
	S30	10.15±0.4	22.57±0.7	45.52±1.1
100% HSPI	S60	12.32±0.4	27.24±0.7	53.92±1.5
	Native	0.10±0.1	0.36±0.1	9.36±0.8
	S0	10.80±0.5	21.23±1.2	39.19±1.6
	S30	6.98±0.1	13.89±0.3	26.14±1.2
100% SPI	S60	6.68±0.1	13.63±0.4	26.34±1.6

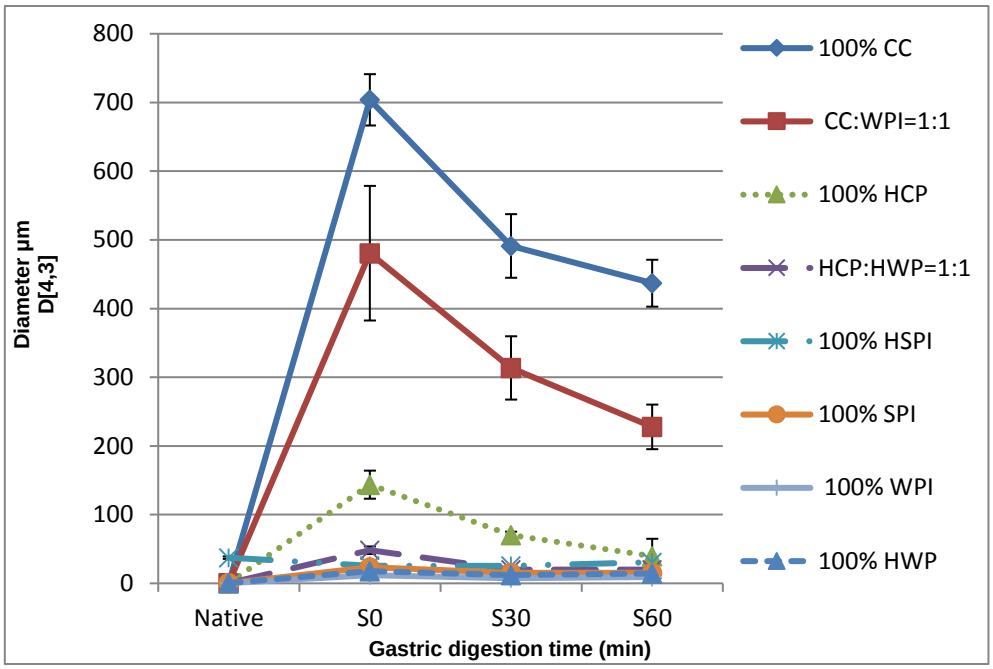


Fig 5.5 Volume mean D[4,3] diameter of native and gastric digested samples of hydrolysed and non-hydrolysed infant milk formulae

The breakdown of the agglomerates by pepsin also resulted in a decrease of the volume mean D[4,3] diameter over 60 min of gastric digestion (Fig. 5). A similar result with commercial infant formula has also been reported by Prakash et al.(2014). At the time of mixing with simulated gastric fluid, D[4,3] increased compared to that of native samples for most of the formulae. With higher amount of caseins in the formulae, larger D[4,3] was observed due to the agglomeration of caseins in the gastric environment (pH 4). Formulae containing hydrolysed caseinate also showed larger D[4,3] because of the availability of some native caseins. However, D[4,3] for formulae with whey proteins and SPI (both hydrolysed and non-hydrolysed) were the smallest and remained unchanged during gastric digestion. This could be due to the conditions in stomach (pH 4, 37^0C) that is not appropriate for whey proteins and soy proteins to precipitate (Adepoju, Longe, Odeinde, Elemo, & Erukainure, 2012; Bramaud, Aimar, & Daufin, 1997). In contrast, during the intestinal phase, particle size distribution of the intestinal digesta remains unchanged similar to the native sample as all the protein agglomerates dissolved because of alkalising the intestinal digesta to pH 8.0. Data for native samples are not shown in Fig 5.4.

5.3.5. Microstructural changes

The gastric and intestinal digestions of the infant formulae with hydrolysed and non-hydrolysed proteins were followed with CLSM. Fig 5.6 [a-d] and Fig 5.6 [A-D] compare the change in microstructure at the beginning and the end of gastric digestion only (the particle size of native and intestinal samples were too small to be captured through CLSM and therefore has not been presented). At the start of the gastric phase (Fig 5.6, S0) hydrolysed and non-hydrolysed proteins are in large agglomerates as confirmed by Fig 5.4 and Fig 5.5. After one hour of proteolysis in the stomach (S60), the large agglomerates of hydrolysed dairy proteins become smaller as compared to that in S0.

In contrast, the confocal micrographs suggested that the size of fat globules during one hour of gastric digestion and two hours of intestinal digestion remained unchanged (Fig 5.7) (data for formulae with non-hydrolysed proteins not shown). This is due to the absence of gastric and pancreatic lipases (Nguyen et al., 2015). When making the infant formula samples, the fat was homogenized during which the surface-active proteins will be adsorbed at the interface of fat particles, forming fat globule membrane. It is expected the proteins in the fat globular membrane will be digested resulting in destabilisation and coalescence of fat droplets. However, the confocal micrographs showed no fat coalescence or free fat smear during the gastrointestinal phase. This is possibly due to the immediate re-adsorption of the surface active proteolytic products at the interface of fat particles in stomach phase. Also the surface-active peptides produced during the digestion process are adsorbed at the interface of fat particles in the absence of lipase that would have affected the behaviour of fat particles. Similar results were also noted by Li et al.(2013); Gallier et al.(2012); Ye et al.(2011) who show that the fat globule membrane was stable during gastric proteolysis. They also postulated that peptides generated by any proteolysis of membrane proteins would be adsorbed into the fat globule membrane, preventing the coalescence of fat globules. Nevertheless, in the intestinal phase, the stabilization of fat globules is due to the replacement of peptides or remaining proteins by bile acids at the fat globule membrane (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011).

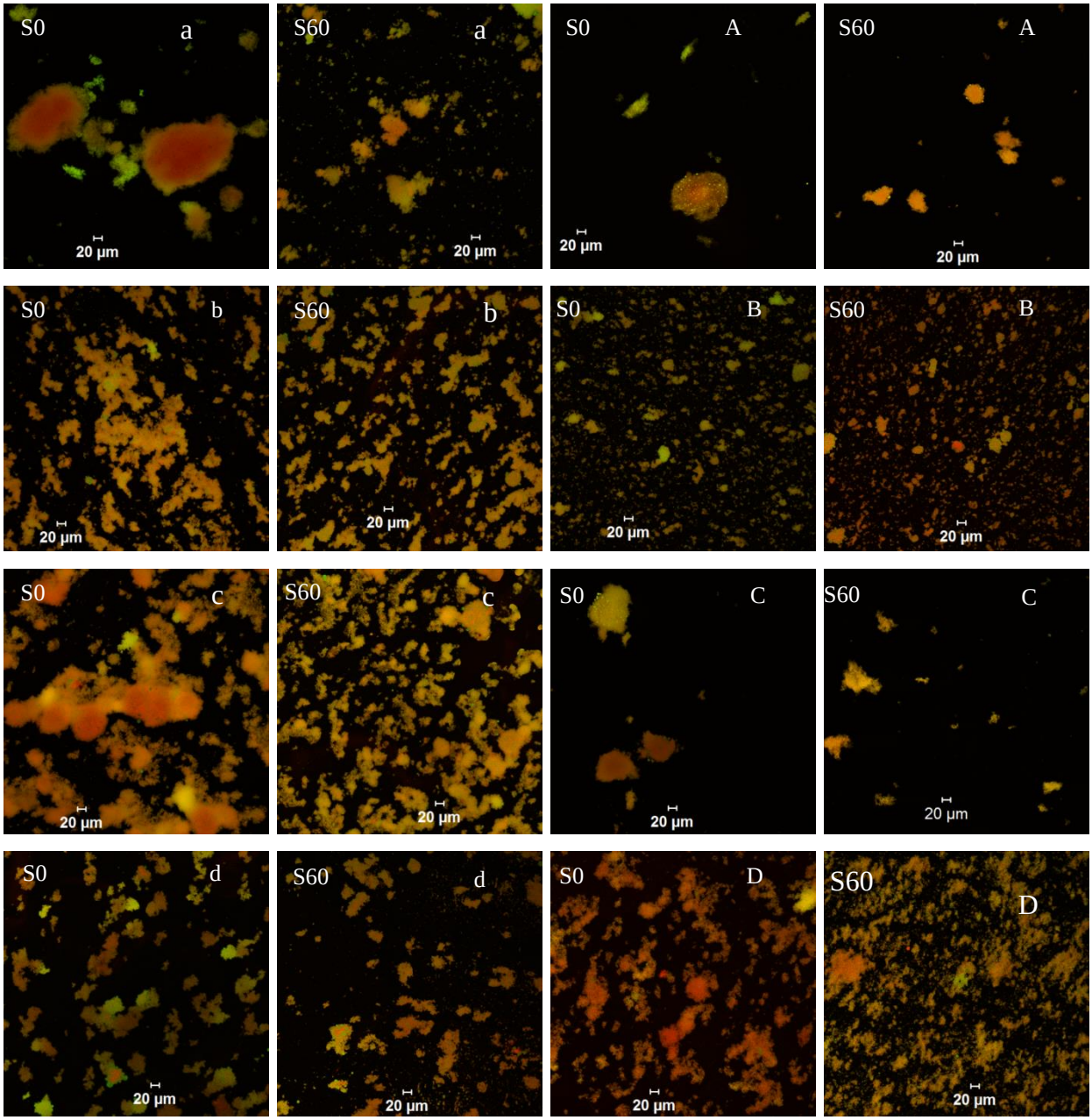


Fig 5.6 CLSM of protein agglomerates in digested samples at beginning (S0) and 60 min (S60) of the gastric digestion with formulae:100% HCP (a) - 100% CC (A), 100% HWP (b) - WPI 100% (B), HWP:HPC=1:1 (c) - WPI:CC=1:1 (C), 100% HSPI 100% (d) –100% SPI (D)

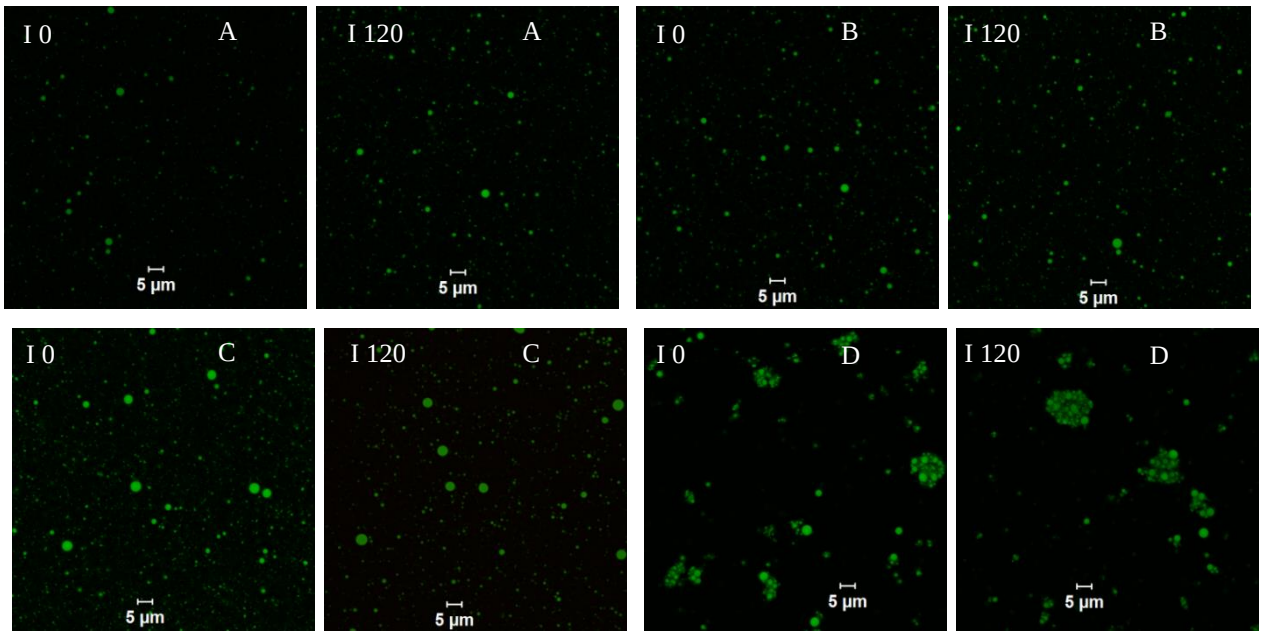


Fig 5.7 CLSM of fat globules in digested samples at beginning (I0) and (I120) of the intestinal digestion of formulae 100% HCP (A), 100% HWP (B), HWP:HCP=1:1 (C), 100% HSPI (D).

In the intestinal phase with all the protein agglomerates in the digesta dissolved, the confocal micrographs could not be obtained for the small particles. This is coherent with the particle size distribution results presented in Table 5.2. Similar results were observed in our previous study (Nguyen et al., 2015).

Overall, this study demonstrates an improvement in digestibility of hydrolysed proteins used in infant formula compared to non-hydrolysed proteins. Hydrolysed dairy and hydrolysed soy protein formulae with significantly higher availability of amino acids and small peptides, but less intact proteins than non-hydrolysed proteins, are completely digested at the intestinal phase. There are also differences in digestibility of non-hydrolysed proteins such as dairy and soy proteins, whey and caseins, and even among whey proteins (α-lactalbumin, β-lactoglobulin, and glycomacropeptide). The difference in structure of these proteins: soy proteins with higher content of β-sheet promoting protein self-aggregation, caseins with open tertiary structure, compact globular structure of α-lactalbumin molecules, self-assembling ability of glycomacropeptide (depends on pH condition), possibly results in the difference in their protease susceptibility.

Difference in particle size distribution and micro-structure among formulae during gastric digestion is mainly influenced by the intact caseins content. Formulae without caseins observed less protein

agglomeration over gastric digestion. No coalescence of fat globules was observed at all for formulae made of hydrolysed and non-hydrolysed proteins through *in vitro* gastric and intestinal digestion for all formulae made of hydrolysed and non-hydrolysed proteins due to the absence of lipases.

5.4. Conclusions

The above results obtained from the static *in vitro* digestion, simulating the infant gastrointestinal tract suggest non-hydrolysed dairy proteins to be first partially hydrolysed by pepsin following which they are further digested by proteases in the intestine. Difference in protein structure is a possible reason leading to the differences in their digestibility. Hydrolysed dairy and hydrolysed soy protein formulae show a significantly higher availability of amino acids and small peptides and less intact proteins than those in non-hydrolysed proteins. An improvement in digestibility of formulae with hydrolysed dairy and hydrolysed soy proteins is observed.

With the absence of lipases, the particle size of fat globules during the gastrointestinal digestion for all formulae remains unchanged due to the stabilisation of fat membrane. For more understanding about how fat emulsion is digested by lipases under the influence of proteases, an *in vitro* infant lipolysis is being conducted. It is clear that partially hydrolysed infant formula is increasingly used worldwide, a full investigation about long-term effect of hydrolysed proteins on infants' development is essential.

5.6. References

Adepoju, P., Longe, A., Odeinde, O., Elemo, G., & Erukainure, O. (2012). Investigation into the coagulating properties of acid and enzyme coagulated soy protein precipitate. *Food and Public Health*, 2(5), 127-130.

Agostoni, C., Axelsson, I., Goulet, O., Koletzko, B., Michaelsen, K. F., Puntis, J., . . . Szajewska, H. (2006). Soy protein infant formulae and follow-on formulae: a commentary by the ESPGHAN Committee on Nutrition. *Journal of Pediatric Gastroenterology and Nutrition*, 42(4), 352-361.

Agostoni, C., Terracciano, L., Varin, E., & Fiocchi, A. (2014). The nutritional value of protein-hydrolysed formulae. *Critical Reviews in Food Science and Nutrition*, 56(1), 65-69.

Allergy, A. S. O. C. I. A. (2010). Infant feeding advise, from https://www.allergy.org.au/images/stories/aer/infobulletins/2010pdf/ASCIA_Infant_Feeding_Advice_2010.pdf

Alles, M. S., Scholtens, P. A., & Bindels, J. G. (2004). Current trends in the composition of infant milk formulas. *Current Paediatrics*, 14(1), 51-63.

Australia, G. o. W. (2014). Child and antenatal nutrition manual infant formula, from http://www.pmh.health.wa.gov.au/health/docs/Infant%20Formula.pdf

Bos, C., Metges, C. C., Gaudichon, C., Petzke, K. J., Pueyo, M. E., Morens, C., . . . Tomé, D. (2003). Postprandial kinetics of dietary amino acids are the main determinant of their metabolism after soy or milk protein ingestion in humans. *The Journal of Nutrition*, 133(5), 1308-1315.

Bramaud, C., Aimar, P., & Daufin, G. (1997). Optimisation of a whey protein fractionation process based on the selective precipitation of α-lactalbumin. *Le Lait*, 77(3), 411-423.

Carbonaro, M., Maselli, P., & Nucara, A. (2012). Relationship between digestibility and secondary structure of raw and thermally treated legume proteins: a Fourier transform infrared (FT-IR) spectroscopic study. *Amino Acids*, 43(2), 911-921.

Carbonaro, M., Maselli, P., & Nucara, A. (2015). Structural aspects of legume proteins and nutraceutical properties. *Food Research International*, 76, 19-30.

Dupont, C., Hol, J., & Nieuwenhuis, E. E. (2015). An extensively hydrolysed casein-based formula for infants with cows' milk protein allergy: tolerance/hypo-allergenicity and growth catch-up. *British Journal of Nutrition*, 113(07), 1102-1112.

Dupont, D., Mandalari, G., Mollé, D., Jardin, J., Rolet-Répécaud, O., Duboz, G., . . . Mackie, A. R. (2010). Food processing increases casein resistance to simulated infant digestion. *Molecular Nutrition & Food Research*, 54(11), 1677-1689.

Farías, M., Martinez, M., & Pilosof, A. (2010). Casein glycomacropeptide pH-dependent self-assembly and cold gelation. *International Dairy Journal*, 20(2), 79-88.

Gallier, S., Tate, H., & Singh, H. (2012). In Vitro Gastric and Intestinal Digestion of a Walnut Oil Body Dispersion. *Journal of Agricultural and Food Chemistry*, 61(2), 410-417.

Gallier, S., Ye, A., & Singh, H. (2012). Structural changes of bovine milk fat globules during in vitro digestion. *Journal of Dairy Science*, 95(7), 3579-3592.

Goedhart, A. C., & Bindels, J. G. (1994). The composition of human milk as a model for the design of infant formulas: recent findings and possible applications. *Nutrition Research Reviews*, 7, 1-24.

Hall, B., Chesters, J., & Robinson, A. (2012). Infantile colic: a systematic review of medical and conventional therapies. *Journal of Paediatrics and Child Health*, 48(2), 128-137.

Hays, T., & Wood, R. A. (2005). A systematic review of the role of hydrolyzed infant formulas in allergy prevention. *Archives of Pediatrics and Adolescent Medicine*, 159(9), 810-816.

Hernell, O. (2011). Human milk vs. cow's milk and the evolution of infant formulas. Paper presented at the Milk and Milk Products in Human Nutrition, Copenhagen.

Hernell, O., & Lönnerdal, B. (2003). Nutritional evaluation of protein hydrolysate formulas in healthy term infants: plasma amino acids, hematology, and trace elements. *The American Journal of Clinical Nutrition*, 78(2), 296-301.

Holt, C., Carver, J. A., Ecroyd, H., & Thorn, D. C. (2013). Invited review: Caseins and the casein micelle: Their biological functions, structures, and behavior in foods. *Journal of Dairy Science*, 96(10), 6127-6146.

Hsu, H., Vavak, D., Satterlee, L., & Miller, G. (1977). A multienzyme technique for estimating protein digestibility. Journal of Food Science, 42(5), 1269-1273.

Jakobsson, I., Borulf, S., Lindberg, T., & Benediktsson, B. (1983). Partial hydrolysis of cow's milk proteins by human trypsins and elastases in vitro. *Journal of Pediatric Gastroenterology and Nutrition*, 2(4), 613-616.

Kaur, L., Maudens, E., Haisman, D. R., Boland, M. J., & Singh, H. (2014). Microstructure and protein digestibility of beef: The effect of cooking conditions as used in stews and curries. *LWT-Food Science and Technology*, 55(2), 612-620.

Koletzko, B., Baker, S., Cleghorn, G., Neto, U. F., Gopalan, S., Hernell, O., . . . Ding, Z. Y. (2005). Global standard for the composition of infant formula: Recommendations of an ESPGHAN Coordinated International Expert Group. *Journal of Pediatric Gastroenterology and Nutrition*, 41(5), 584-599.

Koletzko, B., Saris, W., Flynn, A., Palou, A., Wal, J., Jackson, A., . . . Turck, D. (2003). Report of the Scientific Committee on Food on the Revision of Essential Requirements of Infant Formulae and Follow-on Formulae. Brussels, European Commission.

Koletzko, S., Niggemann, B., Arato, A., Dias, J., Heuschkel, R., Husby, S., . . . Staiano, A. (2012). Diagnostic approach and management of cow's-milk protein allergy in infants and children: ESPGHAN GI Committee practical guidelines. *Journal of Pediatric Gastroenterology and Nutrition*, 55(2), 221-229.

Lacroix, M., Bos, C., Léonil, J., Airinei, G., Luengo, C., Daré, S., . . . Tomé, D. (2006). Compared with casein or total milk protein, digestion of milk soluble proteins is too rapid to sustain the

anabolic postprandial amino acid requirement. The American Journal of Clinical Nutrition, 84(5), 1070-1079.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227(5259), 680-685.

Li, J., Ye, A., Lee, S. J., & Singh, H. (2013). Physicochemical behaviour of WPI-stabilized emulsions in in vitro gastric and intestinal conditions. *Colloids and Surfaces B: Biointerfaces*, 111, 80-87.

Maldonado-Valderrama, J., Wilde, P., Macierzanka, A., & Mackie, A. (2011). The role of bile salts in digestion. *Advances in Colloid and Interface Science*, 165(1), 36-46.

Moore, S. (1968). Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. *Journal of Biological Chemistry*, 243(23), 6281-6283.

More, J. (2013). Infant, Child and Adolescent Nutrition: A Practical Handbook: CRC Press.

Moughan, P. J., Pedraza, M., Smith, W. C., Williams, M., & Wilson, M. N. (1990). An evaluation with piglets of bovine milk, hydrolyzed bovine milk, and isolated soybean proteins included in infant milk formulas. I. Effect on organ development, digestive enzyme activities, and amino acid digestibility. *Journal of Pediatric Gastroenterology and Nutrition*, 10(3), 385-394.

Moure, A., Domínguez, H., & Parajó, J. C. (2005). Fractionation and enzymatic hydrolysis of soluble protein present in waste liquors from soy processing. *Journal of Agricultural and Food Chemistry*, 53(19), 7600-7608.

Nagano, T., Tamaki, E., & Funami, T. (2008). Influence of guar gum on granule morphologies and rheological properties of maize starch. *Carbohydrate Polymers*, 72(1), 95-101.

Nguyen, T., Bhandari, B., Cichero, J., & Prakash, S. (2015). Gastro intestinal digestion of dairy and soy proteins in infant formulas: An in vitro study. *Food Research International*, 76, 348-358.

O'Connor, N. R. (2009). Infant formula. *American Family Physician Journal*, 79(7), 565-570.

Pasupuleti, V. K., & Braun, S. (2008). State of the art manufacturing of protein hydrolysates. Protein hydrolysates in biotechnology (pp. 11-32): Springer.

Pike, A. C., Brew, K., & Acharya, K. R. (1996). Crystal structures of guinea-pig, goat and bovine α-lactalbumin highlight the enhanced conformational flexibility of regions that are significant for its action in lactose synthase. *Structure*, 4(6), 691-703.

Prakash, S., Ma, Q., & Bhandari, B. (2014). Rheological behaviour of selected commercially available baby formulas in simulated human digestive system. *Food Research International*, 64, 889-895.

Sharma, R., Rajput, Y. S., & Mann, B. (2013). Chemical and functional properties of glycomacropeptide (GMP) and its role in the detection of cheese whey adulteration in milk: a review. *Dairy Science & Technology*, 1(93), 21-43.

Sindayikengera, S. (2006). Nutritional evaluation of caseins and whey proteins and their hydrolysates from Protamex. Journal of Zhejiang University Science B, 7(2), 90-98.

Swaisgood, H. E. (1993). Review and Update of Casein Chemistry 1, 2. *Journal of Dairy Science*, 76(10), 3054-3061.

Tsumura, K., Saito, T., Tsuge, K., Ashida, H., Kugimiya, W., & Inouye, K. (2005). Functional properties of soy protein hydrolysates obtained by selective proteolysis. *LWT-Food Science and Technology*, 38(3), 255-261.

van de Velde, F., Weinbreck, F., Edelman, M. W., van der Linden, E., & Tromp, R. H. (2003). Visualisation of biopolymer mixtures using confocal scanning laser microscopy (CSLM) and covalent labelling techniques. *Colloids and Surfaces B: Biointerfaces*, 31(1), 159-168.

van Riemsdijk, L. E., Sprakel, J. H., van der Goot, A. J., & Hamer, R. J. (2010). Elastic networks of protein particles. *Food Biophysics*, 5(1), 41-48.

Ventura, A. K., San Gabriel, A., Hirota, M., & Mennella, J. A. (2012). Free amino acid content in infant formulas. *Nutrition & Food Science*, 42(4), 271-278.

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

CHAPTER 6 *IN VITRO* LIPOLYSIS OF DAIRY AND SOY BASED INFANT MILK FORMULAE

6.1. Introduction

Infant formula is considered as mother's milk substitution for the babies who cannot access mother's milk due to various reasons. Lipids in mother's milk and formulas provide nearly half of the total energy to babies. The lipids contain n-6 and n-3 fatty acids such as linoleic acid (C18:2, n-6) and α-linoleic acid (C18:3, n-3) that are essential for infant brain and eye development (Hermoso et al., 2010; Joeckel & Phillips, 2009). The activity of digestive lipases in infants is lower compared to adults, but infants have three to five times higher lipid intake per kilogram of bodyweight (Andersson, Hernell, Bläckberg, Fält, & Lindquist, 2011). Lipid digestion in infants takes place both in the stomach and small intestine.

Gastric lipolysis plays a more important role in fat digestion in infants than in adults (Carey, Small, & Bliss, 1983; Hamosh et al., 1981). This is because pH in the infant stomach (4.0-5.0) is less acidic than that in adults, which is closer to the optimum pH of gastric lipase (3.5-6.0) (Liao, Hamosh, & Hamosh, 1984). Also gastric lipase does not require bio-surfactants (bile salts), and it is not inhibited by milk fat globule membranes (Hamosh, 1996; Hernell, Blackberg, & Bernback, 1988; Ville, Carriere, Renou, & Laugier, 2002). These properties allow gastric lipase to hydrolyse triglycerides in the infant stomach much easier than in adults. The level of gastric lipase in infants is similar or even higher to the level found in adults and that possibly compensates for the low activity of pancreatic lipases and explains why infants can consume a high dietary fat (Armand et al., 1995; Armand et al., 1996; Sarles, Moreau, & Verger, 1992). It has been reported that gastric lipase in animal infants can digest up to 25-60% of total lipids as compared to 10-25% in human adults (Abrahamse et al., 2012; Hamosh, 2006). There is limited data about physiological lipid digestion in human infants due to the restricted access to clinically invasive procedures (Abrahamse et al., 2012).

The duodenal digestion of lipids in infants is predominantly by pancreatic lipase-related to protein 2 (PLRP2) and bile salt-stimulated lipase (BSSL), while pancreatic lipase (PTL) is the key lipase involved with the duodenal lipid digestion in adults (Andersson et al., 2011; Lindquist & Hernell, 2010). However, the exact activity of PLRP2 and BSSL in infants is still not clear (Andersson et al., 2011). PTL and BSSL cannot hydrolyse the core of triglycerides because of their inability to penetrate

into milk fat globules (Cohen, Morgan, & Hofmann, 1971; Roman et al., 2007). Therefore, bile salts play an important role in emulsifying the lipid in the duodenum before being hydrolysed by pancreatic lipases, but pancreatic lipase activity and bile salt concentration in infants are very low compared to adults (Lindquist & Hernell, 2010). In term infants, the activity of pancreatic lipase and bile salt concentration are approximately 5-10% which is around 50% of adults' figures, respectively (Lebenthal, Lee, & Heitlinger, 1983).

In order to mimic the composition of fat in mothers' milk with a high content of long chain polyunsaturated fatty acids (LCPUFAs), vegetable oils are currently used as a lipid source in manufacturing infant formula (Bourlieu, Bouzerzour, et al., 2015; Nguyen, Bhandari, Cichero, & Prakash, 2015a). However, the structure of fat globules in infant formula is different from mothers' milk in terms of droplet size and membrane components. The oil droplet size distribution in infant formula is in the range of 0.1-1 μm which is smaller than in mothers' milk 0.1-10 μm (Michalski, Briard, Michel, Tasson, & Poulain, 2005). Also, due to the way the fat globules in mothers' milk are made and secreted from the mammary gland cells, the fat droplets in mothers' milk are covered by a phospholipid trilayered membrane composed of phospholipids, proteins and cholesterol (Gallier et al., 2015). However, oil droplets in infant formula are stabilized by an absorbed proteins layer based on caseins, whey proteins, whey protein aggregates, and even soy proteins on the surface of oil droplets (Bourlieu, Ménard, et al., 2015; Reis, Holmberg, Watzke, Leser, & Miller, 2009). Because enzyme lipases act on the insoluble emulsified substrates, lipid digestion process may depend on the lipase adsorbed on to the surface of the emulsified droplets (Armand et al., 1997; Porter, Trevaskis, & Charman, 2007). It is well documented that the rate and extent of lipolysis is controlled not by the enzymes' level but their ability to access the interface of the oil/water (Golding et al., 2011). This means that the digestibility of lipids in infant formula could be affected by the structure of the emulsion such as the surface area, and the composition of their interfacial layer surrounding the oil droplets(Bourlieu, Bouzerzour, et al., 2015; Bourlieu, Ménard, et al., 2015; Reis et al., 2009). One would therefore expect that if the absorbed protein layer of the oil droplet surface is hydrolysed by digestive proteases, the lipase could access the droplet core easier, resulting in an increase in the rate of the lipolysis process. Bourlieu, Ménard, et al. (2015) suggested the presence of pepsin destabilised the emulsions due to proteolysis during the *in vitro* gastric phase. This study used infant formula emulsions from standardized cow's milk with different homogenization pressure and pasteurization treatment conditions. It remains unclear how infant formulae emulsions with vegetable oils stabilised by dairy proteins and plant proteins behave under the simulated infant gastrointestinal digestion.

Therefore, the objective of this study is to estimate the effect of protease hydrolysis on lipid digestion in an *in vitro* infant GI tract using hydrolysed and non-hydrolysed proteins (dairy and soy proteins) in infant milk formulae. This was achieved by determining the oil droplet size and distribution, released free fatty acids, and micro-structural changes during an *in vitro* infant gastrointestinal digestion.

6.2. Materials and method

6.2.1. Materials

6.2.1.1. Enzymes and chemicals

The following enzymes and chemicals were used for the experiment: lipase DF 15 (180 units/mg, stable pH 4.0-7.0, optimum pH 6.0-7.0, obtained from Amano Enzyme Inc., Japan) was used as analogue gastric lipase. Other enzymes were obtained from Sigma-Aldrich, Castle Hill, New South Wales, Australia and stored at the recommended temperature of -20°C. Other enzymes included: Lipase from porcine pancreas (EC 232.619.9, 59578 units/mg). Pepsin from porcine gastric mucosa (EC 3.4.23.1, 3840 units/mg protein, one unit will produce a change in A280 of 0.001 per min at pH 2.0 at 37°C, measured as TCA-soluble products using haemoglobin as substrate). Trypsin from bovine pancreas (EC 3.4.21.4, 13165 units/mg protein, one unit will produce a change in A253 of 0.001 per minute at pH 7.6 at 25°C using Nα-Benzoyl-L-arginine Ethyl Ester (BAEE) as a substrate. Chymotrypsin from bovine pancreas (EC 3.4.21.1, 54.49 units/mg protein, one unit will hydrolyze 1.0 μmol of N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE) per min at pH 7.8 at 25°C as stated by manufacturer).

Porcine pancreatic colipase, sodium taurocholate, pepstatin, trypsin-chymotrypsin inhibitor, and orlistat were obtained from Sigma-Aldrich, Castle Hill, New South Wales, Australia. They were stored at recommended temperature (-20°C). The other ingredients used in the study such as lactose, sodium chloride, hydrochloric acid, sodium hydroxide, and sodium azide were of analytical grade.

6.2.1.2. Dairy and soy proteins

Whey protein isolate (WPI 85.1% protein, 1.0% fat, 1.2% carbohydrate, all w/w) and calcium caseinate (CC, 86.7% protein, 1.0% fat, 0.15% carbohydrate, all w/w) were purchased from Total Foodtec Pty Ltd (Australia).

Hydrolysed whey protein (HWP, 81.9% protein, 0.5% fat, 2.67% carbohydrate all w/w) and hydrolysed casein protein (HCP, 86.7% protein, 0.9% fat, 0.2% carbohydrate, all w/w) were purchased from Total Foodtec Pty Ltd. and Myopure Pty, Australia.

Soy protein isolate (SPI, 82.4% protein, 4.5% fat, less than 1.0% carbohydrate) purchased from Food Manufacturers Pty., Australia. Hydrolysed SPI (HSPI) was made from SPI by pepsin at a concentration of 22.75 U/mg of total protein, at 37°C for 1 hour. The hydrolysed mixture was alkalised to pH 8.0-8.5 by 0.1N NaOH in order to inactivate the enzyme pepsin. The residue of NaOH was then neutralised by 0.01N HCl followed by freeze-drying for 72 hours (Pasupuleti & Braun, 2008).

Sunflower vegetable oil was obtained from the local supermarket.

6.2.2. Method

6.2.2.1. Preparation of infant formulae

The procedure for making infant formulae was as described by Nguyen, Bhandari, Cichero, and Prakash (2015b) except for the homogenisation step which was done using a micro-fluidiser (Microfluidics, Newton Massachusetts, Model 110L). 100 mL of liquid formula containing 1.5 g of protein, 4.0 g of lipid and 6.5 g of lactose was chosen based on the recommendation for infant formula by the European Union (Koletzko et al., 2005). Preliminary screening of the commercial infant formula available in Australia that contained hydrolysed proteins was conducted and it was found that the hydrolysed whey, hydrolysed casein, and hydrolysed soy protein were the commonly used hydrolysed proteins in the formulas. This study was focused on 4 formulae: WPI:CC=1:1, HWP:HCP =1:1, 100% SPI, and 100% HSPI.

6.2.2.2. In vitro infant lipid digestion

The lipase from Rhizopus oryzae was chosen to substitute gastric lipase in this study. Some previous studies used gastric lipase from human and animals such as dogs and rabbits (Amara et al., 2014; Bourlieu, Ménard, et al., 2015; Carrière et al., 2000). Animal gastric lipase has similar properties to human gastric lipase. However, due to ethical issues and clinically invasive procedures, access to human and animals enzymes is limited. Although fungal lipase has high specificity to the sn-1 and sn-3 position of triglyceride while human gastric lipase only cleaves at sn-3, there is no better analogue gastric lipase than fungal lipase so far (Ménard et al., 2014).

A static *in vitro* digestion unit equipped with water bath, and overhead stirrer carried out the digestion trials. Two water-jacketed reaction vessels in the unit were connected to a water bath that provided a constant circulation of warm water in and out of the reaction vessel and maintained a constant temperature of 37°C. A glass stirrer connected to an overhead stirrer continuously mixed the *in vitro* digesta at 250 rpm. The values for activity of analogue gastric lipase (21 units/mL), activity of pancreatic lipase (200 units/mL), molar ration between colipase and pancreatic lipase was 2:1, bile salts (4mM) were chosen based on the data published by Minekus et al. (2014) and Carrière et al. (2000). Levels of proteases and bile salts are described in Nguyen et al. (2015b). In this study, the gastric pH 4.5 was used that is closer to the optimum pH of analogue gastric lipase and in the appropriate range for the infant gastric conditions. The intestinal pH is 6.5.

Digested samples were inhibited by trypsin-chymotrypsin inhibitor and orlistat to inhibit twice the amount of trypsin and chymotrypsin, analogue gastric lipase and pancreatic lipase. Digested samples were neutralised before measuring the particle size, with the remainder stored at -20°C for Gas Chromatography analysis.

6.2.2.3. Particle size distribution

Particle size distribution of native and digested milk samples was measured before and during *in vitro* gastric and duodenal digestions by Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershine, UK). The refractive index values used for dispersed phase (oil droplets), and continuous phase (water) were 1.47 and 1.33, respectively. Value of d(0.1), d(0.5), d(0.9), and D[4,3] were recorded. D[4,3] is a volume mean of the population which is sensitive to the presence of large

particles. Mean particle sizes and distribution were determined as the average of three repeated measurements from duplication. Digested samples were neutralised to pH 7.0 by 1M NaOH to dissolve the protein aggregation, stabilized for one hour before the measurement. In this research study, particle size was measure to determine the change of fat globules' size. During the gastric phase, due to the acidic pH, aggregation proteins affected the size data. This is the reason why digested samples were neutralized before measuring the particle size.

6.2.2.4. Confocal Laser Scanning Microscopy (CLSM)

The physical status of oil droplets in native and digested samples was observed by Zeiss LSM 700 Confocal Laser Scanning Microscope. Protein were stained with Rhodamine B (0.01% w/w in ethanol) and excited with the laser light at a wavelength 540 nm (Nagano, Tamaki, and Funami, 2008; van de Velde, Weinbreck, Edelman, van der Linden, and Tromp, 2003; van Riemsdijk, Sprakel, van der Goot, and Hamer, 2010). Nile red (0.01% w/w in ethanol) was used to stain triglycerides and excited with the laser light wavelength of 515-530 nm (Gallier, Ye, &Singh, 2012; Ye, Cui, and Singh, 2011).

For slide preparation, 500 μL of infant formula samples was mixed with 20 μL of Rhodamine B and 20 μL of Nile red solution by using vortexer (Ratex VM1) for 5 sec. Samples were stained for at least 10 minutes. 2μL of stain samples were loaded onto 26x76 mm slides (Sail Brand) and then covered with 18x18 mm cover slip (Menzel Glaser). The edges of the cover slips were coated with a transparent nail polish to fix the sample position and prevent the sample from drying. The observations of fat globules and the protein aggregates were done with a magnification lens at 63x and 10x, respectively.

6.2.2.5. Free fatty acid analysis by Gas Chromatography

Lipid extraction and isolation of free fatty acids (FFAs) from digested samples was adopted from De Jong and Badings (1990). 5 mL of digesta was mixed with 5 mL ethanol, 0.5 mL H2SO4 (2.5M), 100 μL internal standard solution (heptadecanoic acid 10% w/v). FFAs were then extracted by adding 7.5 mL ether:heptane =1:1 (v/v) to the mixture, and mixed for 30 minutes. After centrifuging at 2500 rpm for 2 minutes, the upper organic phase was taken. The extraction procedure was done twice with another 7.5 mL ether:heptane =1:1 (v/v).

FFA isolation was carried out with an aminopropyl column (StrataNH2, 200mg/3 mL Phenomenex, Australia). The column was first conditioned with 10 mL heptane then the lipid extract was added to the column. 5 mL of chloroform:2-propanol (2:1, v/v) was applied to elute the neutral lipids. The FFAs were eluted with 5 mL diethyl ether containing 2% formic acid. The collected FFAs fraction was concentrated using nitrogen gas and resuspended in 1 mL diethyl ether. FFAs were analysed by gas chromatography (Shimazu, GC-2010 Plus) equipped with an auto-sampler and a flame-ionization detector (FID) using a FFAP-DB column 30.0 m x 0.53 mm i.d, coating diameter (df = 1.0 μm). The carrier gas was helium at a flow rate of 16.1 mL/min, and oven temperature was varied from 200 ^{0}C C to 240 ^{0}C. 0.5 μL of samples was injected in duplicate.

6.2.2.6. Data analysis

The samples were measured in duplicate from duplication (4 times). This meant each formula was conducted a duplicate of the in vitro experiment, then each digesta sample was extracted and measured twice. Each time point of the in vitro digestion has 4 values for data analysis. Experimental data were assessed by ANOVA to determine the significant differences among the means at 95% confidence level. The treatment means were considered to be significantly different when $P<0.05$.

6.3. Results and discussion

6.3.1 Particle size distribution and confocal micrographs

6.3.1.1 Dairy protein based formulae

The particle size distribution of fat globules in their native state and during gastrointestinal lipolysis digestion is as seen in Fig 6.1 (a-d), Fig 6.1 (A-D), and Fig 6.3. The figures show the fat droplet size in infant formulae change during *in vitro* gastrointestinal digestion. The fat droplet size increases over the gastric phase then decreases over the intestinal phase in both matrices - hydrolysed by lipases alone and by lipases in the presence of proteases. The native state and gastric starting point (S0) of fat globules present a bimodal distribution for the dairy protein formulae with a size range from 0.02 to 3.0 μm (Fig 6.1). During gastric digestion, droplet size remarkably increased up to 20 μm. There is an agreement between the results of particle size distribution and CLMS images (Fig. 6.3) that

indicate the lipid coalescence happened due to destabilization of fat droplets under lipolysis. It is to be noted that protein aggregation was dissolved by neutralising the digesta samples prior to particle size analysis (part 2.2.3).

With WPI:CC=1:1 formula, the fat globules show a bimodal distribution during lipolysis without proteases. However, the distribution interchanges with a decrease in the volume of small population (0.06-0.6 μm) and an increase in the volume of large population (0.6-3 μm) indicating lipid coalescence (Fig 6.1a, Fig 6.3). This pattern was much more pronounced in the presence of proteases (Fig 6.1A, Fig 6.3).

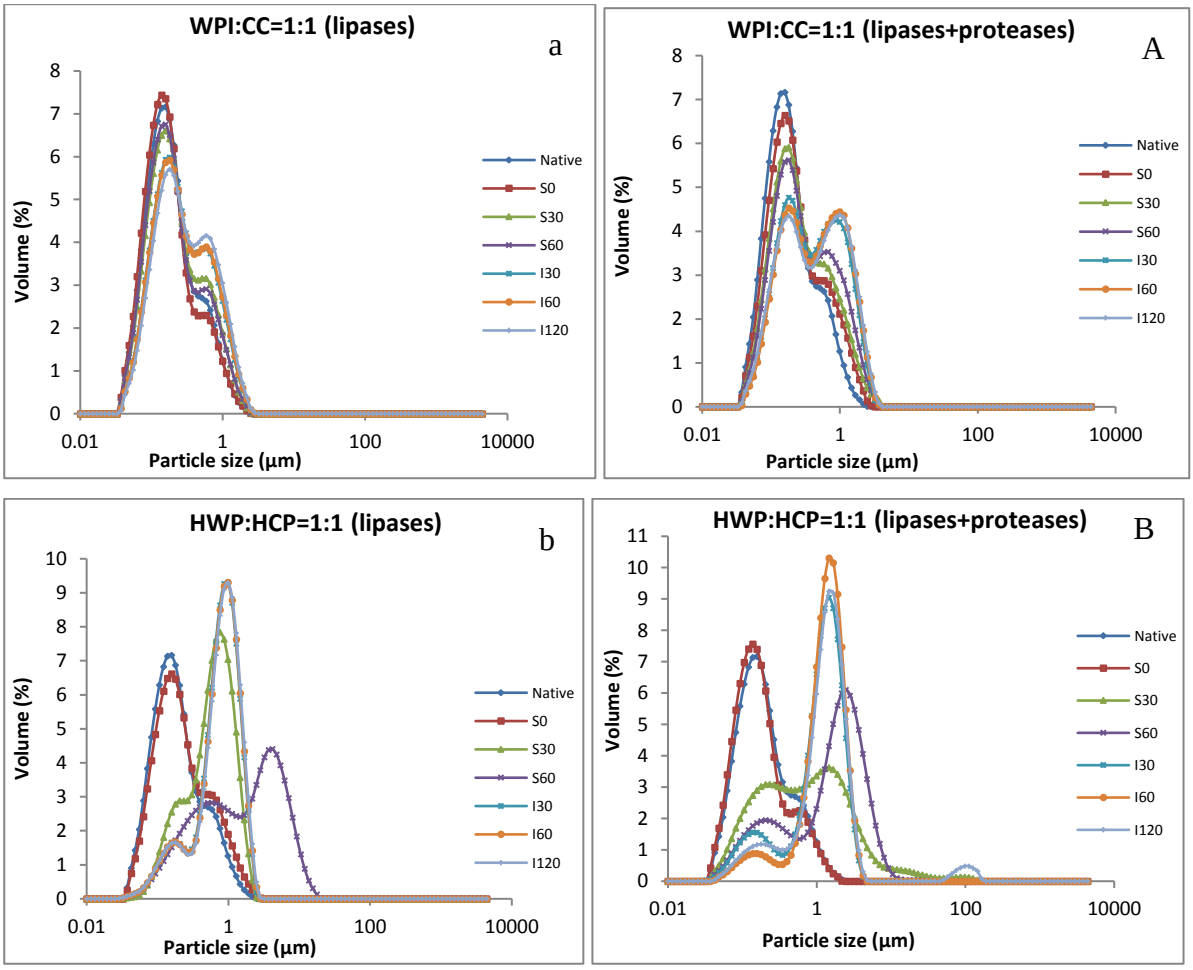


Fig 6.1 Particle size distribution of oil droplets of the four infant milk formulae emulsions WPI:CC=1:1, HWP:HCP=1:1 under in vitro gastrointestinal digestion without (a, b) and with proteases (A, B), respectively.

In comparison to non-hydrolysed formula WPI:CC=1:1, the change in particle size distribution of fat droplets in hydrolysed protein formula HWP:HCP=1:1 during gastrointestinal digestion with and without proteases are more obvious [Fig 6.1 (b-B), Fig 6.3]. The particle size distribution of the fat globules during the gastric phase remains bimodal, with a population of large particle size (0.6-3 µm) dominating after 30 minutes of gastric digestion (S30) unlike the population of small size (0.06-0.6 µm) that was observed at initial stage (S0). After one hour in the stomach, large populations of fat globules with bigger droplet size 3-20 µm were observed. However, in the intestinal phase, the fat globules of large population reduced to 0.6-6 µm and the distribution remained stable over the intestinal phase (Fig 6.1 b). A very similar pattern was also noticed during the lipid digestion of HWP:HCP =1:1 in the presence of proteases (Fig 6.1B, Fig 6.3).

6.3.1.1 Soy protein formulae

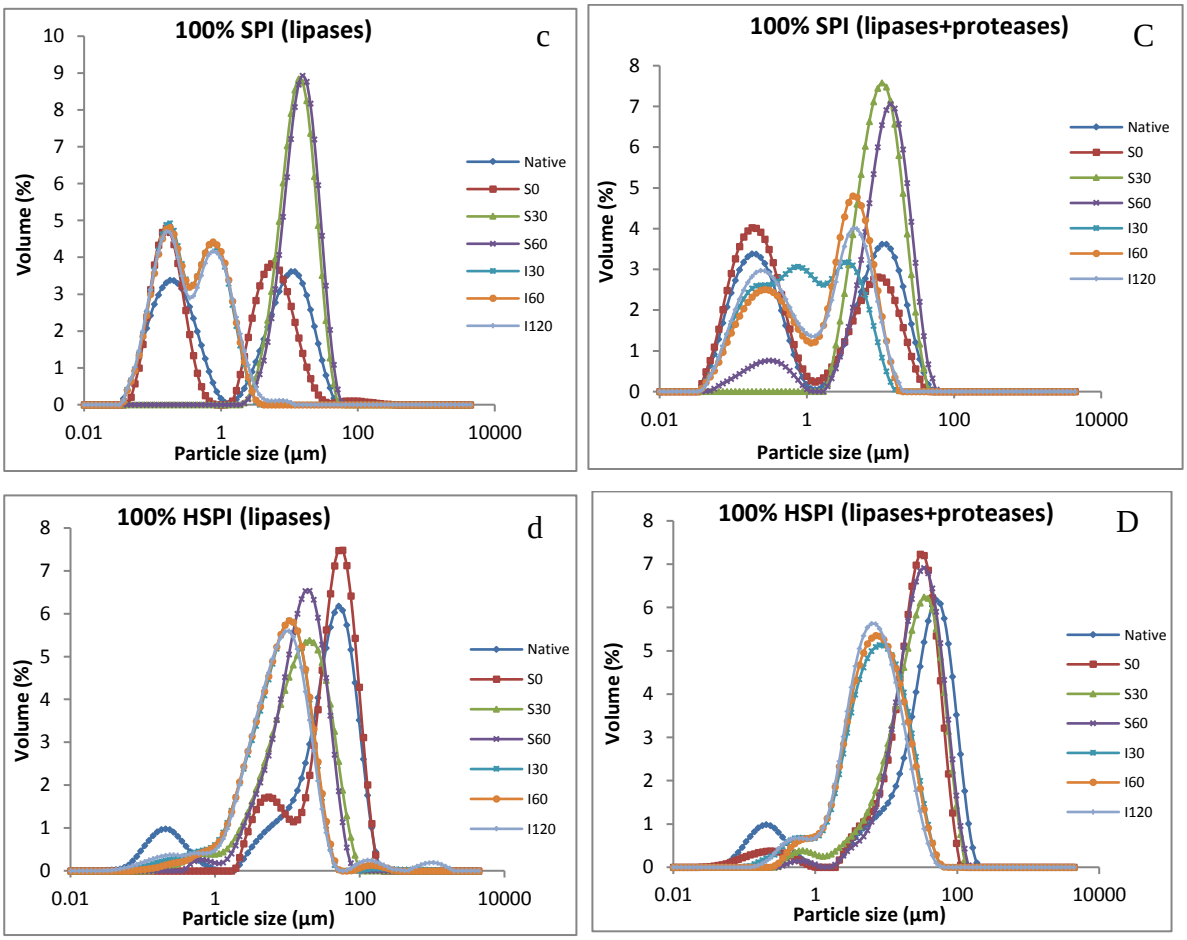


Fig 6.2 Particle size distribution of oil droplets of the four infant milk formulae emulsions 100% SPI, 100% HSPI under *in vitro* gastrointestinal digestion without (c, d) and with proteases (C, D), respectively.

The native and S0 fat globules present in 100% SPI and 100% HSPI have a bimodal distribution with a small population of 0.6-1.0 μm and a large population of 1-80 μm (for 100% SPI), 3-200 μm (for 100% HSPI) [Fig 6. 2 (c-d)].

During 1 hour of lipolysis without proteases in the gastric phase, the fat droplets showed a unimodal distribution with particles between 3-90 μm for 100% SPI, but remained bimodal for 100% HSPI. However, in the intestinal phase, the particle size distribution of fat globules was bimodal again with size reduced to less than 20 μm for 100% SPI and less than 80 μm for 100% HSPI.

The increase in fat droplet size during 60 minutes of gastric digestion (after adjusting the pH of the digesta to 7.0) suggests the occurrence of coalescence although some minor flocculation was still observed. Golding et al. (2011) reported that during gastric digestion, the emulsion can flocculate due to the acidic condition removing the electric charge of the absorbed protein layer surrounding the oil droplets. The emulsion flocculation is reversible, but not the coalescence process in the GI tract. Therefore, it could be that the gastric analogue lipase, after penetrating through the interface of infant formula's emulsions, hydrolyses the lipid causing coalescence. The particle distribution results are in agreement with CLSM images with oil coalescence observed after 60 minutes in stomach (Fig 6.4). The finding from Golding et al. (2011) contradicts our previous particle size results, which are remarkably larger in gastric digested samples than at the beginning (S0). This could be due to the reason that unlike our study Golding et al.'s samples were not neutralised by alkaline solution before the analysis (Nguyen et al., 2015b; Nguyen, Bhandari, Cichero, & Prakash, 2016).

The change of the volume-weighted mean diameter D[4,3] of all formulae over gastrointestinal digestion (Fig 6.3) confirms the limited effect of proteases in *in vitro* lipolysis digestion. A similar result with restricted impact of pepsin on gastric lipolysis has been reported recently by Bourlieu, Ménard, et al. (2015). Singh (2011) suggested that the degradation of the adsorbed proteins by pepsin can lead to a loss of positive charge on the droplet surface and a reduction of the droplet layer. The produced peptides might reabsorb on the droplets' surface but these absorbed peptides cannot provide adequate electro-static repulsions nor steric stabilisation as intact proteins (Bourlieu, Ménard, et al., 2015; Nguyen et al., 2015b). Therefore, the emulsion is highly susceptible to aggregation, flocculation and coalescence (Bourlieu, Ménard, et al., 2015; Singh, 2011). This could be the reason why D[4,3] of gastric lipid digestion in the presence of proteases is larger than the corresponding D[4,3] without proteases (Fig 6.3).

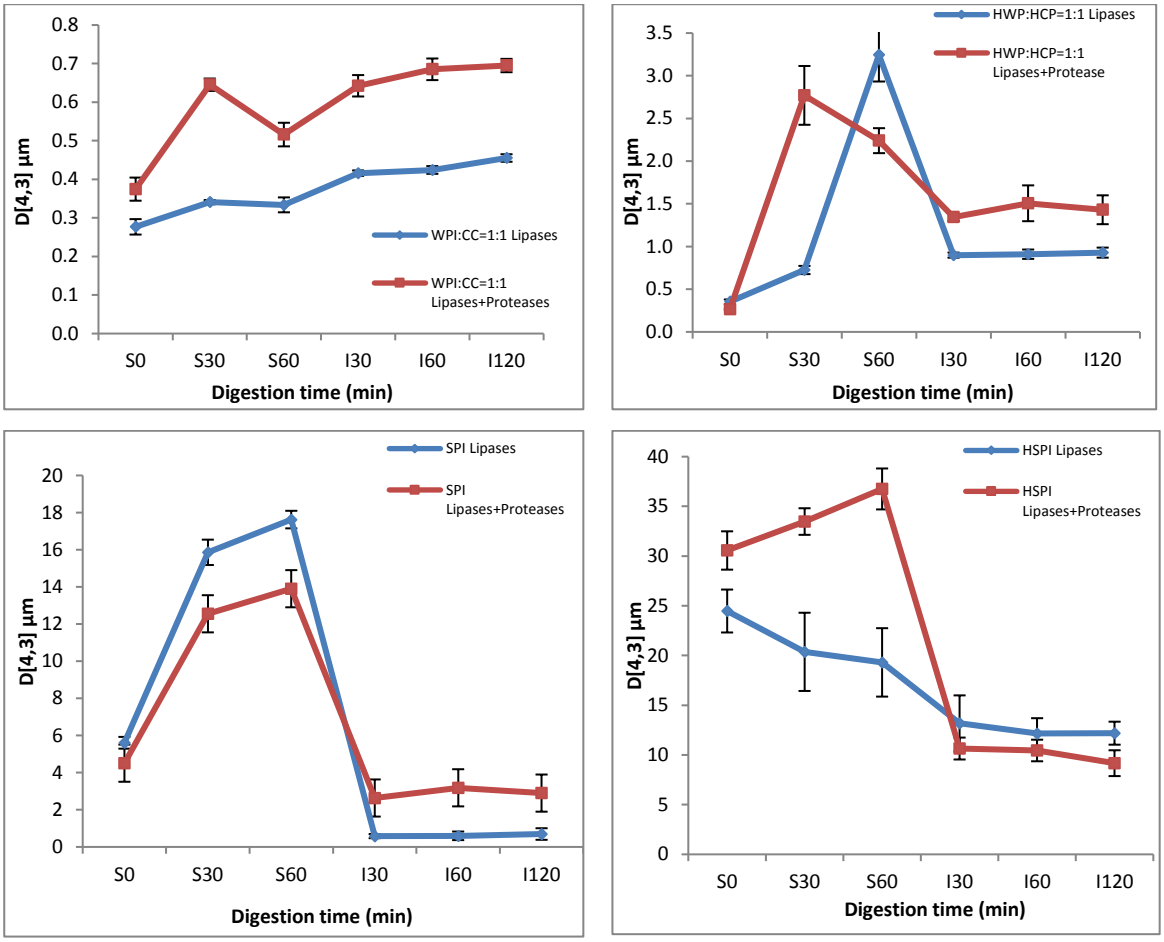


Fig 6.3 Volume mean D[4,3] diameter of native and digested samples of infant milk formulae emulsions WPI:CC=1:1, HWP:HCP=1:1, 100% SPI, and 100% HSPI.

In the small intestine, there is a significant decrease in particle size for most of the infant formula emulsions after 30 minutes of digestion for both the matrices. This is due to a possible destabilization of protein aggregation, droplet flocculation of gastric digesta during the intestinal condition (pH 7). Trypsine and chymotrypsine further hydrolyse proteins of digesta from the stomach. Caseinate can be completely digested but not whey and soy proteins (Nguyen et al., 2015b). The composition of the absorbed protein layer can be changed due to the produced peptides and polypeptides. It has been reported that these peptides and polypeptides that re-absorb at the interface of the droplet surface cannot provide sufficient electrostatic repulsions nor steric stabilization as the intact proteins that could lead to some extent of aggregation (Bourlieu, Ménard, et al., 2015). In addition, the available peptides and polypeptides in the intestinal phase are less surface active than the intact proteins; they are easily displaced by bile salts (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011) that

quickens the lipolysis. However, there is not much change in D[4,3] for all emulsions except WPI:CC=1:1. The reason for this is still unclear.

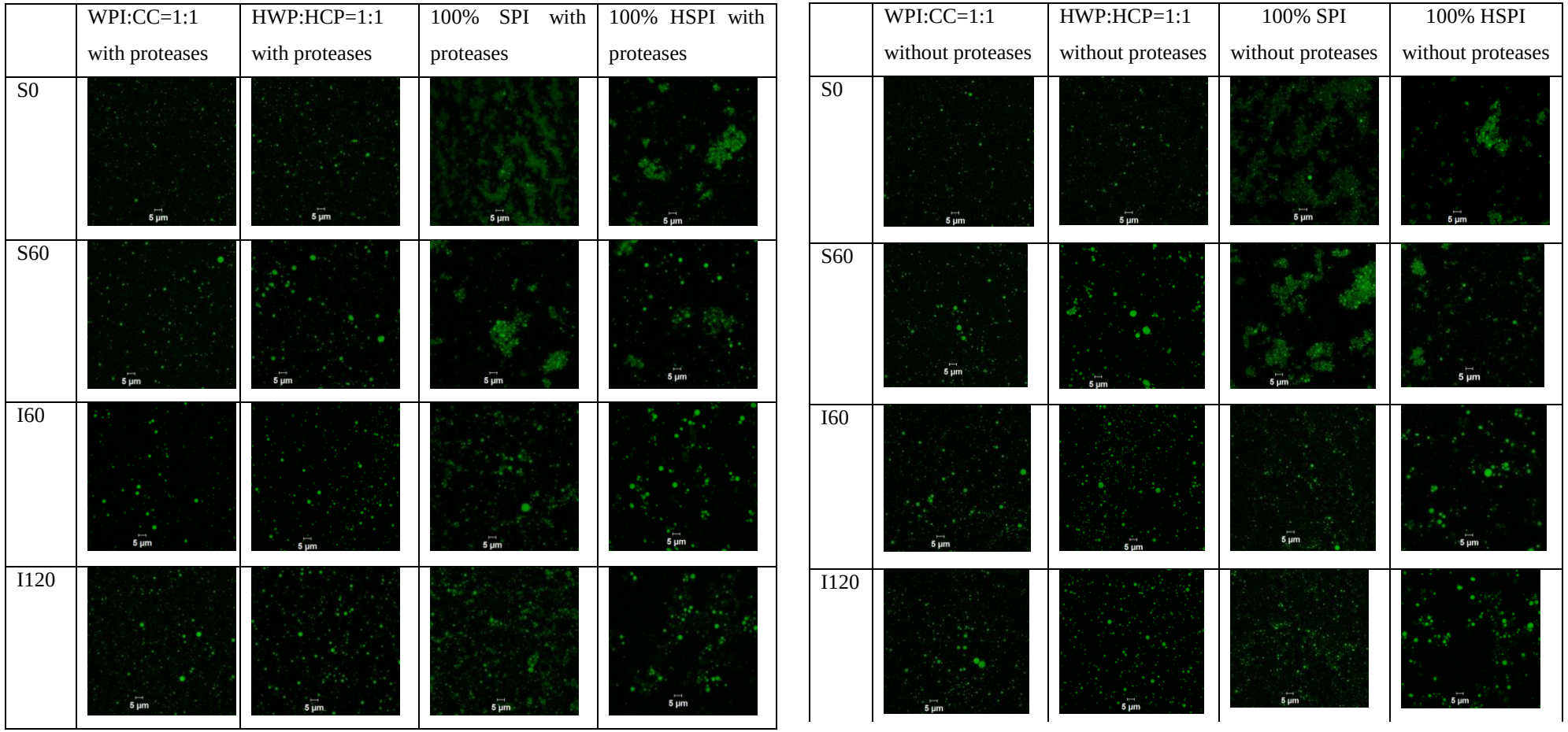


Fig 6.4 CLSM of oil droplets in digested samples at beginning (S0), 60 minutes gastric digestion (S60), 60 minutes intestinal digestion (I60) and 120 minutes intestinal digestion (I120) of the infant formulae emulsions WPI:CC=1:1, HWP:HCP=1:1, 100% SPI, and 100% HSPI.

6.3.2 Total free released fatty acids from dairy and soy based infant formula

Fig 6.5 (a) and Fig 6.5 (b) compares the level of FFA released during lipid digestion with and without proteases. It is clear that total released FFA is slow in the stomach, but faster during the intestinal phase. Also, the released FFA levels are higher in the matrices without proteases. After 60 minutes of gastric digestion for all formulae, total released FFA is less than 10 µmol/mL (without proteases) and less than 9 µmol/mL (with proteases). However, this amount increases up to 40 µmol/mL (without proteases) and 20 µmol/mL (with proteases) after 60 minutes in intestinal phase; and increases up to 50 µmol/mL and 30 µmol/mL, respectively after 120 minutes. Although the lipase analogue of fungal origin does not require any cofactor for efficient activity (Jaeger & Reetz, 1998; Saxena et al., 1999) and gastric pH 4.5 was in the stable pH range for lipase, there are some other factors that could be responsible for the slow release of FFA by analogue gastric lipase when compared to pancreatic lipases. This could be explained by the structural change of the emulsions in the gastric phase. At acidic pH 4.5 in the stomach, caseins and soy proteins aggregate together leading to a reduction of the surface area of the oil-water interface. This could happen with infant formula emulsion based on non-hydrolysed proteins WPI:CC =1:1 and 100% SPI. The oil droplets could be entrapped in the protein aggregation network that has been reported recently by Nguyen et al. (2015b) and Ye, Cui, and Singh (2011). During pepsinolysis in the stomach, partially hydrolysed soy proteins and caseinate are observed and most of the whey is resistant to pepsin digestion (Nguyen et al., 2015b), meaning that some intact proteins still remain on the interfacial layer surrounding oil droplets. As mentioned in section 3.1.1 the polypeptides and peptides hydrolysed from the surface layer are again surface-active and do not move into the aqueous phase proteins, but are absorbed at the surface area of oil droplets. In addition, with the use of vegetable oil with a high level of long chain FFA, the fully long chain FFA liberated after lipolysis can accumulate at the oil-water interface. The released long chain FFA may also contribute to the limited penetration of gastric lipase to oil droplets (Gargouri et al., 1986; Pafumi et al., 2002). The above factors could prohibit the rate of lipid digestion of analogue gastric lipase in the stomach phase.

In the intestinal phase, the amount of released FFA is considerably higher as compared to that in the stomach. This was observed with all formulae in both the matrices and can be explained by the fact that pancreatic lipase and analogue gastric lipase are active in intestinal conditions. Bile salts in the intestine as a bio-surfactant can displace some of absorbed intact proteins, polypeptides, and peptides in oil droplet surface. In addition, colipase aided pancreatic lipase bind easier to the interface and also

stabilize pancreatic lipase in its open conformation at the interface (Brockman, 2002). Bile salts and colipase help pancreatic lipase become accessible to lipid substrate (Mun, Decker, & McClements, 2007). Meanwhile, analogue gastric lipase with an optimal pH 6.0-7.0 is able to hydrolyse lipids at intestinal pH (7.0), considerably contributing to the increased amount of FFA released in the small intestine.

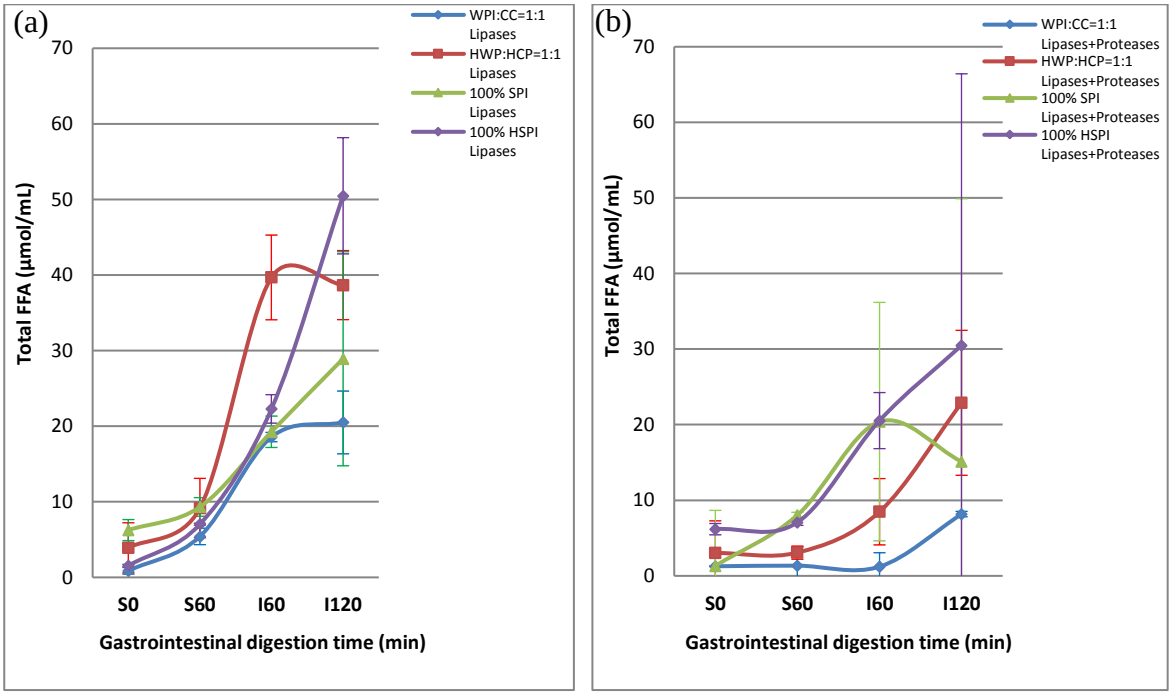


Fig 6.5 Total FFA released *in vitro* gastrointestinal digestion infant of infant milk formulae emulsions WPI:CC=1:1, HWP:HCP=1:1, 100% SPI, and 100% HSPI without proteases (a) and with proteases (b).

In comparison to the destabilisation of infant formula emulsions with hydrolysed and non-hydrolysed proteins, the rate of lipid digestion seems higher for hydrolysed protein emulsions than non-hydrolysed ones for both digestion matrices. It is well documented that not only the structure but also the composition of the interfacial layer surrounding the oil droplets plays an important role in the accessibility of lipase to the oil/water interface. The produced peptides and polypeptides could lead to aggregation (Bourlieu, Ménard, et al., 2015) because they cannot provide sufficient electrostatic repulsions nor steric stabilization as the intact protein. In addition, due to the less significantly surface active peptides and polypeptides than the intact proteins, peptides and polypeptides are easily displaced by bile salts (Maldonado-Valderrama et al., 2011). This can help pancreatic lipase become

more accessible to lipids in emulsions stabilized by hydrolysed proteins (formulae HWP:HCP =1:1 and 100% HSPI) than by non-hydrolysed proteins (WPI:CC =1:1 and 100% SPI).

Obtained results in Fig 6.5 also shows that the infant formula emulsions stabilized by soy proteins have a trend of higher rate of lipolysis than dairy proteins. This was supported by Nik, Wright, and Corredig (2011), however the exact reason for this is still unclear.

6.4. Conclusions

The results from the static *in vitro* digestion system, simulating infant gastrointestinal tract, suggest fat droplet size increases over the gastric phase but then decreases during the intestinal phase in both digestion matrices with lipases-only and lipases in the presence of proteases. This change was more noticeable if lipases work in concert with proteases or using formulae based on non-hydrolysed proteins. The obtained results suggest that digestive proteases had an insignificant effect on lipolysis of infant formulae.

Total FFA released slowly in the stomach, but faster during the intestinal phase, and was obtained with higher concentration in the matrices without proteases or in formulae with hydrolysed proteins. This difference in the *in vitro* lipolysis between hydrolysed and non-hydrolysed infant formulas would be a suggestion for manufacturers to consider the lipid content in infant formula based on hydrolysed proteins. However, the *in vitro* gastric lipolysis with fungal lipase may demonstrate a lower digestion rate than in the intestinal phase and does not represent the in vivo lipid digestion in healthy infants. Mammal gastric lipase would be the best substitute for human gastric lipase for the *in vitro* study although the availability of mammal lipase is still restricted.

6.5. References

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

Amara, S., Patin, A., Giuffrida, F., Wooster, T. J., Thakkar, S. K., Bénarouche, A., . . . Molinari, S. (2014). In vitro digestion of citric acid esters of mono-and diglycerides (CITREM) and CITREM-containing infant formula/emulsions. *Food & Function*, 5(7), 1409-1421.

Andersson, E.-L., Hernell, O., Bläckberg, L., Fält, H., & Lindquist, S. (2011). BSSL and PLRP2: key enzymes for lipid digestion in the newborn examined using the Caco-2 cell line. *Journal of Lipid Research*, 52(11), 1949-1956.

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

Armand, M., Hamosh, M., Mehta, N. R., Angelus, P. A., Philpott, J. R., Henderson, T. R., . . . Hamosh, P. (1996). Effect of human milk or formula on gastric function and fat digestion in the premature infant1. *Pediatric Research*, 40(3), 429-437.

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

Bourlieu, C., Bouzerzour, K., Ferret-Bernard, S., Bourgot, C. L., Chever, S., Ménard, O., . . . Bonhomme, C. (2015). Infant formula interface and fat source impact on neonatal digestion and gut microbiota. *European Journal of Lipid Science and Technology*, 117(10), 1500-1512.

Bourlieu, C., Ménard, O., De La Chevasnerie, A., Sams, L., Rousseau, F., Madec, M.-N., . . . Bouhallab, S. (2015). The structure of infant formulas impacts their lipolysis, proteolysis and disintegration during in vitro gastric digestion. *Food Chemistry*, 182, 224-235.

Brockman, H. (2002). Colipase-induced reorganization of interfaces as a regulator of lipolysis. *Colloids and Surfaces B: Biointerfaces*, 26(1), 102-111.

Carey, M. C., Small, D., & Bliss, C. (1983). Lipid digestion and absorption. *Annual Review of Physiology*, 45(1), 651-677.

Carrière, F., Renou, C., Lopez, V., De Caro, J., Ferrato, F., Lengsfeld, H., . . . Verger, R. (2000). The specific activities of human digestive lipases measured from the in vivo and in vitro lipolysis of test meals. *Gastroenterology*, 119(4), 949-960.

Cohen, M., Morgan, R. G., & Hofmann, A. F. (1971). Lipolytic activity of human gastric and duodenal juice against medium and long chain triglycerides. Gastroenterology, 60(1), 1-15.

De Jong, C., & Badings, H. T. (1990). Determination of free fatty acids in milk and cheese procedures for extraction, clean up, and capillary gas chromatographic analysis. *Journal of High Resolution Chromatography*, 13(2), 94-98.

Gallier, S., Vocking, K., Post, J. A., Van De Heijning, B., Acton, D., Van Der Beek, E. M., & Van Baalen, T. (2015). A novel infant milk formula concept: Mimicking the human milk fat globule structure. *Colloids and Surfaces B: Biointerfaces*, 136, 329-339.

Gargouri, Y., Pieroni, G., Rivière, C., Lowe, P. A., Saunière, J.-F., Sarda, L., & Verger, R. (1986). Importance of human gastric lipase for intestinal lipolysis: an in vitro study. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 879(3), 419-423.

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

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

Hamosh, M. (2006). Enteral lipid digestion and absorption. Neonatal nutrition and metabolism, 2nd edn. Cambridge University Press, Cambridge, 350-368.

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

Hermoso, M., Tabacchi, G., Iglesia-Altaba, I., Bel-Serrat, S., Moreno-Aznar, L. A., García-Santos, Y., . . . Koletzko, B. (2010). The nutritional requirements of infants. Towards EU alignment of reference values: the EURRECA network. *Maternal & Child Nutrition*, 6, 55-83.

Hernell, O., Blackberg, L., & Bernback, S. (1988). Digestion and absorption of human milk lipids. Paper presented at the Bristol-Myers nutrition symposia.

Hsu, H., Vavak, D., Satterlee, L., & Miller, G. (1977). A multienzyme technique for estimating protein digestibility. *Journal of Food Science*, 42(5), 1269-1273.

Jaeger, K.-E., & Reetz, M. T. (1998). Microbial lipases form versatile tools for biotechnology. *Trends in Biotechnology*, 16(9), 396-403.

Joeckel, R. J., & Phillips, S. K. (2009). Overview of infant and pediatric formulas. *Nutrition in Clinical Practice*, 24(3), 356-362.

Koletzko, B., Baker, S., Cleghorn, G., Neto, U. F., Gopalan, S., Hernell, O., . . . Ding, Z. Y. (2005). Global standard for the composition of infant formula: Recommendations of an ESPGHAN Coordinated International Expert Group. *Journal of Pediatric Gastroenterology and Nutrition*, 41(5), 584-599.

Lebenthal, E., Lee, P., & Heitlinger, L. (1983). Impact of development of the gastrointestinal tract on infant feeding. *The Journal of Pediatrics*, 102(1), 1-9.

Liao, T. H., Hamosh, P., & Hamosh, M. (1984). Fat digestion by lingual lipase: mechanism of lipolysis in the stomach and upper small intestine. *Pediatric Research*, 18(5), 402-409.

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

Maldonado-Valderrama, J., Wilde, P., Macierzanka, A., & Mackie, A. (2011). The role of bile salts in digestion. *Advances in Colloid and Interface Science*, 165(1), 36-46.

Ménard, O., Cattenoz, T., Guillemin, H., Souchon, I., Deglaire, A., Dupont, D., & Picque, D. (2014). Validation of a new in vitro dynamic system to simulate infant digestion. *Food Chemistry*, 145(0), 1039-1045.

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

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., . . . Dupont, D. (2014). A standardised static in vitro digestion method suitable for food-an international consensus. *Food & Function*, 5(6), 1113-1124.

Mun, S., Decker, E. A., & McClements, D. J. (2007). Influence of emulsifier type on in vitro digestibility of lipid droplets by pancreatic lipase. *Food Research International*, 40(6), 770-781.

Nguyen, T. T. P., Bhandari, B., Cichero, J., & Prakash, S. (2015a). A comprehensive review on in vitro digestion of infant formula. *Food Research International*, 76, 373-386.

Nguyen, T. T. P., Bhandari, B., Cichero, J., & Prakash, S. (2015b). Gastro intestinal digestion of dairy and soy proteins in infant formulas: An in vitro study. *Food Research International*, 76, 348-358.

Nguyen, T. T. P., Bhandari, B., Cichero, J., & Prakash, S. (2016). In vitro digestion of infant formulations with hydrolysed and non-hydrolysed proteins from dairy and soybean. *Food & Function*, 7(12), 4908-4919.

Nik, A. M., Wright, A. J., & 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), 321-330.

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

Pasupuleti, V. K., & Braun, S. (2008). State of the art manufacturing of protein hydrolysates Protein hydrolysates in biotechnology (pp. 11-32): Springer.

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

Reis, P., Holmberg, K., Watzke, H., Leser, M., & Miller, R. (2009). Lipases at interfaces: a review. *Advances in Colloid and Interface Science*, 147, 237-250.

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

Sarles, J., Moreau, H., & Verger, R. (1992). Human gastric lipase: ontogeny and variations in children. *Acta Paediatrica*, 81(6-7), 511-513.

Saxena, R., Ghosh, P., Gupta, R., Davidson, W. S., Bradoo, S., & Gulati, R. (1999). Microbial lipases: potential biocatalysts for the future industry. *Current Science*, 77(1), 101-115.

Singh, H. (2011). Aspects of milk-protein-stabilised emulsions. *Food Hydrocolloids*, 25(8), 1938-1944.

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

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

CHAPTER 7 *IN VITRO* DIGESTION OF CARBOHYDRATES IN INFANT MILK FORMULAE

7.1 Introduction

Carbohydrates are the second most important source of energy for infants after lipids, contributing to 35–55% of the total energy of the infants' diet (Fanaro & Vigi, 2012). Mothers' milk contains both digestible carbohydrates (lactose), and indigestible oligosaccharides (gluco-oligosaccharides, maltodextrin-like oligosaccharides (Engfer, Stahl, Finke, Sawatzki, & Daniel, 2000)), but only digestible carbohydrates are approved by authorities to be added to infant formula like lactose, maltose, maltodextrins, glucose syrup, precooked starch or gelatinised starch which are naturally free of gluten (Koletzko et al., 2003; Thompkinson & Kharb, 2007). Glucose, saccharose and fructose are not recommended to be added to infant formula due to the high osmotic activity of glucose or a negative impact on infants who have fructose intolerance. The recommended total carbohydrates in infant formula is in the range 9-14 g/100 kcal as per Directive 2006/141/EC (Panel, 2014).

Lactose is the main carbohydrate in milk with mothers' milk containing approximately 6-7 g of lactose per 100 mL of milk (Nguyen, Bhandari, Cichero, & Prakash, 2015a). Lactose can be used as the sole carbohydrate source in infant formula but the amount should not exceed the total recommended carbohydrates for infant formula, minimum being at 4.5 g/100kcal (Nguyen et al., 2015a; Panel, 2014). Lactose is digested slowly in the brush border intestine than other carbohydrates, the remaining undigested lactose will be fermented into lactic acid in the large intestine (Engfer et al., 2000; Thompkinson & Kharb, 2007). This lactic acid produced from undigested lactose is able to maintain the pH 5.5-6.0 in the large intestine that helps to protect infants from infection. Although low levels of lactase activity in pre-term neonates have been reported, lactase in full-term infants is sufficient to digest lactose into galactose and glucose (Lebenthal, Lee, & Heitlinger, 1983).

Maltose, maltodextrins, and glucose syrup are the products of corn-starch hydrolysis. They are permitted to be added to infant formulas due to the sufficiency of maltase and amyloglucosidase or glucoamylase, (the enzymes that are able to digest maltose) in infants and their ability to prevent the osmolality increase in the formula (Fanaro & Vigi, 2012; Koletzko et al., 2005). It is suggested that maltodextrins with 5-9 glucose units are added to infant formulas as the amyloglucosidase in the human intestine has the specificity on this chain length of maltodextrin (Koletzko et al., 2003).

However, the chain length of maltodextrins is not regulated and maltodextrins formed from 1-30 glucose units are currently being used (Coppa et al., 1993).

Pregelatinized starch is being used as a thickening agent in pumped mothers' milk and infant formula for infants who are at risk of aspiration or reflux (Bosscher et al., 2000; Cichero, Nicholson, & September, 2013). However, only a small amount of the starch are recommended as additives to baby food due to insufficient amylase enzymes during infancy to assist digestion (Koletzko et al., 2003). Modified starches are suggested as they offer desirable thickening properties during cooling down and storage (Nguyen et al., 2015a). Therefore, pregelatinized starch (naturally free of gluten) are preferred in infant formula (Thompkinson & Kharb, 2007) with the recommended amount no more than 2 g/100 mL or no more than 30% of total carbohydrates (Koletzko et al., 2003). Although very low or no α-amylase activity has been found in the duodenal fluids of infants under 4 months of age, some extent of amyloglucosidase is available in the small intestinal mucosa of newborns and infants (>50% adults' level) (Lebenthal, Lee, et al., 1983). Amyloglucosidase is a brush border enzyme that can digest starch directly to glucose which helps infants digest a moderate amount of starch (Lebenthal, Lee, et al., 1983).

Locust bean gum (LBG) is a galactomannan polysaccharide which is also permitted to be used as a thickening agent in infant food (Meunier et al., 2014). In the European Union, LBG can be used up to 1 g/L in follow-on formula, but no maximum level has been advised for infant formula (Koletzko et al., 2003). In Australia and New Zealand, LBG is approved to add up to 1 g/L in infant formula and up to 1 g/100 g in other infant foods (Australian Government, 2008), (Meunier et al., 2014).

Commercially different carbohydrates are being used in normal and specialised infant formulas such as lactose in normal formulas, locust bean gum, corn starch or potato starch in anti-reflux formulas, glucose syrup instead of lactose in lactose-free formulas. With the presence of maltodextrin, locust bean gum, or starch, the rheology of the infant formula will change. It has been reported that thickened infant formula has a viscosity up to 320 mPa.s as compared to unthickened infant formula with a viscosity of 2 mPa.s (shear rate of $50s^{-1}$, 37°C) (Cichero, Nicholson, & Dodrill, 2011). However, there is limited information about the behaviour of these carbohydrates during their passage through the infant digestive tract. The aim of this study is to compare the digestibility of different type of carbohydrates: lactose, corn starch, locust bean gum (carob bean gum) and glucose syrup, which are

commonly added in infant formulas; and also study the flow behaviour of the infant formula with these added carbohydrates.

7.2 Materials and method

7.2.1 Materials

7.2.1.1 Enzymes and chemicals

The following enzymes and chemical were used for the experiment:

Lactase Godo YNL-2 (EC 3.2.1.23, 50000 U/g) derived from selected strain of the yeast Kluyveromyces lactis, was supplied by Connell Bros Company Australasia Pty. Ltd (Victoria, Australia). Other enzymes were obtained from Sigma-Aldrich, Castle Hill, New South Wales, Australia such as Amyloglucosidase (EC 3.2.1.3, 321 U/mL). Pepsin from porcine gastric mucosa (EC 3.4.23.1, 3840 units/mg protein, one unit will produce a change in A280 of 0.001 per min at pH 2.0 at 37°C, measured as TCA-soluble products using haemoglobin as substrate). Trypsin from bovine pancreas (EC 3.4.21.4, 13165 units/mg protein, one unit will produce a change in A253 of 0.001 per minute at pH 7.6 at 25°C using Nα-Benzoyl-L-arginine Ethyl Ester (BAEE) as a substrate. Chymotrypsin from bovine pancreas (EC 3.4.21.1, 54.49 units/mg protein, one unit will hydrolyze 1.0 μmol of N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE) per min at pH 7.8 at 25°C as stated by the manufacturer).

Glucose (HK) assay kit GAHK-20, sodium taurocholate, pepstatin, and trypsin-chymotrypsin inhibitor were obtained from Sigma-Aldrich, Castle Hill, New South Wales, Australia. Sodium glycodeoxycholate was obtained from Merck, Kilsyth, Victoria, Australia. They were stored between 2-8°C.

The other ingredients used in the study such as lactose, sodium chloride, hydrochloric acid, and sodium hydroxide were of analytical grade.

7.2.1.2. Dairy proteins and carbohydrates

Whey protein isolate (WPI 85.1% protein, 1.0% fat, 1.2% carbohydrate) and calcium caseinate (CC, 86.7% protein, 1.0% fat, 0.1% carbohydrate) were purchased from Total Foodtec Pty Ltd (Australia).

Glucose syrup was obtained from AFIS (Sydney, Australia) with DE 42-43, dry solids 80.5-82.5%. Locust bean gum (LBG) was obtained from The Melbourne Food Ingredient Depot (Victoria, Australia). Corn starch and sunflower vegetable oil were obtained from a local supermarket.

7.2.2. Method

7.2.2.1. Preparation of infant formula

Preparation of infant milk formulae and procedure for *in vitro* infant digestion are as described by Nguyen et al. (2015b). Infant formulae used in this study has the common ratio of whey and casein WPI:CC = 6:4. Carbohydrates were used with concentration: lactose (65 g/L), glucose syrup (70 g/L), gelatinized corn starch (20 g/L), LBG (0.5 g/L). The amount of these carbohydrates added in the infant milk formulae was as recommend by Koletzko et al. (2003) and Bhatia and Greer (2008) in their research work. Corn starch was pregelatinized by heating 10% corn starch solution in water at 80°C for 15 minutes during preparation of the infant formulae.

7.2.2.1. In vitro infant carbohydrate digestion

In this study, lactase from the yeast Kluyveromyces lactis was chosen to substitute the brush border lactase in the in vivo digestion procedure. Because there is no information reported about the lactase activity in any *in vitro* models, we decided to choose the lactase level for in vivo infant digestion based on the lactase activity that was employed to produce free lactose milk in recent studies. To make lactose-hydrolysed milk, 0.1% of lactase with 5000 U/g was added into raw milk and incubated at 4°C for 40 hours (Choi, Lee, & Won, 2007). Based on this level of lactase, some trials were done with lactase concentration between 0.1-1.0 g/ L. Lactase 1.0 g/L was chosen to apply in the *in vitro* infant digestion procedure as a clear increase in released glucose was observed in the digesta samples after 2 hours of digestion. Amyloglucosidase was used at 0.28 U/mg of starch (Shrestha et al., 2010).

A static *in vitro* digestion unit equipped with water bath, and overhead stirrer carried out the digestion trials. Two water-jacketed reaction vessels in the unit were connected to a water bath that provided a constant circulation of warm water in and out of the reaction vessel and maintained a constant temperature of 37°C. A glass stirrer connected to an overhead stirrer continuously mixed the *in vitro* digesta at 250 rpm. Levels of proteases and bile salts are described in Nguyen et al. (2015b).

Digested samples were immediately placed in boiling water for 5 minutes to inactivate the enzymes (Slaughter, Ellis, & Butterworth, 2001; Warren, Zhang, Waltzer, Gidley, & Dhital, 2015). The supernatant after fat removal was then neutralised by 0.1 M HCl and protein precipitate was removed immediately by centrifugation at 4000 rpm, 4°C for 20 minutes. Collected supernatant phase was then purified with a 0.45 μm syringe filter to remove large peptides (Ventura, San Gabriel, Hirota, & Mennella, 2012). Samples were stored at -20°C for glucose assay.

7.2.2.2. Glucose assay

Released D-glucose in the digested samples was analysed with the hexokinase/glucose-6-phosphate-dehydrogenase assay (HK assay). In this method, glucose is phosphorylated by adenosine triphosphate (ATP) in the reaction catalyzed by hexokinase. Glucose- 6-phosphate (G6P) is then oxidized to 6-phosphogluconate in the presence of oxidized nicotinamide adenine dinucleotide (NAD) in a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). During this oxidation, an equimolar amount of NAD is reduced to NADH. The consequent increase in absorbance at 340 nm is directly proportional to glucose concentration.

Absorbance was measured by spectrophotometer with quartz cuvettes (HELL-200-66501-MP, Shimadzu) at a wavelength of 340 nm.

7.2.2.3. Rheological measurement

The rheological properties of the samples were characterized at 37°C using a Discovery HR1 rheometer (TA instruments UH Ltd., U.K.), fitted with 60 mm cone plate and a gap of 50 μm. The flow behaviour of the infant formula samples was determined by shear rate sweep (0.1-100 s^{-1}).

7.2.2.4. Statistical analysis

The values for glucose assay were measured in triplicate from duplication. Experimental data were assessed by ANOVA tests to determine the significant differences among the means at 95% confidence level. The treatment means were considered to be significantly different when $P<0.05$.

7.3. Results and discussion

7.3.1. Flow behaviour of infant milk formulae

The flow behaviour of the infant milk formulae using different carbohydrate sources: lactose, glucose syrup, corn starch and LBG are presented in Fig 7.1. It is clear that the viscosity of all formulae significantly decreased as the shear rate increased suggesting shear thinning behaviour. The formulae without corn starch or LBG, but with 100% lactose and 100% glucose syrup, had much lower viscosity (between 22-25 mPa.s at 0.1 s^{-1}) compared to the formulae with thickeners (above 37 mPa.s at 0.1 s^{-1}). At lower concentration of LBG 0.5 g/L as compared to 20.0 g/L for corn starch, LBG exhibited a considerably higher viscosity between the two formulae.

As can be seen in Fig 7.1, for lactose formulae, adding starch only increases the viscosity to 37.46 mPa.s while adding LBG, it increases the viscosity to up to 54.74 mPa.s at the starting shear rate 0.1 second-1. Glucose syrup formulae also showed the similar pattern of viscosity when corn starch and LBG were added, with the viscosity increasing to 47.36 mPa.s for corn starch and 55.94 mPa.s for LBG. These results were in agreement with findings from Carlos A González-Bermúdez, Frontela-Saseta, López-Nicolás, Ros-Berruezo, and Martínez-Graciá (2014) who suggested LBG provides the highest viscosity when added to the infant formula compared with corn starch. The reasons why LBG can provide higher viscosity compared to pregelatinized starch could be due to the combined effect of difference in molecular weight, molecular structure between LBG and starch, and also the physical interaction of these thickeners with proteins components in the matrix (Carlos Alberto González-Bermúdez et al., 2015; Saha & Bhattacharya, 2010; Syrbe, Bauer, & Klostermeyer, 1998).

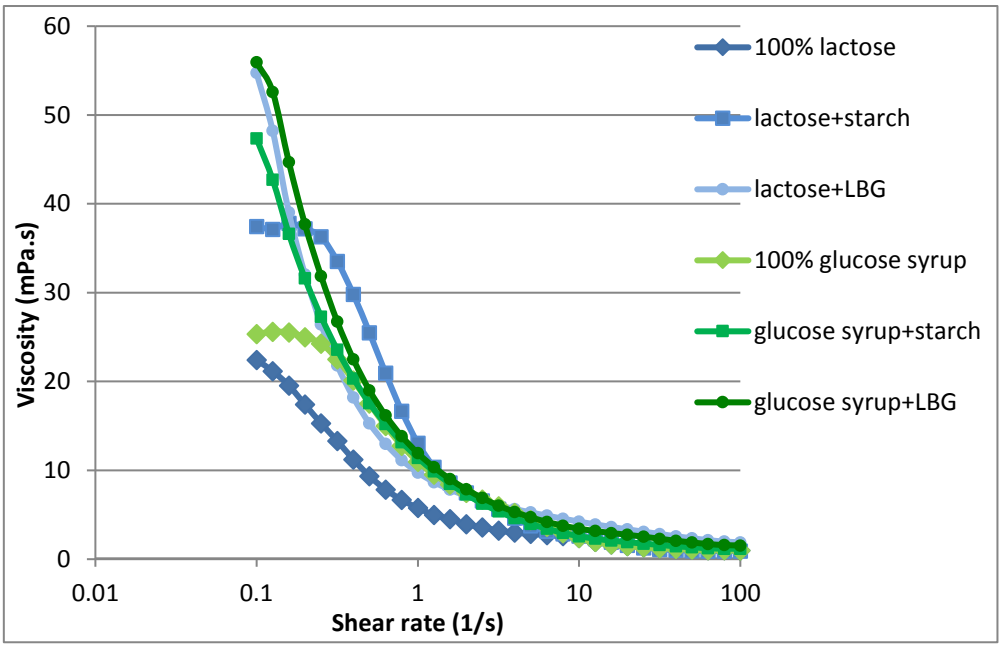


Fig 7.1 Viscosity changing of infant formulas over a shear rate of 0.1 to 100 s^{-1} at 37°C of infant milk formulae using 100% lactose, lactose + starch, lactose + LBG, 100% glucose syrup, glucose syrup + starch, glucose syrup + LBG.

7.3.2 Digestion of carbohydrates in infant milk formulae

The digestion rate of different carbohydrates used in infant milk formulae is determined by the extent of released glucose in the digesta samples during the digestion in the gastric (S0, S60) and intestinal phases (I0-I120), and results are presented in Fig 7.3. There was no glucose released during the gastric phase for all the infant formulae, but the amount of glucose increased remarkably in the intestinal phase (except formulae contain glucose syrup).

In the gastric phase, carbohydrate levels for all formulae remained unchanged and no glucose was released as there are no carbohydrases available in the *in vitro* infant stomach. The gastric phase in the *in vitro* model just imitates the physiological digestion process in infants; therefore, the digestion of carbohydrates in our study resembled the infant in vivo digestion.

In the small intestine phase, lactose in the infant milk formulae is hydrolysed by lactase enzymes resulting in the release of glucose. The formula with 100% lactose contained 12.73 mg and 14.25 mg of glucose per mL of digesta after 30 min and 120 min of digestion in the small intestine. This result

is similar to Dutra Rosolen, Gennari, Volpato, and Volken de Souza (2015) who also used lactase to hydrolyse lactose in milk and obtained around 17 mg/mL glucose after 2 hours of incubation.

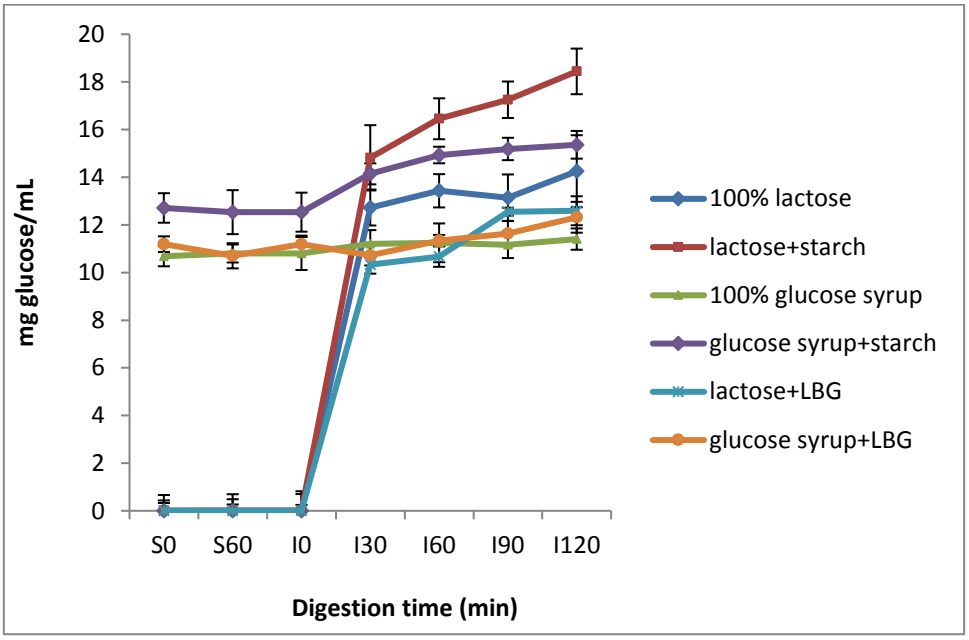


Fig 7.2a Comparison of glucose released (mg glucose/mL) during *in vitro* digestion after 0 and 60 min in the stomach (S0, S60) and the intestinal phase (I0, I30, I60, I90, I120) of infant milk formulae using 100% lactose, lactose + starch, 100% glucose syrup, glucose syrup + starch, lactose + LBG, glucose syrup + LBG.

For the infant milk formulae containing glucose syrup, during the gastric phase, a remarkably higher amount of glucose was observed as compared to the formulae containing lactose. At the beginning of gastric digestion (S0), with formulae containing glucose syrup, the glucose level reached 11-13 mg/mL and remained stable at this concentration during the gastric (stomach) phase. In contrast, during 2 hours in the small intestine, there is not much glucose released for the formulae with glucose syrup only. It is worth noting that glucose syrup in this study is a product of starch hydrolysing with DE42-43 which means the amount of glucose in the formulae is quite high right from the start of digestion. This leads to the high initial glucose concentration in the gastric phase. In the small intestine phase, however, our findings are not in agreement with Lebenthal, Heitlinger, et al. (1983) who reported glucose syrups were well hydrolysed during the infant *in vitro* digestion. This disparity in results could be due to the different type of glucose syrups employed; DE10-24 used by Lebenthal et al. had a much lower glucose content than DE42-43 which was used in our study. Also, different methods of glucose assay were used that might lead to different results - nicotinamide adenine

dinucleotide reduction method-Flozyme assay was used in their study (Lebenthal, Heitlinger, et al., 1983) while hexokinase/glucose-6-phosphate-dehydrogenase assay-HK assay was used in our study.

For infant milk formulae containing starch, the results show that starch was well digested by enzyme amyoglucosidase. After two hours of digestion in the small intestine, the released glucose for formulae lactose + starch and glucose syrup + starch are 18.44 and 15.36 mg/mL, respectively. These are much higher as compared to glucose release of 14.25 and 11.40 mg/mL for formulae with 100% lactose and 100% glucose syrup, respectively. Since, there is no amylase available in the *in vitro* small intestine, starch is obviously digested by amyloglucosidase. It was reported that amyloglucosidase which can digest starch directly to glucose, is the enzyme mainly in charge of starch digestion in infants due to the limited amylase activity (Lebenthal, Lee, et al., 1983).

Fig 7.2a shows that LBG did not contribute to the released lactose levels during the digestion of lactose + LBG and glucose syrup + LBG formulae in the *in vitro* small intestine. It is clear that the lactose levels were lower in the digesta of the formulae with LBG than in the ones without LBG. This can be explained by the fact that LBG is a galactomannan polysaccharide, not digested in the small intestines due to its structure, only a small portion of galactomannan could be fermented by intestinal microbiota (Meunier et al., 2014). The galactomannans present in LBG are a component of dietary fibres which are resistant to digestive juices or enzymes in the human gastrointestinal tract (Trowell et al., 1976). Carlos Alberto González-Bermúdez et al. (2015) also observed LBG remaining stable during the digestion process of infant foods. Also, it is possible that the viscous formulae of LBG could reduce the interaction between lactose and lactase enzyme in the intestinal phase.

In order to compare the effect of the emulsion on the digestibility of carbohydrates, an *in vitro* digestion of the control formulae was conducted (same concentration of carbohydrates but without proteins and vegetable oils). As can be seen in Fig 7.2b, lactose and starch were digested quicker in control formulas than in infant formulas. For the control formula containing 100% lactose, after 60 and 120 minutes of the intestinal digestion, glucose release was 15.05 and 16.20 mg/mL, higher than 13.43 and 14.25 mg/mL, respectively, for infant formula with 100% lactose. The same pattern was observed in the formulae with lactose + starch, glucose syrup + starch. The lower amount of released glucose during digestion of infant formula is possibly due to the presence of proteins and vegetable oils in the digesta. The emulsion could be a restraining factor for the interaction between the substrates and the enzymes.

However, the glucose released in infant milk formulae with 100% glucose syrup and glucose syrup + LBG were similar to the control, the resistance to digestive enzymes of glucose syrup and LBG in the small intestine being the main reason.

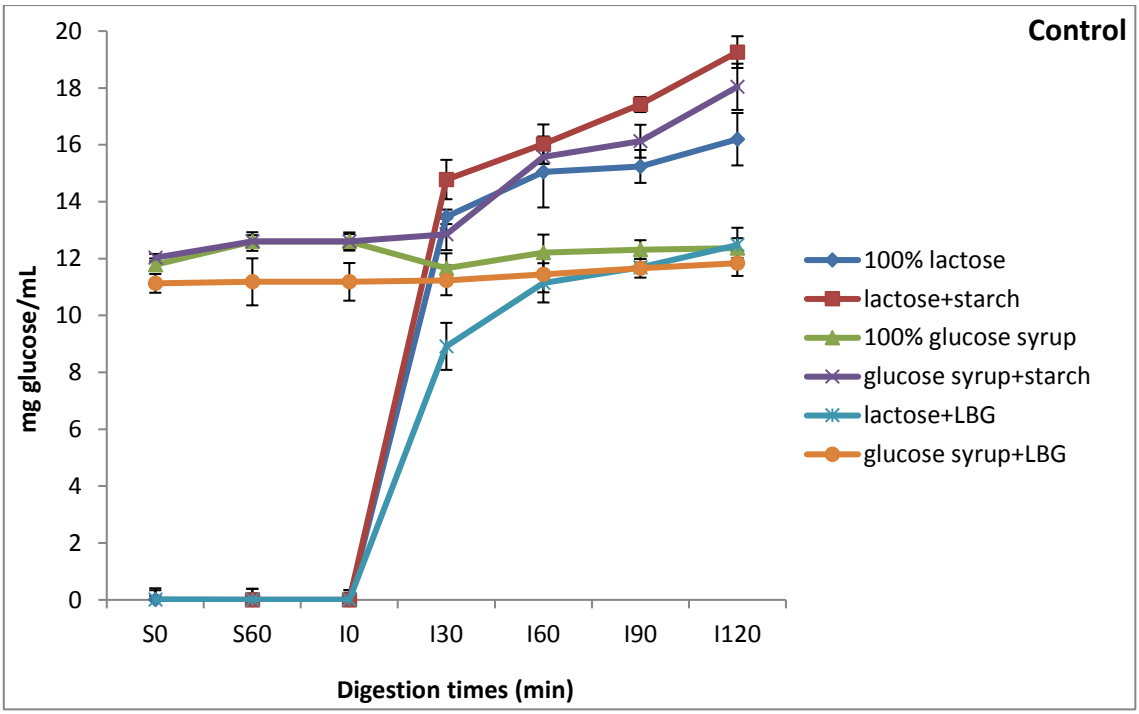


Fig 7.2b Comparison of glucose released (mg glucose/mL) during *in vitro* digestion after 0 and 60 min in the stomach (S0, S60) and the intestinal phase (I0, I30, I60, I90, I120) of control formulae with 100% lactose, lactose + starch, 100% glucose syrup, glucose syrup + starch, lactose + LBG, glucose syrup + LBG.

7.4. Conclusion

The results from the static *in vitro* digestion of infant formula emulsions based on whey, caseins, and vegetable oils, simulating the infant gastrointestinal tract, suggest that no carbohydrate was digested in the gastric phase because no carbohydrase enzymes are available in the infant stomach. In the intestinal digestion, the formulae with lactose gave a much higher amount of released glucose than the formulae with glucose syrup. Also, precooked starch and LBG provide a higher viscosity for infant formula using lactose or glucose syrup as the main carbohydrates. Precooked starch was well digested during the intestinal digestion, but not LBG due to its resistance to the digestive enzymes. This suggests starch is a good thickener option in term of digestibility for infants with aspiration or

reflux issues. The higher viscosity of infant milk formulae due to thickener adding could has some effects on the digestibility of ingredients because of the limited interaction between the ingredients and digestive enzymes.

7.5. References

Australia Government, Commonwealth Law. (2008). Australia New Zealand Food Standards Code - Standard 1.1A.1 - Transitional Standard for Infant Formula Products. Retried from https://www.legislation.gov.au/Details/F2008B00801.

Bhatia, J., & Greer, F. (2008). Use of soy protein-based formulas in infant feeding. *Pediatrics*, 121(5), 1062-1068.

Bosscher, D., Van Caillie-Bertrand, M., Van Dyck, K., Robberecht, H., Van Cauwenbergh, R., & Deelstra, H. (2000). Thickening infant formula with digestible and indigestible carbohydrate: availability of calcium, iron, and zinc in vitro. *Journal of Pediatric Gastroenterology and Nutrition*, 30(4), 373-378.

Choi, S., Lee, S., & Won, H. (2007). Development of lactose-hydrolyzed milk with low sweetness using nanofiltration. *Asian Australian Journal of Animal Sciences*, 20(6), 989.

Cichero, J., Nicholson, T., & Dodrill, P. (2011). Liquid barium is not representative of infant formula: characterisation of rheological and material properties. *Dysphagia*, 26(3), 264-271.

Cichero, J., Nicholson, T. M., & September, C. (2013). Thickened milk for the management of feeding and swallowing issues in infants a call for interdisciplinary professional guidelines. *Journal of Human Lactation*, 0890334413480561.

Coppa, G. V., Gabrielli, O., Pierani, P., Catassi, C., Carlucci, A., & Giorgi, P. L. (1993). Changes in carbohydrate composition in human milk over 4 months of lactation. Pediatrics, 91(3), 637-641.

Dutra Rosolen, M., Gennari, A., Volpato, G., & Volken de Souza, C. F. (2015). Lactose hydrolysis in milk and dairy whey using microbial β-galactosidases. *Enzyme Research*, 2015.

Engfer, M. B., Stahl, B., Finke, B., Sawatzki, G., & Daniel, H. (2000). Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. *The American Journal of Clinical Nutrition*, 71(6), 1589-1596.

Fanaro, S., & Vigi, V. (2012). Feeding the Term Infant: Human Milk and Formulas Neonatology (pp. 290-297): Springer.

González-Bermúdez, C. A., Castro, A., Perez-Rea, D., Frontela-Saseta, C., Martínez-Graciá, C., & Nilsson, L. (2015). Physicochemical properties of different thickeners used in infant foods and their relationship with mineral availability during in vitro digestion process. *Food Research International*, 78, 62-70.

González-Bermúdez, C. A., Frontela-Saseta, C., López-Nicolás, R., Ros-Berruezo, G., & Martínez-Graciá, C. (2014). Effect of adding different thickening agents on the viscosity properties and in vitro mineral availability of infant formula. *Food Chemistry*, 159, 5-11.

Koletzko, B., Baker, S., Cleghorn, G., Neto, U. F., Gopalan, S., Hernell, O., . . . Ding, Z. Y. (2005). Global standard for the composition of infant formula: Recommendations of an ESPGHAN Coordinated International Expert Group. *Journal of Pediatric Gastroenterology and Nutrition*, 41(5), 584-599.

Koletzko, B., Saris, W., Flynn, A., Palou, A., Wal, J., Jackson, A., . . . Turck, D. (2003). Report of the scientific committee on food on the revision of essential requirements of infant formulae and follow-on formulae. Brussels, European Commission.

Lebenthal, E., Heitlinger, L., Lee, P., Nord, K., Hodge, C., Brooks, S., & George, D. (1983). Corn syrup sugars: in vitro and in vivo digestibility and clinical tolerance in acute diarrhea of infancy. *The Journal of Pediatrics*, 103(1), 29-34.

Lebenthal, E., Lee, P., & Heitlinger, L. (1983). Impact of development of the gastrointestinal tract on infant feeding. *The Journal of Pediatrics*, 102(1), 1-9.

Meunier, L., Garthoff, J. A., Schaafsma, A., Krul, L., Schrijver, J., van Goudoever, J. B., . . . Vandenplas, Y. (2014). Locust bean gum safety in neonates and young infants: an integrated review of the toxicological database and clinical evidence. *Regulatory Toxicology and Pharmacology*, 70(1), 155-169.

Nguyen, T. T. P., Bhandari, B., Cichero, J., & Prakash, S. (2015a). A comprehensive review on in vitro digestion of infant formula. *Food Research International*, 76, 373-386.

Nguyen, T. T. P., Bhandari, B., Cichero, J., & Prakash, S. (2015b). Gastro intestinal digestion of dairy and soy proteins in infant formulas: An in vitro study. *Food Research International*, 76, 348-358.

Panel, E. N. (2014). Scientific opinion on the essential composition of infant and follow-on formulae. *EFSA Journal*, 12, 3760.

Saha, D., & Bhattacharya, S. (2010). Hydrocolloids as thickening and gelling agents in food: a critical review. *Journal of Food Science and Technology*, 47(6), 587-597.

Shrestha, A. K., Ng, C. S., Lopez-Rubio, A., Blazek, J., Gilbert, E. P., & Gidley, M. J. (2010). Enzyme resistance and structural organization in extruded high amylose maize starch. *Carbohydrate Polymers*, 80(3), 699-710.

Slaughter, S. L., Ellis, P. R., & Butterworth, P. J. (2001). An investigation of the action of porcine pancreatic α-amylase on native and gelatinised starches. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1525(1), 29-36.

Syrbe, A., Bauer, W., & Klostermeyer, H. (1998). Polymer science concepts in dairy systems—an overview of milk protein and food hydrocolloid interaction. *International Dairy Journal*, 8(3), 179-193.

Thompkinson, D., & Kharb, S. (2007). Aspects of infant food formulation: Comprehensive reviews, *Food Science and Food Safety*, 6(4), 79-102.

Trowell, H., Southgate, D. T., Wolever, T. S., Leeds, A., Gassull, M., & Jenkins, D. A. (1976). Dietary fibre redefined. *The Lancet*, 307(7966), 967.

Ventura, A. K., San Gabriel, A., Hirota, M., & Mennella, J. A. (2012). Free amino acid content in infant formulas. *Nutrition & Food Science*, 42(4), 271-278.

Warren, F. J., Zhang, B., Waltzer, G., Gidley, M. J., & Dhital, S. (2015). The interplay of α-amylase and amyloglucosidase activities on the digestion of starch in in vitro enzymic systems. *Carbohydrate Polymers*, 117, 192-200.

CHAPTER 8 *IN VITRO* INFANT DIGESTION OF MOTHERS' MILK IN COMPARISON WITH INFANT FORMULAE

8.1. Introduction

Human mothers' milk is the ideal food for infants because of the availability of the perfect nourishment for infants' growth and development. Mothers' milk supplies the well-balanced nutrition, growth factors, and immune components that have valuable impacts on infants' digestion, immune system, and cognitive development (Hernell, 2011; Lönnerdal, 2013). An immunoglobulin sIgA in mothers' milk plays an important part in protecting the newborn against infections from intestinal tract diseases (Belderbos et al., 2012; Floris, Lambers, Alting, & Kiers, 2010). 100 mL of mothers' milk contains the average composition 0.9-1.2 g of protein, 3.2-3.6 g of lipid, and 6.7-7.8 g of lactose (Ballard & Morrow, 2013; Bosscher et al., 2000; Jensen, 1995).

However, when mothers' milk is not accessible, infant formula based on bovine milk or soymilk becomes the best second option (Martin, Ling, & Blackburn, 2016). Because of the differences in composition of proteins, fats, and carbohydrates between human milk, bovine milk, and soymilk, there have been attempts to duplicate the nutritional composition of mothers' milk in order to make infant formula closer to human milk as much as possible (Goedhart & Bindels, 1994; Martin et al., 2016). For examples: whey:casein ratio in infant formula has been adjusted according to mothers' milk, lactoferrin and α-lactalbumin has been added, a mixture of vegetable oils has been used as a lipid source in infant formula.

The composition and type of the ingredients added to make infant formula and their microstructure and physico-chemical properties (such as chemical make-up, dispersed phase size and other inter-molecular interactions), will be different to mothers' milk. These factors may influence the digestibility of the infant formula and be different from the digestibility of human milk. Therefore, it will be important to understand the digestibility of human milk in order to compare with the infant formula. To understand the digestibility and structural changes of infant formula in the gastrointestinal tract, an *in vitro* digestion model for infants was applied in previously published work by this research group (Nguyen, Bhandari, Cichero, & Prakash, 2015a, 2016); however, there are not many studies reported on the *in vitro* digestion of mothers' milk. Recently, an *in vitro* dynamic digestion model for term newborns was set up by Oliveira, Bourlieu, et al. (2016) and Deglaire et al.

(2016) for proteolysis and lipolysis in mothers' milk. This model used pepsin, gastric lipase from rabbit gastric extract for the gastric phase; and porcine pancreatin for intestinal phase. Oliveira, Bourlieu, et al. (2016) found that β-casein was digested better than α-lactalbumin after 120 min of gastric digestion; also, mothers' milk proteins were rapidly digested after 30 min in intestinal phase. For lipid digestion, these authors reported that the lipolysis of pasteurized mothers' milk was significantly lower than raw mother's milk. This suggests pasteurization limits the lipolysis in mothers' milk after and during digestion as the two endogenous lipases in mothers' milk that facilitate lipids hydrolysis - pancreatic lipase-related to protein 2 (PLRP 2) and bile salt-stimulated lipase (BSSL), are completely inactivated during pasteurization (Oliveira, Bourlieu, et al., 2016).

The aim of this work is to determine the digestibility of human milk by the same method employed for dairy and soy based infant formulae. This will allow observation of the differences between the human milk and infant formula in regard to the digestibility. Our study employed the *in vitro* static digestion model as described previously in Chapter 3 (Nguyen, Bhandari, Cichero, & Prakash, 2015b) that offers advantages such as low cost, easy sampling and operation to compare the digestibility of proteins, lipids, and lactose in mothers' milk to dairy and soy infant milk formulae under the influence of all the digestive enzymes (proteases, lipase, lactase, and amyloglucosidase). The previous chapters in this thesis have investigated the *in vitro* digestibility of important ingredients such as: non-hydrolysed and hydrolysed dairy and soy proteins, lipids, and carbohydrates, currently used in infant formula. This study determined proteins hydrolysates, free fatty acids, and glucose released during the digestion of mothers' milk and infant formulae. Also, the particle size distribution and confocal images of the digesta were also determined.

8.2 Materials and method

8.2.1. Materials

8.2.1.1. Enzymes and chemicals

Pepsin from porcine gastric mucosa (EC 3.4.23.1, 3840 units/mg protein, one unit will produce a change in A280 of 0.001 per min at pH 2.0 at 37°C, measured as TCA-soluble products using haemoglobin as substrate). Trypsin from bovine pancreas (EC 3.4.21.4, 13165 units/mg protein, one unit will produce a change in A253 of 0.001 per minute at pH 7.6 at 25^0C using Nα-Benzoyl-L-

arginine Ethyl Ester (BAEE) as a substrate. Chymotrypsin from bovine pancreas (EC 3.4.21.1, 54.49 units/mg protein, one unit will hydrolyze 1.0 μmol of N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE) per min at pH 7.8 at 25°C as stated by manufacturer). Lipase DF 15 (180 units/mg, stable pH 4.0-7.0, optimum pH 6.0-7.0, obtained from Amano Enzyme Inc., Japan) was used as analogue gastric lipase. Lipase from porcine pancreas (EC 232.619.9, 59578 units/mg). Lactase Godo YNL-2 (EC 3.2.1.23, 50000 U/g) supplied by Connell Bros Company Australasia Pty. Ltd (Victoria, Australia). Amyloglucosidase (EC 3.2.1.3, 321 U/mL). Other enzymes were obtained from Sigma-Aldrich, Castle Hill, New South Wales, Australia.

Porcine pancreatic colipase, sodium taurocholate, pepstatin, trypsin-chymotrypsin inhibitor, orlistat, and Glucose (HK) assay kit GAHK-20 were obtained from Sigma-Aldrich, Castle Hill, New South Wales, Australia. The other ingredients used in the study such as lactose, sodium chloride, hydrochloric acid, sodium hydroxide, and sodium azide were of analytical grade.

All the enzymes and chemicals were stored at the manufacturer's recommended temperature.

8.2.1.2. Mothers' milk, dairy and soy proteins for infant formula

Three lactating mothers nursing their babies of about 3-6 months volunteered to donate their milk samples for the study. The ethics approval for this study was granted by Research and Innovation Human Ethics Committee, The University of Queensland, Australia (approval number 2016000313). The milk was collected in frozen state. After thawing at 4°C for 12 hours, the samples from the three volunteers were mixed together and stored at -20°C until the day of the digestion trial. Total storage time was approximately 12 months. The mothers' milk had 0.72 % total protein (whey:caseins=3.5:1), 6.3% lactose and 2.8% fat.

Whey protein isolate (WPI 85.1% protein, 1.0% fat, 1.2% carbohydrate) and calcium caseinate (CC, 86.7% protein, 1.0% fat, 0.1% carbohydrate) were purchased from Total Foodtec Pty Ltd (Australia). Soy protein isolate (SPI, 82.4% protein, 4.5% fat, less than 1.0% carbohydrate) was purchased from Food Manufacturers Pty., Australia.

Sunflower vegetable oil was obtained from the local supermarket.

8.2.2. Method

8.2.2.1. Preparation of infant formulae

The procedure for making infant formulae was as described by Nguyen et al. (2015b) except the homogenisation step which was done using a micro-fluidiser (Microfluidics, Model No. LM10, Quadro Engineering, Canada) (Fig 6.1). 100 mL of infant milk formulae containing 1.5 g of protein, 4.0 g of lipid and 6.5 g of lactose was chosen based on the recommendation for infant formulae by the European Union (Koletzko et al., 2005). This recommendation meets the requirements of providing a safe and nutritional adequacy for healthy infants rather than mimics exactly the composition of mothers' milk. This study used two formulae using dairy and soy proteins: WPI:CC=6:4, 100% SPI.

8.2.2.2. In vitro infant digestion with all digestive enzymes

A static *in vitro* digestion unit equipped with water bath, and overhead stirrer carried out the digestion trials. Two water-jacketed reaction vessels in the unit were connected to a water bath that provided a constant circulation of warm water in and out of the reaction vessel and maintained a constant temperature of 37°C. A glass stirrer connected to an overhead stirrer continuously mixed the *in vitro* digesta at 250 rpm. All the enzymes added with the activity was described in previous chapters. The gastric phase had pepsin (22.75 U/mg) and simulated gastric lipase (21 units/mL). The intestinal phase had trypsin 3.45 U/mg protein; α-chymotrypsin 0.04 U/mg protein, pancreatic lipase (200 units/mL), molar ration between colipase and pancreatic lipase was 2:1, bile salts (4mM), lactase (1.0 g/L), amyloglucosidase was used with the activity equivalent to 0.28 U/mg of starch (Chapter 7).

Digested samples were inhibited by trypsin-chymotrypsin inhibitor and orlistat to inhibit twice the amount of trypsin and chymotrypsin, analogue gastric lipase and pancreatic lipase. Lactase and amyloglucosidase were inhibited by putting the samples in ice for 5 minutes. Then the samples were stored at -20°C for further analysis (SDS-PAGE, Gas Chromatography and glucose content).

8.2.2.4. Gel electrophoresis (SDS-PAGE)

Gel electrophoresis was described in details in Chapter 4. Degradation of proteins during *in vitro* digestion based on molecular weight of proteins and polypeptides was assessed by SDS-PAGE (Nguyen et al., 2015). The protein breakdown of the digesta over the gastric and intestinal phase was analysed by reducing SDS-PAGE running on a Mini Protean 3 cell (Bio-Rad) for 37 min at 200V. The assay was performed according to the protocol described by Laemmli (1970), using 4-20% Tris-HCl precast gel, protein ladder. Each volume of sample was mixed with four volume of sample buffer (0.0625M Tris-HCl buffer pH 6.8), 40% glycerol, 2% SDS, 0.04% bromophenol blue, and β-mercaptoethanol (19:1, v/v). The mixture was heated at 95°C for 5 min then loaded to the wells. 10 µL was loaded for both gastric and intestinal phase samples.

8.2.2.5. Particle size distribution

Particle size distribution of native and digested milk samples were measured before and during the *in vitro* gastric and intestinal digestions by a Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershine, UK). The refractive index value for the dispersed phase was 1.35 (milk), and for the continuous phase was 1.33 (water). Samples were diluted in deionised water in the measurement cell of the equipment until the obscuration reached 15%. Mean particle sizes and distribution were determined as the average of three repeated measurements from duplication.

8.2.2.6. Confocal Laser Scanning Microscopy (CLSM)

The physical arrangement of protein and fat globules of native and digested sample were observed by Zeiss LSM 700 Confocal Laser Scanning Microscope. Protein were stained with Rhodamine B (1% w/w in MiliQ water) and excited with the laser light at a wavelength 540 nm (Nagano, Tamaki, and Funami, 2008; van de Velde, Weinbreck, Edelman, van der Linden, and Tromp, 2003; van Riemsdijk, Sprakel, van der Goot, and Hamer, 2010). Nile red (0.1% w/w in acetone) was used to stain triglycerides and excited with the laser light wavelength of 515-530 nm (Gallier, Ye, &Singh, 2012; Ye, Cui, and Singh, 2011).

For slide preparation, 100 µl of infant formula samples was mixed with 25 µl of Rhodamine B or 10 µl of Nile red solution by using vortexer (Ratex VM1) for 5 sec. Samples were stained at least 10

minutes. 10 µl of stain samples was loaded onto 26x76 mm slides (Sail Brand) and then covered with 18x18 mm cover slip (Menzel Glaser). The edges of the cover slips were coated with a transparent nail polish to fix the sample position and prevent 5the sample from drying. The observations for fat globules and the breakdown of protein aggregation was done with a magnification lens at 63x and 10x, respectively.

8.2.2.7. Free fatty acid analysis by Gas Chromatography

Lipid extraction and isolation of free fatty acids (FFAs) from digested samples was adopted from De Jong and Badings (1990). 5 mL of digesta was mixed with 5 mL ethanol, 0.5 mL H_2SO_4 (2.5M), 100 µL internal standard solution (heptadecanoic acid 10% w/v). FFAs were then extracted by adding 7.5 mL ether:heptane =1:1 (v/v) to the mixture, and mixed for 30 minutes. After centrifuging at 2500 rpm for 2 minutes, the upper organic phase was taken. The extraction procedure was done twice with another 7.5 mL ether:heptane =1:1 (v/v).

FFA isolation was carried out with an aminopropyl column (StrataNH2, 200mg/3 mL Phenomenex, Australia). The column was first conditioned with 10 mL heptane then the lipid extract was added to the column. 5 mL of chloroform:2-propanol (2:1, v/v) was applied to elute the neutral lipids. The FFAs were eluted with 5 mL diethyl ether containing 2% formic acid. The collected FFAs fraction was concentrated using nitrogen gas and resuspended in 1 mL diethyl ether. FFAs were analysed by gas chromatography (Shimazu, GC-2010 Plus) equipped with an auto-sampler and a flame-ionization detector (FID) using a FFAP-DB column 30.0 m x 0.53 mm i.d, coating diameter (df = 1.0 µm). The carrier gas was helium at a flow rate of 16.1 mL/min, and oven temperature was varied from 200°C to 240°C. 0.5 µL of samples was injected in duplicate.

8.2.2.7. Glucose assay

Released D-glucose in the digested samples was analysed with the hexokinase/glucose-6-phosphate-dehydrogenase assay (HK assay). Glucose is phosphorylated by adenosine triphosphate (ATP) in the reaction catalyzed by hexokinase. Glucose- 6-phosphate (G6P) is then oxidized to 6-phosphogluconate in the presence of oxidized nicotinamide adenine dinucleotide (NAD) in a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). During this oxidation, an equimolar

amount of NAD is reduced to NADH. The consequent increase in absorbance at 340 nm is directly proportional to glucose concentration.

Absorbance was measured by spectrophotometer with quartz cuvettes (HELL-200-66501-MP, Shimadzu) at a wavelength of 340 nm.

8.2.2.8. Statistical analysis

The value for glucose assay and released free fatty acids for pH drop were measured in triplicate from duplication. Experimental data were assessed by ANOVA tests to determine the significant differences among the means at 95% confidence level. The treatment means were considered to be significantly different when $P<0.05$.

8.3. Results and discussion

8.3.1. Protein digestion

8.3.1.1. Digestion of proteins in mothers' milk and in infant formulae based on bovine milk proteins

Fig 8.1 (A-B) presents the PAGE patterns of mothers' milk and infant formulae with WPI:CC = 4:6 at 0, 30 and 60 min of gastric digestion and at 0, 30, 60, 120 min of intestinal digestion. As can be seen from Fig 8.1 (A & B), in comparison to bovine proteins, mothers' milk proteins contain the bands for lactoferrin, serum albumin, β-casein, α-casein, α-lactalbumin (with the theoretical molecular weight of 78, 69, 24, 23, and 14.4 kDa, respectively), but not for β-lactoglobulin (18 kDa).

After 60 minutes of digestion in the stomach, around 90% of β-casein and 60% of α-casein in mothers' milk were digested; but for infant formula using WPI and CC from bovine milk, most of the caseins were digested. Most of the whey proteins resisted proteolysis by pepsin in the simulated stomach with only 20% of α-lactalbumin in mothers' milk and 10% in infant formula being digested. Also, nearly 90% of β-lactoglobulin, 80% of serum albumin, and 60% of lactoferrin resisted proteolysis by pepsin in the stomach. Similar results for proteolysis of β-casein, serum albumin and lactoferrin in mothers' milk under an *in vitro* dynamic digestion system for term-newborn was reported previously (Oliveira,

Deglaire, et al., 2016) with only approximately 50% of β-casein, 40% of lactoferrin and 20% of serum albumin were hydrolysed in the gastric phase.

The higher density of β-casein than α-casein shown in the PAGE pattern [Fig. 8.1(A)] reconfirms that β-casein is the main caseins in mothers' milk (O'Callaghan, O'Mahony, Ramanujam, & Burgher, 2011). The gastric digestion of β-casein in mothers' milk is also in agreement with Dallas et al. (2014), who reported β-casein in human milk quickly broke down more so than other proteins in the gastric digestion phase. However, the behaviour of caseins in stomach contradicts with the result obtained by Nguyen et al. (2015b) with less than 20% of caseins being hydrolysed. There is no clear explanation for the difference, but the different conditions under which the *in vitro* stomach digestion was carried out in the two studies need to be taken into account with pepsin, analogue gastric lipase, gastric pH of 4.5 used in this study (that is closer to the optimum pH of analogue gastric lipase) as compared to only pepsin and gastric pH of 4.0 used by Nguyen et al. (2015b). A further study on this is needed to investigate it further.

In the intestinal phase, after 30 minutes of exposure to trypsin and chymotrypsin enzymes, caseins were well hydrolysed, the bands for caseins become very faint for mother's milk and totally disappear for infant formulae [Fig 8.1 A-C)]. Oliveira, Deglaire, et al. (2016) and Oliveira, Bourlieu, et al. (2016) also reported that caseins in mothers' milk were totally digested after 30 minutes of intestinal digestion. For infant formulae, similar results for digestion of caseins was observed by Nguyen et al. (2015b). Whey proteins in mothers' milk, except serum albumin remained resistant during the intestinal phase; lactoferrin and α-lactalbumin were completely digested which is in agreement with Oliveira, Deglaire, et al. (2016) and Oliveira, Bourlieu, et al. (2016). For infant formulae, β-lactoglobulin was completely digested, but not serum albumin. Although present in minor concentration in infant formulae, the bovine serum albumin seems not affected by the digestive proteases in the *in vitro* gastrointestinal tract.

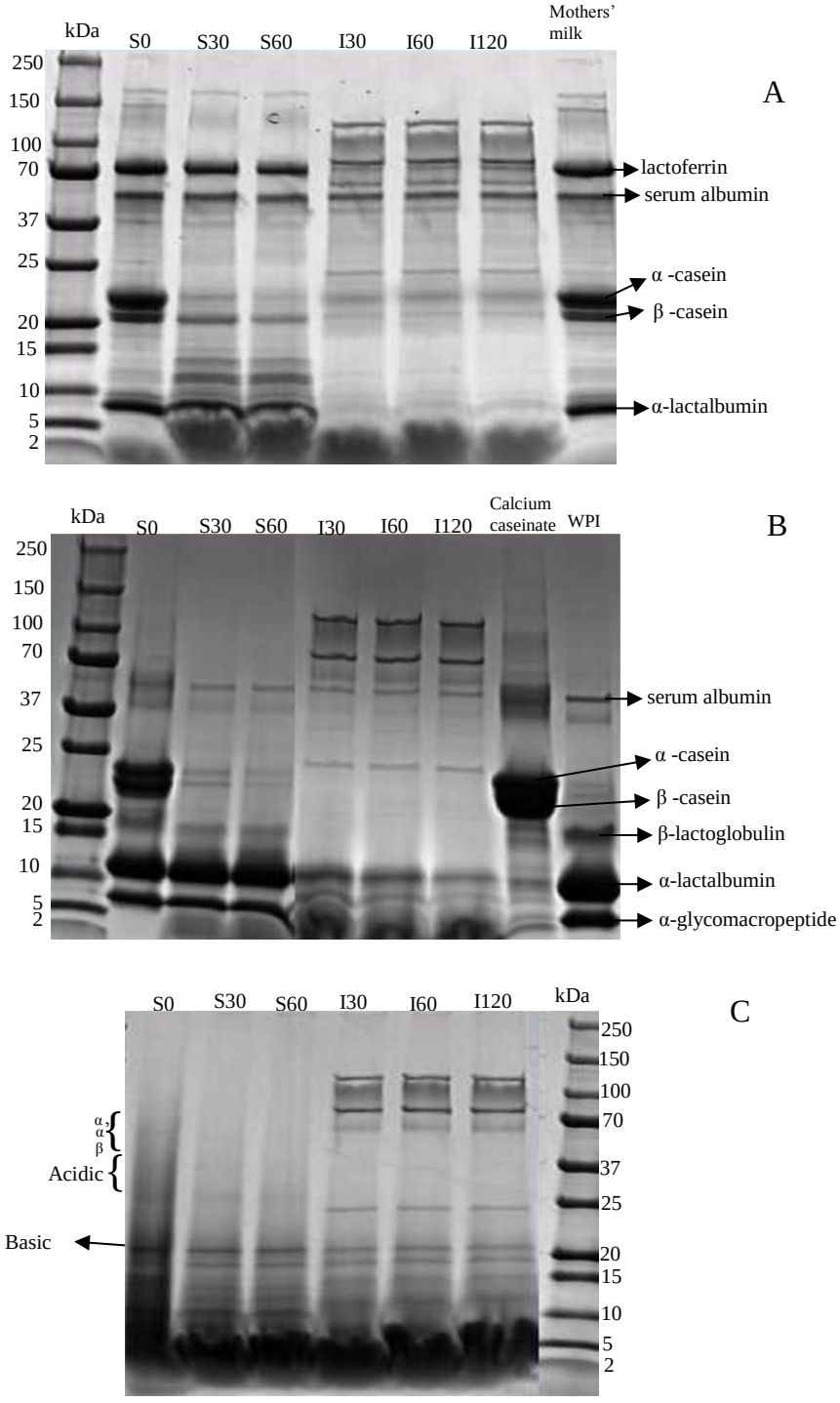


Fig 8.1 Reducing SDS-PAGE analysis of in vitro digested samples of mothers' milk (A) and two infant formulae: WPI:CC=6:4 (B), 100% SPI (C) during the gastric phase from 0 min (S0) to 60 min (S60) and the intestinal phase from 0 min (I0), 30 min (I30) to 120 min (I120).

It is clear that caseins are more quickly digested in the in vitro gastrointestinal tract than whey proteins in both mothers' milk and infant milk formulae. This difference in digestibility can be due to the difference in the structure and composition of amino acids in caseins and whey. Due to the high degree of phosphorylation, caseins (especially β-casein) have an open tertiary structure (Greenberg, Groves, & Dower, 1984; Holt, Carver, Ecroyd, & Thorn, 2013) which is sensitive to proteolysis. In contrast, whey proteins contains high amount of sulphur-containing amino acids (such as methionine, cystein, lysine, threoin and tryptophan) that create disulphide bonds making whey proteins a compact structure that restricts the action of digestive proteases (Lacroix et al., 2006).

8.3.1.2. Digestion of soy proteins in infant formula

The sequential PAGE patterns of soy based infant formulae after 60 minutes of gastric digestion with pepsin, analogue gastric lipase and 120 minutes of intestinal digestion with trypsin, chymotrypsin, pancreatic lipase, colipase, lactase, amyloglucosidase and bile salts are as shown in Fig. 8.1(C). Soy proteins contain basic polypeptides (18-20 kDa) and acidic polypeptides (31-45 kDa), and to some extent α, α′, β conglycinin (76, 72, 53 kDa) (Brooks & Morr, 1985; Thanh & Shibasaki, 1977). The intensity of the bands for these soy proteins decreased with increasing incubation time in the stomach and intestine [Fig. 8.1(C)] indicating partial hydrolysis of these proteins in the *in vitro* gastrointestinal tract. Similar results reported by Nguyen et al. (2015b) and Nguyen et al. (2016) suggest that soy proteins have the lowest digestibility compared to bovine milk proteins. Thus, it is also less digestible than mother's milk. The structure and processing of soy proteins could be partially responsible for their low digestibility. It has been reported that the secondary structure of soy proteins is dominated by β-sheets while milk proteins are rich in α-helix. The β-sheet structure of soy protein is highly hydrophobic and stimulates protein aggregation that limits the solubility and digestibility of soy proteins. Also, heat treatment during processing causes β-sheet aggregation among soy protein molecules that resists digestion of soy proteins (Carbonaro, Maselli, & Nucara, 2012, 2015). Here it is also worth noting that the properties of soy proteins could vary among products and manufacturers that result in their different ability to digest.

8.3.2. Lipid digestion in mother's milk and infant formulae

The total FFA released and FFA profile characterized by GC during the gastrointestinal digestion of mothers' milk and infant formulae are displayed in Fig 8.2 and Fig 8.3 (A, B, C), respectively. As

can be seen in Fig 8.2, total released FFA increased in the stomach, but the rate of increase is even faster in the first hour of intestinal digestion. After 60 minutes of gastric digestion, total released FFA is only around 9 μmol/mL for WPI:CC=4:6 and 13 μmol/mL for 100% SPI, but reaches up to 28 μmol/mL at I60 for both the formulae. The lipolysis of mothers' milk is also quite slow in the gastric digestion, but it increases remarkably and double the amount of FFA is released at I60 min. The next hour in the intestine, the lipolysis of mother milk and infant formulae kept increasing but at a slower rate. The reasons for the slower lipid digestion rate in the stomach than the intestine has been described in Chapter 6 (section 6.3.2). Due to the structural change of the emulsions under the gastric condition, caseins and soy proteins precipitate and aggregate at gastric pH 4.5 that limits the surface area of the oil-water interface. The oil droplets could be entrapped in the protein aggregation network as reported by Nguyen et al. (2015b) and Ye, Cui, and Singh (2011). In addition, the released polypeptides and peptides from the surface layer during the protein digestion are surface active and might be reabsorbed at the surface area of the oil droplets. Also, lipids present in mothers' milk and sunflower oils have large amount of long chain FFA which accumulate at the oil-water interface. This might reduce the penetration ability of gastric lipase to oil droplets (Gargouri et al., 1986; Pafumi et al., 2002). The above reasons could reduce the rate of lipolysis in the simulated stomach phase.

In the intestinal phase, lipid digestion in the first hour was faster for mothers' milk and two infant formulae. As bile salts work as a bio-surfactant, they are able to displace some of absorbed intact proteins, polypeptides, and peptides in the surface of oil droplets; colipase is a co-enzyme that assists pancreatic lipase binding easily to the oil-water interface making the pancreatic lipase more accessible to the substrate lipid (Brockman, 2002). Also, analogue gastric lipase at optimum pH (6.0-7.0), is still active in the intestinal condition that leads to a considerable contribution to the increased amount of FFA release in the small intestine. However, lipolysis in the next hour slowed down for mothers' milk and infant formulae, similar to the results obtained by Oliveira, Deglaire, et al. (2016) for mothers' milk. This could possibly be due to the decrease in substrate concentration or might be that the complex interaction of the increased digestive products that are available in the digesta such as intact proteins, peptides and polypeptides, released FFA) and other enzymes (lactase, amyloglucosidase) over the oil-water interface.

Fig 8.2 also shows the total released FFA, the levels of FFA at the beginning of the stomach phase (S0) were remarkably high in mothers' milk. In contrast, it is almost unavailable in infant formulae that used sunflower oil as the lipid source. Fig. 8.3 gives more details about the FFA profile of

mothers' milk and infant formulae. The levels of all the released FFA in S0 are much higher in mothers' milk, but almost absent in both infant formulae. It is worth noting that human mothers' milk contains endogenous lipases such as bile salt-stimulated lipase (BSSL) and lipoprotein lipase (LPL) (Freed et al., 1989; Henderson, Fay, & Hamosh, 1998). These endogenous lipases facilitate lipids hydrolysis during mothers' milk storage that leads to some extent of FFA already available before mothers' milk get digested. This is supported by the results from Oliveira, Deglaire, et al. (2016) and Oliveira, Bourlieu, et al. (2016) who concluded that the lipolysis in raw mothers' milk is higher than their holder pasteurized counterpart because the endogenous lipases were inactivated by pasteurization.

The profile of FFA for mothers' milk also showed the higher level of medium chain fatty acid C_{10}, C_{12}, C_{14} while they are totally absent or present at a very low level in infant formulae (Fig.8.3). Recent results from Oliveira, Deglaire, et al. (2016) and Oliveira, Bourlieu, et al. (2016) also show a considerable amount of medium chain FFA released during the *in vitro* digestion of mothers' milk. It has been reported that medium chain triglycerides in mothers' milk contribute up to 10% of the total fat and released medium fatty acid can be absorbed through the gastric mucosa (Lemarie et al., 2016; Łoś-Rycharska, Kieraszewicz, & Czerwionka-Szaflarska, 2016). In contrast, the source of lipid in infant formulae was sunflower oil that does not contain medium chain fatty acids. This finding is in agreement with Orsavova, Misurcova, Ambrozova, Vicha, and Mlcek (2015).

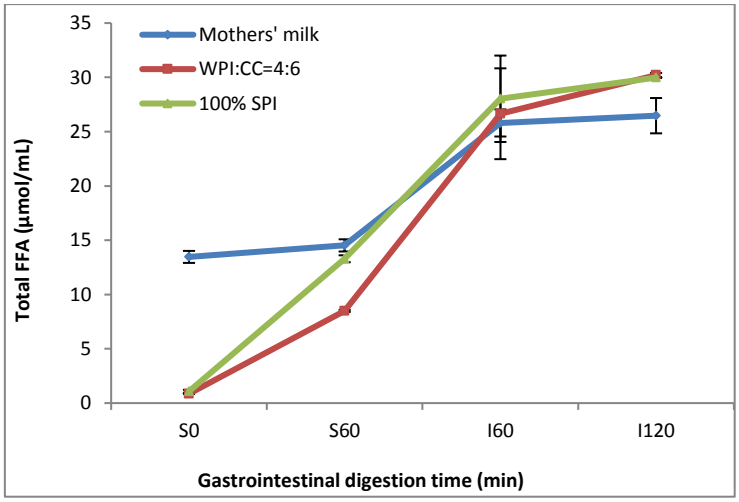


Fig 8.2 Total FFA released in the *in vitro* gastrointestinal digestion of mothers' milk and formula emulsions WPI:CC=6:4 and 100% SPI.

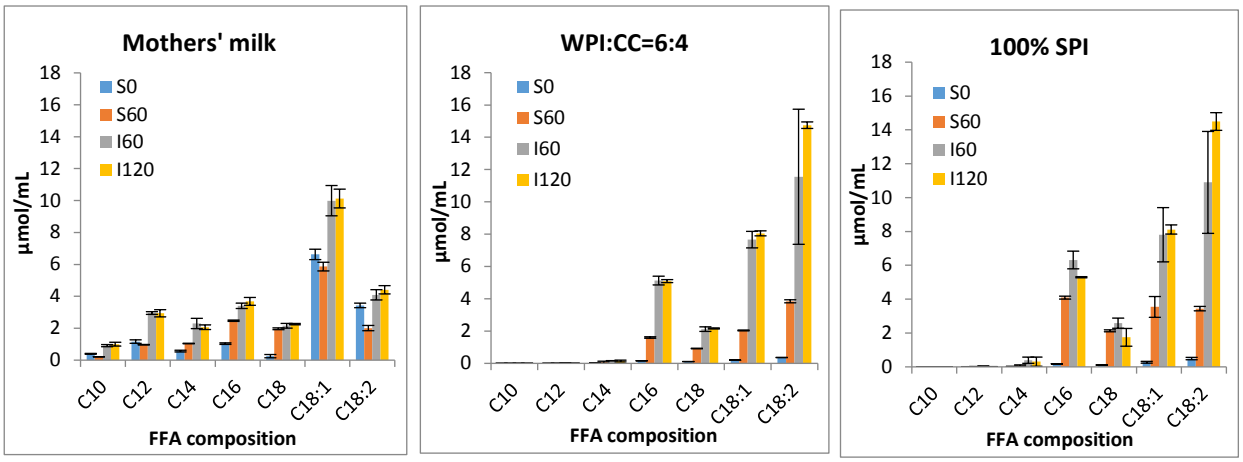


Fig 8.3 FFA composition released from the gastrointestinal digesta samples (S0, S60, I60, I120) in mothers' milk (A) and infant formulae (B, C).

8.3.3. Particle size distribution and confocal micrographs

The change in the digesta particle size during the gastrointestinal digestion can influence its viscosity and dissolvability. Prakash, Ma, and Bhandari (2014) reported that the particle size distribution of infant formula affects the rheological behaviour during the *in vitro* infant formula digestion. Hence, this study compares the particle size distribution of mothers' milk and the two infant formulae using dairy proteins and soy proteins (WPI:CC=6:4 and 100% SPI) during the simulated infant digestion (Fig 8.4).

The particle size distribution of native state of mothers' milk is in size range 0.5-140 µm, larger than 0.04-4 µm and 0.04-10 µm for infant formulae with dairy proteins and soy proteins, respectively. In native state of milk, fat droplets are the main element contributing to the particle size. It has been reported that fat droplets in mature mothers' milk is 4 µm and bigger in colostrum milk (9 µm) (Michalski, Briard, Michel, Tasson, & Poulain, 2005). However, the bigger size distribution of mothers' milk in our study is understandable as the mothers' milk was frozen for nearly 12 months. The freeze-thaw cycles and the possibility of endogenous lipases hydrolysing lipid can cause coalescence of lipid droplets (Fig 8.4-native).

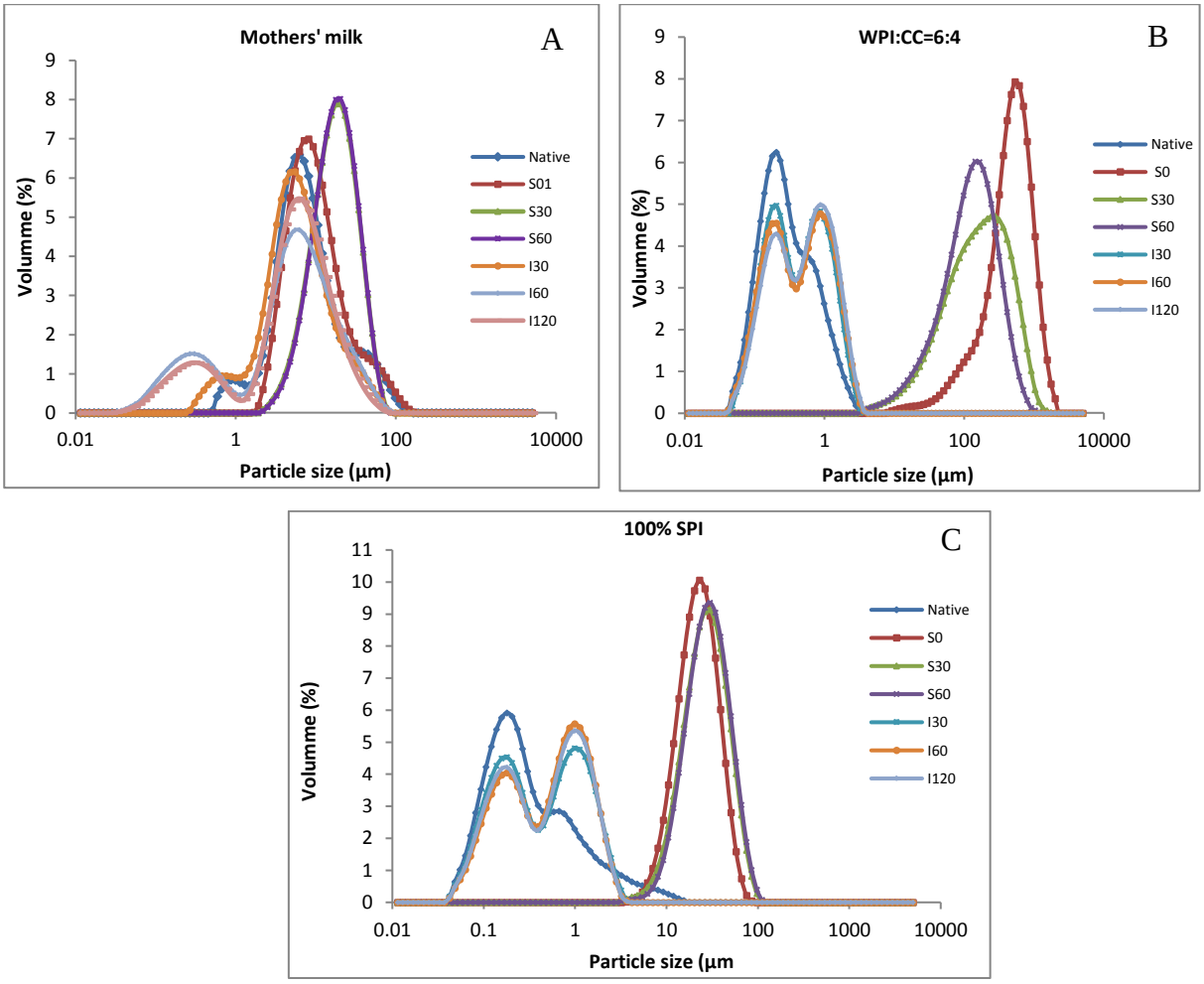


Fig 8.4 Size distribution of native and digested samples under the *in vitro* gastrointestinal digestion of mothers' milk (A) and infant formulae WPI:CC=6:4 (B) and 100% SPI (C)

After adding the simulated gastric fluid to the native milk of infant formulae, the particle size distribution remarkably increased due to the precipitation of caseins and soy proteins at gastric pH 4.5 (Fig 8.4, Fig.8.5-S0). Protein precipitation and aggregation resulted in the immediate increase in the particle size as can be seen in Table 8.1. D(0.9) in the formula WPI:CC=6:4 increased from 0.93 µm (native) to around 1000 µm, d(0.9) in 100% SPI also increased from 1.65 µm (native) to 38.68 µm (S0). A slight increase in particle size from native state to S0 is also observed for mothers' milk, but not as much in the two infant formulae. This might be because the caseins proportion in mothers' milk is less than in the dairy infant formulae (whey:caseins=3.5:1 as compared to 6:4), or might be because of the interaction between the fat droplet coalescence and proteins.

Table 8.1 Particle size distribution of native and gastric digested samples of the mothers' milk and two infant milk formulae WPI:CC=6:4, 100% SPI

Name of formulae	Samples	d(0.1) µm	d(0.5) µm	d(0.9) µm
Mothers' milk	Native	2.2±0.1	5.9±0.1	20.3±1.3
	S0	3.6±0.1	8.6±0.4	31.7±1.2
	S30	7.2±0.7	17.4±1.8	36.9±3.7
	S60	6.8±0.6	16.5±1.7	35.3±3.5
WPI:CC=6:4	Native	0.2±0.1	0.2±0.1	0.9±0.1
	S0	125.9±12.1	469.6±46.3	1001.1±108.6
	S30	28.3±3.6	137.9±21.4	459.7±89.9
	S60	28.9±2.7	119.4±11.1	325.4±44.1
100% SPI	Native	0.1±0.1	0.2±0.1	1.6±0.1
	S0	10.4±0.7	20.8±1.6	38.7±3.4
	S30	11.7±0.3	25.3±0.6	49.9±1.7
	S60	12.69±0.27	26.43±0.49	51.67±1.25

During 60 minutes of gastric digestion, large particle size in the digesta of infant formulae WPI:CC=6:4 break down by pepsin leading to the availability of small and medium particles (Fig 8.5-S60). Similar result was also reported by Nguyen et al. (2015b). However, this was not observed for mothers' milk and soy protein formula whose particle size increase slightly from S0 to S30 and remained unchanged during the gastric digestion. It has been reported the released peptides from the degradation of the adsorbed proteins on the droplet surface by enzyme pepsin might result in a loss of positive charge and a reduction of the droplet layer (Singh, 2011). These absorbed peptides cannot provide adequate electro-static repulsions nor steric stabilisation as intact proteins; therefore they could make the digesta become highly susceptible to aggregation, flocculation, and coalescence (Bourlieu et al., 2015).

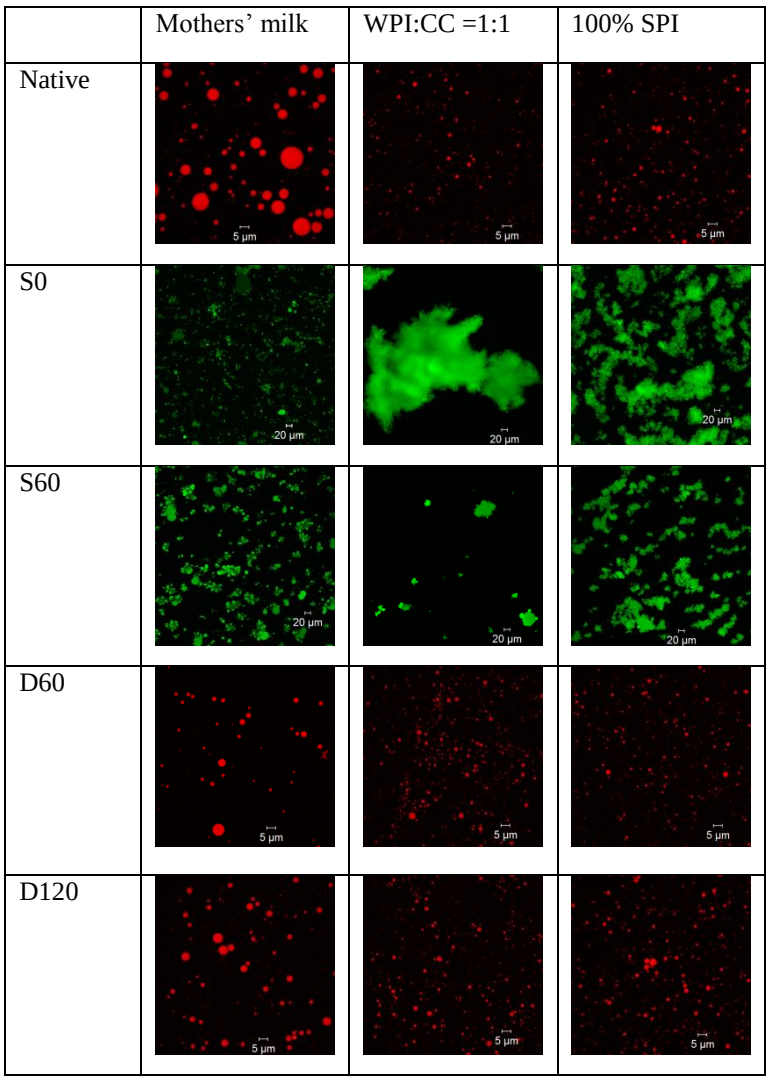


Fig 8.5 CLSM digested samples at beginning (S0), 60 minutes gastric digestion (S60), 60 minutes intestinal digestion (I60) and 120 minutes intestinal digestion (I120) of mothers' milk and infant formula emulsions WPI:CC=6:4 and 100% SPI.

In the intestinal phase, as all protein aggregates dissolved under the intestinal pH 6.5, only fat globules were captured in the images. The particle size decreases remarkably back to a range from 0.04-4 µm for infant formulae and 0.02-75 µm for mothers' milk (Fig 8.5-I60, I120). As compared to the native state of the dairy and soy protein formula, the particle size during the intestinal digestion is still bimodal distribution, but the distribution interchanges with a decrease in the volume of small population (0.04-0.4 µm) and an increase in the volume of large population (0.4-4 µm) indicating lipid coalescence due to lipolysis. Similar trend was obtained for dairy infant formulae during lipid digestion (Chapter 6, section 6.3.1.1). However, different result was observed for mothers' milk with an increase in the volume of small population (0.02-1.1 µm) and a decrease in the volume of large

population (1.1-75 μm). The difference in the particle distribution during the gastrointestinal digestion could be due to the initial difference of emulsion droplet size and the structure of milk fat globule membrane of mothers' milk and infant formulae. Native fat droplets in mothers' milk have bigger size than in infant formula, also native fat droplets in mothers' milk have the milk fat globule membrane (MFGM) that is made of mammalian cell membrane includes phospholipids, sphingolipids, cholesterol, proteins and enzymes (Lopez, Madec, & Jimenez-Flores, 2010). However, MFGM of the dairy and soy infant formulae only contains caseins, whey or soy proteins. This dissimilarity in structure between mothers' milk and infant formula's emulsion could lead to the different behaviour during the *in vitro* digestion.

8.3.4 Carbohydrate digestion in mothers' milk and infant milk formulae

The digestion of lactose in mothers' milk and infant milk formulae (WPI:CC=6:4 and 100% SPI) was determined by the amount of glucose released in the digesta during the gastric phase (S0, S60) and the intestinal phase (I0-I120), and results are presented in Fig 8.6. It is clear that the obtained results for mothers' milk and infant milk formulae had the same pattern which are similar to the results presented previously in Chapter 7. No glucose was released during the gastric digestion, but increased significantly in the small intestine. This is because lactase enzyme which hydrolyses lactose into glucose and galactose is present in the intestine only but not available in the stomach. The similar concentration of lactose in mothers' milk (6.3%) and infant milk formulae (6.5%) led to the similar levels of released glucose during the intestinal digestion. Approximately 13 mg and 15 mg of glucose per mL of digesta were formed after 60 min and 120 min of digestion in the small intestine, respectively. This is in agreement with the study from Dutra Rosolen, Gennari, Volpato, and Volken de Souza (2015) who also used lactase to hydrolyse lactose in milk and obtained around 17 mg/mL glucose after 2 hours of incubation. Also, the observed glucose levels in this study (with the presence of all intestinal digestive enzymes are similar to the results for lactose digestion (Chapter 7) confirming that lactose in mothers' milk and infant milk formulae behave similarly in the simulated infant gastrointestinal tract. This observation could be explained by the fact that lactose has a small molecular size and easily dissolves in the milk emulsion. These properties restrict lactose from being affected by the interaction of other components in the digestion matrixes such as intact proteins, proteins hydrolysates, lipids, FFA, and enzymes.

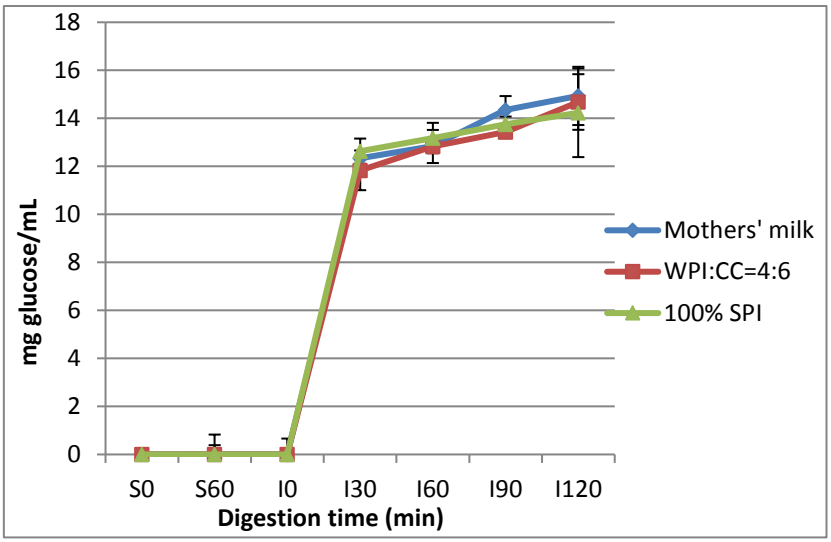


Fig 8.6 Comparison of glucose released (mg glucose/mL) during *in vitro* digestion after 0 and 60 min in the stomach (S0, S60) and the intestinal phase (I0, I30, I60, I90, I120) of mothers' milk, infant milk formula WPI:CC=4:6 and 100% SPI.

8.4. Conclusion

The above results obtained from mothers' milk suggest a difference in protein composition as compared to dairy infant milk formulae. Caseins in mothers' milk contain higher level of β-casein than α-casein; lactoferrin and serum albumin are also available in whey proteins of mothers' milk that is unavailable in infant milk formulae.

This work also investigated and compared the digestibility of mothers' milk and standard infant formulae based on bovine and soy proteins. During the simulated infant gastrointestinal digestion, caseins in mothers' milk and infant milk formulae were quickly and extensively digested than whey proteins. The structure and compositional differences between caseins and whey proteins could significantly affect their digestibility. Soy milk protein was digested slower than both mothers' milk and bovine protein. In terms of digestibility of proteins the order of protein digestion behaviour was: mother's milk > bovine milk > soymilk.

The *in vitro* lipid digestion showed that mothers' milk had a different released fatty acid profile compared to dairy and soy protein infant formula. Medium chain fatty acids were present in mothers' milk, but not in infant milk formulae. A considerably higher level of released FFA was observed in

mothers' milk before undergoing the gastric digestion suggesting that the lipolysis was being facilitated by endogenous lipases during storage.

The particle size result was interesting because confocal image showed that the mothers' milk exhibited smaller particle size and absence of aggregates in the stomach as compared to infant formula. This is attributed to the lower protein aggregation at low pH for mother's milk as this contains mainly beta-casein. Lower particle size can facilitate the enzyme accessibility for hydrolysis of proteins and lipids.

Lactose in mothers' milk or in infant milk formulae based on dairy proteins or soy proteins behaved the same in the *in vitro* infant digestion because the type of lactose is the same and it is in water soluble state without any effect of pH, thus is easily accessible to enzyme.

8.5. References

Ballard, O., & Morrow, A. L. (2013). Human milk composition: nutrients and bioactive factors. [Research Support, N I H, Extramural Research Support, Non-U S Gov't Review]. *Pediatric Clinics North America*, 60(1), 49-74.

Belderbos, M., Houben, M., Van Bleek, G., Schuijff, L., Van Uden, N., Bloemen-Carlier, E., . . . Bont, L. (2012). Breastfeeding modulates neonatal innate immune responses: a prospective birth cohort study. *Pediatric Allergy and Immunology*, 23(1), 65-74.

Bosscher, D., Van Caillie-Bertrand, M., Van Dyck, K., Robberecht, H., Van Cauwenbergh, R., & Deelstra, H. (2000). Thickening infant formula with digestible and indigestible carbohydrate: availability of calcium, iron, and zinc in vitro. *Journal of Pediatric Gastroenterology and Nutrition*, 30(4), 373-378.

Bourlieu, C., Ménard, O., De La Chevasnerie, A., Sams, L., Rousseau, F., Madec, M.-N., . . . Bouhallab, S. (2015). The structure of infant formulas impacts their lipolysis, proteolysis and disintegration during in vitro gastric digestion. *Food Chemistry*, 182, 224-235.

Brockman, H. (2002). Colipase-induced reorganization of interfaces as a regulator of lipolysis. *Colloids and Surfaces B: Biointerfaces*, 26(1), 102-111.

Brooks, J., & Morr, C. (1985). Current aspects of soy protein fractionation and nomenclature. *Journal of the American Oil Chemists' Society*, 62(9), 1347-1354.

Carbonaro, M., Maselli, P., & Nucara, A. (2012). Relationship between digestibility and secondary structure of raw and thermally treated legume proteins: a Fourier transform infrared (FT-IR) spectroscopic study. *Amino Acids*, 43(2), 911-921.

Carbonaro, M., Maselli, P., & Nucara, A. (2015). Structural aspects of legume proteins and nutraceutical properties. *Food Research International*, 76, 19-30.

Dallas, D. C., Guerrero, A., Khaldi, N., Borghese, R., Bhandari, A., Underwood, M. A., . . . Barile, D. (2014). A peptidomic analysis of human milk digestion in the infant stomach reveals protein-specific degradation patterns. *The Journal of Nutrition*, 144(6), 815-820.

Deglaire, A., Oliveira, S. C., Jardin, J., Briard-Bion, V., Emily, M., Ménard, O., . . . Dupont, D. (2016). Impact of human milk pasteurization on the kinetics of peptide release during in vitro dynamic term newborn digestion. *Electrophoresis*, 37(13), 1839-1850.

Dutra Rosolen, M., Gennari, A., Volpato, G., & Volken de Souza, C. F. (2015). Lactose hydrolysis in milk and dairy whey using microbial β-galactosidases. *Enzyme Research*, 2015.

Floris, R., Lambers, T., Alting, A., & Kiers, J. (2010). Trends in infant formulas: a dairy perspective. In M. W. Griffiths (Ed.), Improving the safety and quality of milk, Volume 2: Improving quality in milk products (pp. 454-474).

Freed, L. M., Berkow, S., Hamosh, P., York, C., Mehta, N., & Hamosh, M. (1989). Lipases in human milk: effect of gestational age and length of lactation on enzyme activity. *Journal of the American College of Nutrition*, 8(2), 143-150.

Gargouri, Y., Pieroni, G., Rivière, C., Lowe, P. A., Saunière, J.-F., Sarda, L., & Verger, R. (1986). Importance of human gastric lipase for intestinal lipolysis: an in vitro study. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 879(3), 419-423.

Goedhart, A. C., & Bindels, J. G. (1994). The composition of human milk as a model for the design of infant formulas: recent findings and possible applications. *Nutrition Research Reviews*, 7, 1-24.

Greenberg, R., Groves, M. L., & Dower, H. J. (1984). Human beta-casein. Amino acid sequence and identification of phosphorylation sites. *Journal of Biological Chemistry*, 259(8), 5132-5138.

Henderson, T. R., Fay, T. N., & Hamosh, M. (1998). Effect of pasteurization on long chain polyunsaturated fatty acid levels and enzyme activities of human milk. *The Journal of Pediatrics*, 132(5), 876-878.

Hernell, O. (2011). Human milk vs. cow's milk and the evolution of infant formulas. Paper presented at the Milk and Milk Products in Human Nutrition, Copenhagen.

Holt, C., Carver, J. A., Ecroyd, H., & Thorn, D. C. (2013). Invited review: Caseins and the casein micelle: Their biological functions, structures, and behavior in foods. *Journal of Dairy Science*, 96(10), 6127-6146.

Jensen, R. G. (1995). Handbook of milk composition: Access Online via Elsevier.

Lacroix, M., Bos, C., Léonil, J., Airinei, G., Luengo, C., Daré, S., . . . Tomé, D. (2006). Compared with casein or total milk protein, digestion of milk soluble proteins is too rapid to sustain the anabolic postprandial amino acid requirement. *The American Journal of Clinical Nutrition*, 84(5), 1070-1079.

Lemarie, F., Cavalier, J.-F., Garcia, C., Boissel, F., Point, V., Catheline, D., . . . Rioux, V. (2016). Effect of preduodenal lipase inhibition in suckling rats on dietary octanoic acid (C8: 0) gastric absorption and plasma octanoylated ghrelin concentration. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1861(9), 1111-1120.

Lönnerdal, B. (2013). Bioactive proteins in breast milk. *Journal of Paediatrics and Child Health*, 49, 1-7.

Lopez, C., Madec, M.-N., & Jimenez-Flores, R. (2010). Lipid rafts in the bovine milk fat globule membrane revealed by the lateral segregation of phospholipids and heterogeneous distribution of glycoproteins. *Food Chemistry*, 120(1), 22-33.

Łoś-Rycharska, E., Kieraszewicz, Z., & Czerwionka-Szaflarska, M. (2016). Medium chain triglycerides (MCT) formulas in paediatric and allergological practice. *Gastroenterology Review*, 11(4).

Martin, C. R., Ling, P.-R., & Blackburn, G. L. (2016). Review of infant feeding: key features of breast milk and infant formula. *Nutrients*, 8(5), 279.

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

Nguyen, T. T. P., Bhandari, B., Cichero, J., & Prakash, S. (2015a). A comprehensive review on in vitro digestion of infant formula. *Food Research International*, 76, 373-386.

Nguyen, T. T. P., Bhandari, B., Cichero, J., & Prakash, S. (2015b). Gastro intestinal digestion of dairy and soy proteins in infant formulas: An in vitro study. *Food Research International*, 76, 348-358.

Nguyen, T. T. P., Bhandari, B., Cichero, J., & Prakash, S. (2016). In vitro digestion of infant formulations with hydrolysed and non-hydrolysed proteins from dairy and soybean. *Food & Function*, 7(12), 4908-4919.

O'Callaghan, D. M., O'Mahony, J. A., Ramanujam, K. S., & Burgher, A. M. (2011). Dehydrated Dairy Products | Infant Formulae. In W. F. Editor-in-Chief: John (Ed.), Encyclopedia of Dairy Sciences (Second Edition) (pp. 135-145). San Diego: Academic Press.

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

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

Orsavova, J., Misurcova, L., Ambrozova, J. V., Vicha, R., & Mlcek, J. (2015). Fatty acids composition of vegetable oils and its contribution to dietary energy intake and dependence of cardiovascular mortality on dietary intake of fatty acids. *International Journal of Molecular Sciences*, 16(6), 12871-12890.

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

Prakash, S., Ma, Q., & Bhandari, B. (2014). Rheological behaviour of selected commercially available baby formulas in simulated human digestive system. *Food Research International*, 64, 889-895.

Singh, H. (2011). Aspects of milk-protein-stabilised emulsions. *Food Hydrocolloids*, 25(8), 1938-1944.

Thanh, V. H., & Shibasaki, K. (1977). Beta-conglycinin from soybean proteins. Isolation and immunological and physicochemical properties of the monomeric forms. *Biochimica et Biophysica Acta (BBA)-Protein Structure*, 490(2), 370-384.

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

CHAPTER 9 GENERAL CONCLUSIONS AND RECOMMENDATIONS

9.1. General conclusions

The general objectives of this project were to understand the *in vitro* digestibility of ingredients such as proteins, lipids, and carbohydrates that were used in infant milk formulae in comparison with mothers' milk; the micro-structural characteristic of infant milk formulae as well as mothers' milk during the simulated infant gastrointestinal digestion. The conditions of the *in vitro* digestion for infants, composition and preparation procedure of infant milk formulae were studied and presented in **Chapter 2**, which was published as a review paper.

Chapter 3 developed and validated a simple *in vitro* static model for the infant gastrointestinal digestion, which comprises two water-jacketed reaction vessels connecting with a water bath, overhead stirrers, and online pH meters. The model digestive unit can maintain a constant temperature of 37°C, continuously mix the *in vitro* digesta, and measure the pH change during the digestion process. Digested samples were easy to access to analyse the key digestion parameters such as particle size, protein digestibility, lipid digestibility, and carbohydrate digestibility, structural and rheological changes. The minimum and maximum capacity that the reaction vessel can accommodate in a digestive experiment was in the range 40-150 mL. This made the *in vitro* digestion unit relatively economical as small amounts of enzymes and chemicals are required. Also, with the the advantages of easy control and operation, this bench-top model was an ideal tool for routine *in vitro* digestion studies which was employed to study and compare the digestibility of ingredients in infant milk formulae and mothers' milk.

Chapter 4 compared the *in vitro* digestibility of dairy and soy proteins in infant milk formulae in the absence of lipases. Soy proteins had lower digestibility than dairy proteins (caseins, β–lactoglobulin) which are partially hydrolysed in the stomach, but completely digested in the small intestine. It has been reported previously that the hydrophobic β-sheet structures of soy proteins encourages protein aggregation, which significantly affects the digestibility of soy proteins.

Chapter 5 investigated the digestibility of hydrolysed proteins used in infant milk formulae. Hydrolysed proteins help infants who are allergic to intact proteins (formulae contain extensively

hydrolysed proteins) or mild digestive issues (formulae contain partially hydrolysed proteins). Infant milk formulae based on hydrolysed dairy and hydrolysed soy proteins showed a significantly higher availability of amino acids and small peptides and less intact proteins than those in non-hydrolysed proteins that suggests an improvement in digestibility of formulae with hydrolysed dairy and soy proteins. In the absence of lipases, the particle size of fat globules during the gastrointestinal digestion remained unchanged due to the stabilisation of fat membrane.

Chapter 6 investigated how fat emulsion being digested by lipases under the influence of proteases; an *in vitro* infant formula lipolysis was conducted. It was clear that fat droplet size increased in the gastric phase, but then decreases during intestinal phase in both digestion matrices, lipases only and lipases in the presence of proteases. Higher level of total FFA was obtained in the matrices without proteases or in milk formulae based on hydrolysed proteins. The difference in the *in vitro* lipolysis between hydrolysed and non-hydrolysed infant milk formulae would be a good recommendation for manufacturers to consider the lipid content in infant formula based on hydrolysed proteins, due to the released fatty acids profile has an important role to play in the adequate growth and development of infants.

Chapter 7 studied the digestibility of the carbohydrate sources (lactose, glucose syrup) and thickening agents (pregelatinized corn starch, locust bean gum) added in infant milk formulae. No carbohydrate was digested in stomach phase because of the unavailability of carbohydrase enzymes; but in the intestinal digestion, lactose gave a much higher amount of released glucose than glucose syrup. Precooked corn-starch and locust bean gum were able to provide a high viscosity for infant milk formulae, reconfirming they can make infant milk become thicker. This finding suggests that starch can be a good thickener option for infants with aspiration or reflux issues in term of digestibility. Locust bean gum thickeners can give a higher viscosity to infant milk formula than precooked starch. However, it is critical to note that the higher viscosity can affect the digestibility of all the ingredients in mothers' milk and infant formulae when thickeners are added, due to the limited interaction between the digestive enzymes and their substances.

Chapter 8 compared the *in vitro* digestibility of main components (proteins, lipids, and lactose) of infant milk formulae with human mothers' milk. The *in vitro* gastrointestinal digestion for infants was studied in the presence of all the digestive enzymes: proteases, lipase, lactase, and amyloglucosidase. The obtained results showed that mothers' milk has a different protein

composition as compared to infant milk formulae based on dairy proteins. Caseins in mothers' milk contains higher concentration of β-casein than α-casein; whey proteins of mothers' milk contain lactoferrin and serum albumin that is normally unavailable in infant milk formulae (this could be a reason explain why some commercial infant formulae products have recently added lactoferrin). During the *in vitro* gastrointestinal digestion, caseins in mothers' milk and infant milk formulae are quickly and extensively digested than whey proteins due to the difference in the structure and composition between caseins and whey proteins. The *in vitro* lipolysis of mothers' milk released free fatty acids profile containing medium chain fatty acids that are not present in dairy and soy protein infant milk formulae. A higher level of released free fatty acids was observed in mothers' milk even before the start of the gastric digestion suggesting that the lipolysis is facilitated by endogenous lipases during storage process.

9.2. Recommendations for future research

- To design an infant formula that is as close as human mothers' milk is a goal since infant formula was invented. From the protein profile of mother's milk, it is clear that mothers' milk has considerable concentrations of lactoferrin, serum albumin that are not available in infant milk formulae based on bovine milk. It is necessary to mimic an infant formulae that has a composition similar to mothers' milk. Hence, future research needs to investigate ways to supplement lactoferrin, serum albumin in infant formulae and an *in vitro* digestion need to be carried out to ensure the outcome of lactoferrin, serum albumin during the infant formula digestion is similar to the outcome of mothers' milk.

- Another remarkable observation is the difference in the ratio of β-casein and α-casein in mothers' milk and infant milk formulae. Further studies are required to understand the contribution of each type of caseins in the amino acid profile in the digesta, following which suggestions could be made whether it is fundamental to adjust the β-casein and α-casein ratio in infant formula.

- Although differences in particle size was undertaken in this study, understanding the rheological characteristics of mothers' milk and infant formulas during *in vitro* gastrointestinal digestion is also needed to ensure that infant formulae has similar behaviour

at particular phase of the digestion; especially the specialized infant formulae for babies with some medical conditions such as dysphagia, gastroesophageal reflux.

- In this project, fungal lipase obtained from *Rhizopus oryzae* was used for the *in vitro* gastrointestinal digestion studies. This fungal lipase has the optimum temperature and the stable pH range that are suitable for the infant gastric conditions, but it is highly specific to the sn-1 and sn-3 positions of triglycerides. Meanwhile, human gastric lipase only cleaves at sn-3. It is better to use a mammal gastric lipase to mimic the infant gastric lipolysis in future research.

- Fresh mothers' milk should be employed and used for experiments in the same day of collection to avoid the lipolysis, which facilitated by endogenous lipases. These lipases are initially present in mothers' milk.