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A comprehensive review on *in vitro* digestion of infant formula

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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, formula-fed infants 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, and McClements, 2011).

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). 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. 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. Figure 1 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 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).

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.

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 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 requires to be done.

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 concentration 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.

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.

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 mother 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).

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 duodenal digestion of lipids. Pancreatic lipase-related to protein 1 (PLRP 1) was detected in 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. 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, xanthan 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 mother milk. In mother 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.

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.

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).

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.

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 (Heine, Radke, Wutzke, Peters, & Kundt, 1996; Lien, 2003, Davis, Harris, Lien, Pramuk, and Trabulsi 2008). 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 (O'Callaghan et al., 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, 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 (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.

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) 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).

Soy protein isolate

Soybased 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 legume 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).

3.2. Lipids

Mothers' milk contains 3.0-4.5% fat that constitutes the principle 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). 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 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).

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).

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.

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 (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 the good effect on the infant gastrointestinal tract, oligosaccharides were expected to supplement 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).

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 preventing 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).

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.

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).

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).

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).

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 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.

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 advise 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 (Figure 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* duodenal 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.

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).

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 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:

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.

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; Vors et al., 2012; Capolino et al., 2011) 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 duodenal 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.

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 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.

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 are gastric pH, the concentration range of digestive enzymes, and bile salts have been elucidated. 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.

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Table 1 Gastrointestinal enzymes in infants and their activity compared to adults

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		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

Adapted from Lebenthal et al.(1983), Hamosh (1996), and Lindquist & Hernell (2010)

Table 2 Gastric pH of 39 infants (one hour after feeding)

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

Adapted from (Miller, 1942)

Table 3 Function of the principle nutrients of human milk in infants

Nutrients	Concentration (g/L)		Function
	Human	**Cow**	
Protein			
Total whey protein	67.3	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

Adapted from Shah (2000), Haug, Hostmark & Harstad, (2007), Lönnerdal and Darragh (2011), Landers and Hartmann (2013).

Table 4 ESPGHAN recommendation about components in infant formula

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			

Adapted from Koletzko et al. (2005)

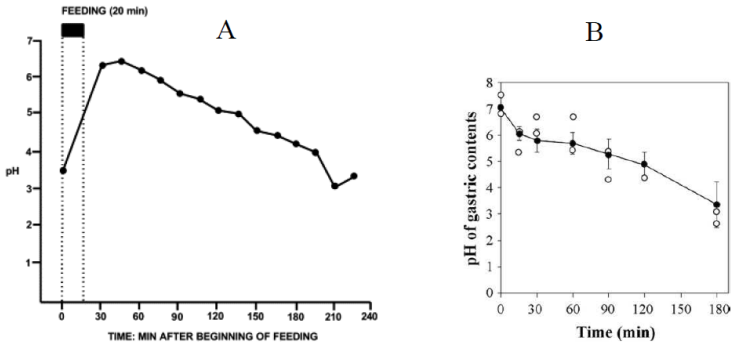

Figure 1 Gastric pH during feeding-mean values of pH of the stomach contents A (as presented in Chatterton et al., 2004) and B (Roman et al., 2007).

Highlights

- Digestion of macronutrients in infants occurs in gastric and intestinal phases.
- *In vitro* models available for infant gastrointestinal digestibility study.
- Main difference between human and infant GI system is availability of some digestive enzymes, their concentration, and less acidic gastric pH.
- Infant formula and breast milk differ in their composition.