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Gastro intestinal digestion of dairy and soy proteins in infant formulas: An *in vitro* study

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Abstract

An *in vitro* digestion simulating infant gastrointestinal tract studied the digestion of caseins, whey and soy proteins, commonly used in infant formulations, in the presence of proteases only (without lipolytic enzymes). 60 minutes of gastric phase and 120 minutes of intestinal phase coupled with gel electrophoresis, confocal microscopy, mastersizer and pH was employed to monitor the degradation of proteins, microstructure, particle size distribution and pH drop of the digesta through the *in vitro* digestion process. Obtained results showed around 20% of caseins and almost no components of whey were hydrolysed after 60 minutes in the simulated stomach. In the simulated duodenal phase, 8% of α–lactalbumin was hydrolysed while caseins and β–lactoglobulin completely digested immediately and 30 minutes respectively after addition of duodenal 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 duodenal 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 duodenal digestion in the given conditions.

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Highlights

- α–lactalbumin and β–lactoglobulin resist *in vitro* infant gastric proteolysis
- β–lactoglobulin and caseins completely hydrolyse in the *in vitro* infant duodenal phase
- Degradation of proteins is highest for formulations with the highest casein fraction
- Glycinin and β-conglycinin partially hydrolyse in the infant *in vitro* digestion
- Pepsinolysis decreases the particle size distribution of the protein aggregates

Keywords

Caseins; whey protein; soy protein isolate; proteolysis; confocal microscopy; particle size

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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 formula supplies 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,

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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

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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 duodenal 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 duodenal 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

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(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 duodenal 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 focusing 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 formulation. With all the above background information the objectives of the current study were designed:

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a) To understand and compare the digestibility of dairy and soy proteins in infant formulations in the absence of lipolytic enzymes.

b) To understand the microstructural changes of infant formulations with an *in vitro* digestive model

2. Materials and method

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. The flow diagram of the bench-top *in vitro* digestion unit is as shown in Fig.1. This model was comprised 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. 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 and 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 and uniform mixing of all the ingredients in the reaction vessel as reported by Pérez et al. (2014) and Oomen et al., (2002) in their studies.

2.2. Enzymes and chemicals

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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^{0}C.

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

2.3 Dairy and soybean proteins

Whey protein isolate (WPI 85.15% protein, 1.0% fat, 1.2% carbohydrate) and calcium caseinate (86.7% protein, 1.01% fat, 0.15% carbohydrate) were purchased from Total Foodtec (Australia). Soy protein isolate (SPI 83.05% protein, 0.5% fat, 3.0% carbohydrate) was purchased from Food Manufacturers Pty (Australia). Sunflower vegetable oil was obtained from a local supermarket.

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2.4 Preparation of infant formulations

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 formulation 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 formulation 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 formulations in our study. The measured quantity of WPI and calcium caseinate 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 formulation, the same protein content of 1.5 g soy protein isolate/100 mL.

The step-by-step preparation of infant formulation is as shown in Fig.2. The mixtures of WPI and calcium caseinate 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 formulation was kept at 4^0C for a maximum two days with the addition of sodium azide

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(0.02% w/v) (Gallier, Ye, and Singh, 2012).

2.5 In vitro infant protein digestion

The bench-top *in vitro* digestive unit as shown in Figure 1 was used to carry out the *in vitro* digestion. The two-step digestion procedure of gastric and duodenal phase was performed in the water-jacketed reactors at 37^0C 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 Figure 3.

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 and its pH adjusted to 4.0 by adding 0.1M HCl. The liquid infant formulation 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^oC (about 15 min), following which the enzyme pepsin was added to give 22.75 U/mg of total protein, 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).

2.5.2 Duodenal digestion

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The duodenal 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 were added to the digesta to give the final concentration of 2 mM. 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 duodenal juice (0.15M NaCl, pH 8.0±0.03) at the temperature of digestion (37^{0}C), and the duodenal 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 duodenal 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).

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 duodenal phase, the pH decreased rapidly below the adjusted value due to the breakdown of protein to amino acids and peptides was measured every

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minute by pH meter for a duration of two hours. Each infant formulation trial was duplicated and three repeated measurements were collected from one formulation. The values used for analysis were taken from an average of three repeated measurements from duplication.

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

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

2.2.6 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 duodenal 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 (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. Gels scanning was done by densitometry and analysed by Quantity One software.

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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 duodenal 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)

2.2.7 Particle size distribution

Particle size distribution of native and digested milk samples were measured before and during *in vitro* gastric and duodenal digestions by Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershine, UK). The refractive index of milk value of 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.

2.2.8 Confocal Laser Scanning Microscopy (CLSM)

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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 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.

2.2.9 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 $P<0.05$.

3. Results and discussion

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3.1. Protein digestion determined by SDS-PAGE

3.1.1. Dairy protein (whey protein and caseins)

Figure 4 (a-c) presents the PAGE patterns of the three different dairy formulations (whey protein and calcium caseinate 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, S60). Similar observations were reported by Sakai et al. (2000). In the duodenal phase, the enzymes trypsin and chymotrypsin completely digested α–casein and β–casein. The bands markedly became faint at point D0 and completely disappeared soon after, between D30- D120, Fig 4 (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, S60). However, in the duodenal 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 formulations [Fig.4 (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).

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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 duodenal 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

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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 gastrointestinal digestion during *in vivo* 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.

3.1.2 Soy protein

The sequential PAGE patterns of soy based infant formulation 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 (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(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 duodenal phase, these proteins indicated by lighter bands from D30 to D120. As hydrolysis progressed, a large amount of small peptides were formed at approximately 20 kDa.

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3.2. Digestibility assay - pH drop method

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

The rate of digestibility is characterized by the extent of the pH drop at 2 hours after enzyme addition in the duodenal phase. Figure 5 demonstrates the difference in digestion of the three dairy infant formulations and the soy protein formulation. Formulations with a whey to caseins ratio of 2:8 shows a maximum pH drop, while soy formulation created the least drop. The pH drop method suggests rapid digestion of the formulation with a higher proportion of caseins which is in agreement with the digestibility rate calculated using equation 1 (Table 1) and the PAGE patterns (Fig. 4c). PAGE patterns for formulations with whey to casein ratios of 6:4 (Fig 4a) and 4:6 (Fig 4b) show faint bands at the start of the duodenal phase while this is not observed in formulations 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

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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. 5 and Table 1, it is clear that soy-based formulation 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

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compare the amount of amino acids and peptides obtained in the gastric and duodenal digestion phases.

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 formulations in their native state and during gastric and duodenal digestion were studied [Fig.6 (a-d)]. The figures clearly suggest a bimodal distribution for all the four formulations 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 2, which shows an increase in particle size immediately after addition of simulated gastric fluid to the four native formulations. With formulation WPI:caseins 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 formulations (Table 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

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possible for it to contribute towards the particle size of the digesta and the large particle size is due to aggregation of proteins.

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 formulations (Fig.7). The higher the amount of caseins (formulation 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 formulation was the smallest. The changes in the particle size distribution of soy protein formulation during the gastric and intestinal digestion was very similar to dairy formulations as observed in Figure 6.

In the duodenal 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 Figure 4.

3.4. Microstructural changes

The gastric and duodenal digestion of the four infant formulations were followed with CLSM (Figs. 8-10) 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 8, S0) the dairy protein (caseins and whey proteins) and soy proteins are in large aggregates as confirmed by Figure 6 and 7. After one hour of proteolysis in the stomach (S60), the large aggregates of milk protein (Figures 8 A-C) and soy protein (Fig.8D) become smaller as compared to that in S0 (Fig.8A-D). However, the confocal micrographs of fat suggested no change in the size of fat globules

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during the one hour gastric digestion and two hour of intestinal digestion. This is due to the absence of gastric and pancreatic lipases (Fig.9 and 10A-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 duodenal 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 duodenal 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 duodenal phase, at pH 6.5, all the protein agglomerates in the digesta dissolved. Hence, confocal images could not be obtained for the small particles.

4. Conclusions

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

Soy-based infant formulations showed the least *in vitro* protein hydrolysis compared to dairy formulations. 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 igestibility 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.

5. Acknowledgments

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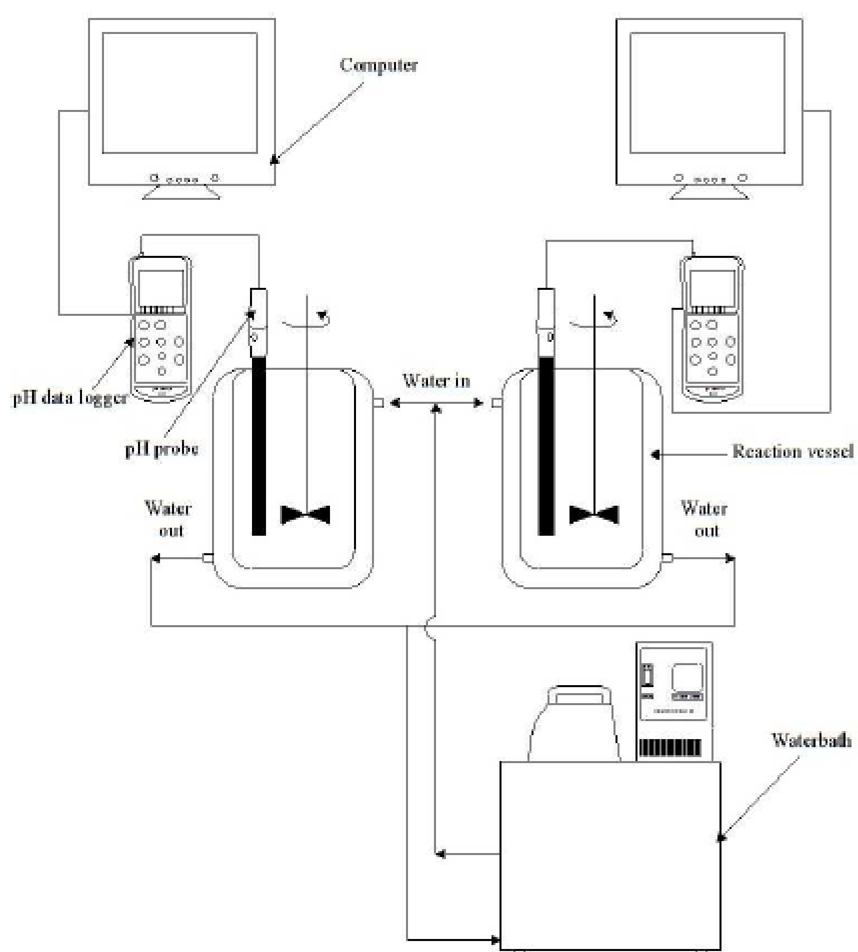

Figure 1

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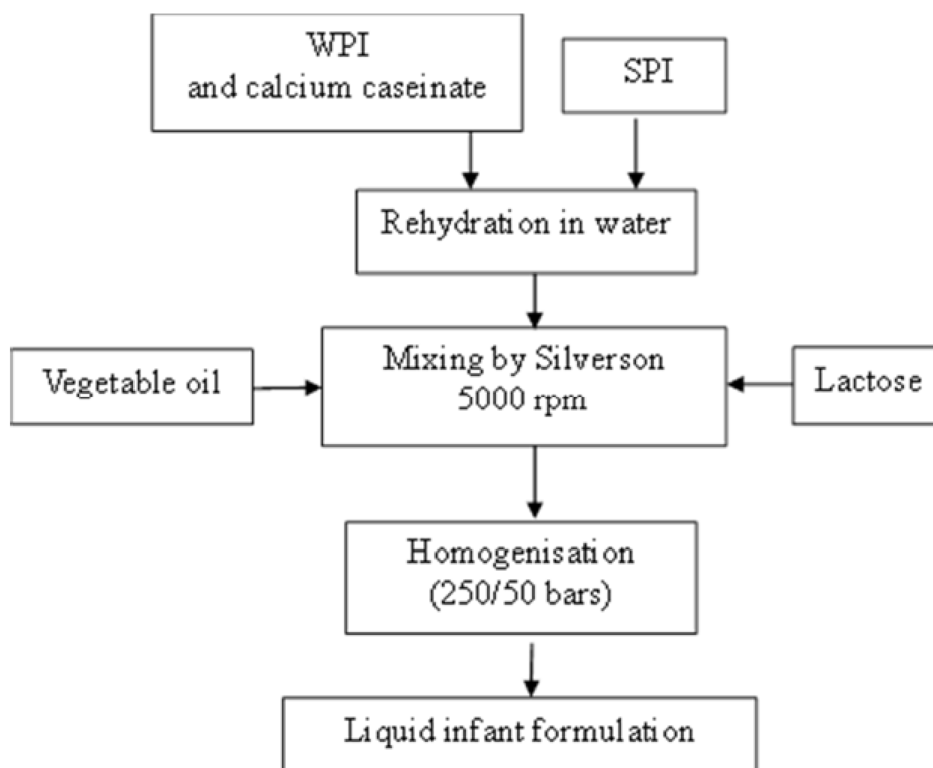

Figure 2

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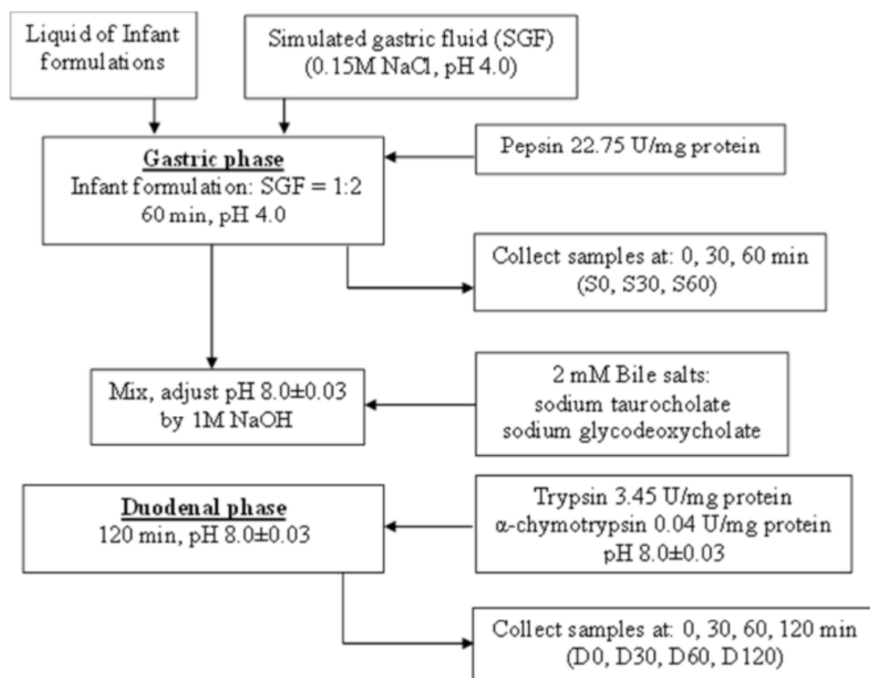

Figure 3

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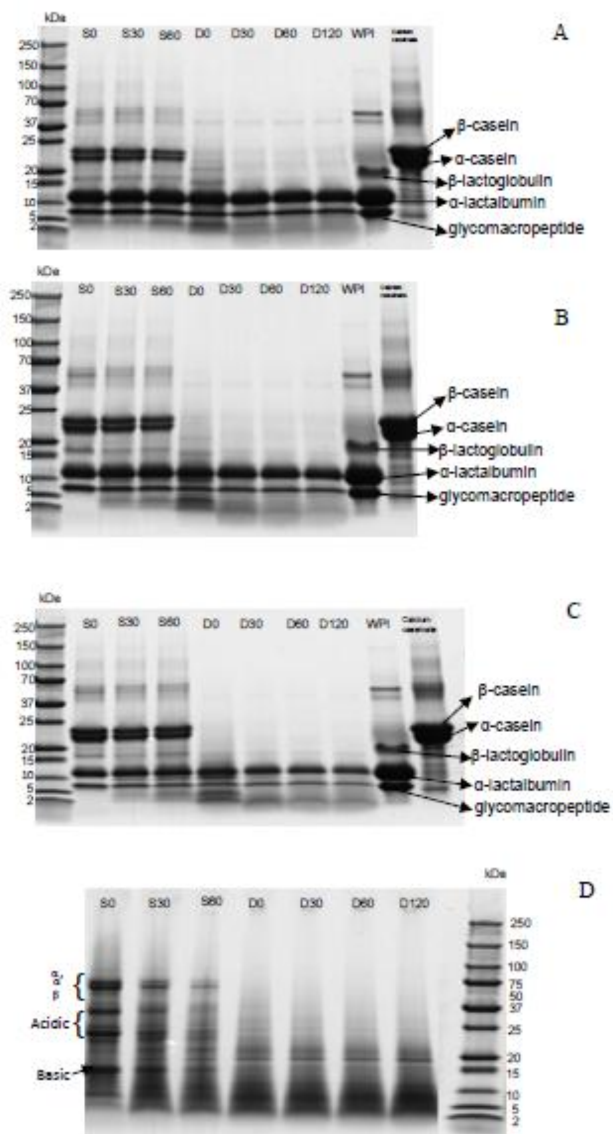

Figure 4

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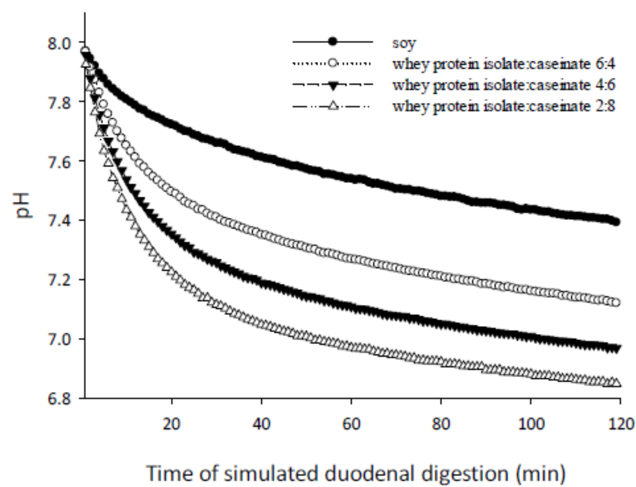

Figure 5

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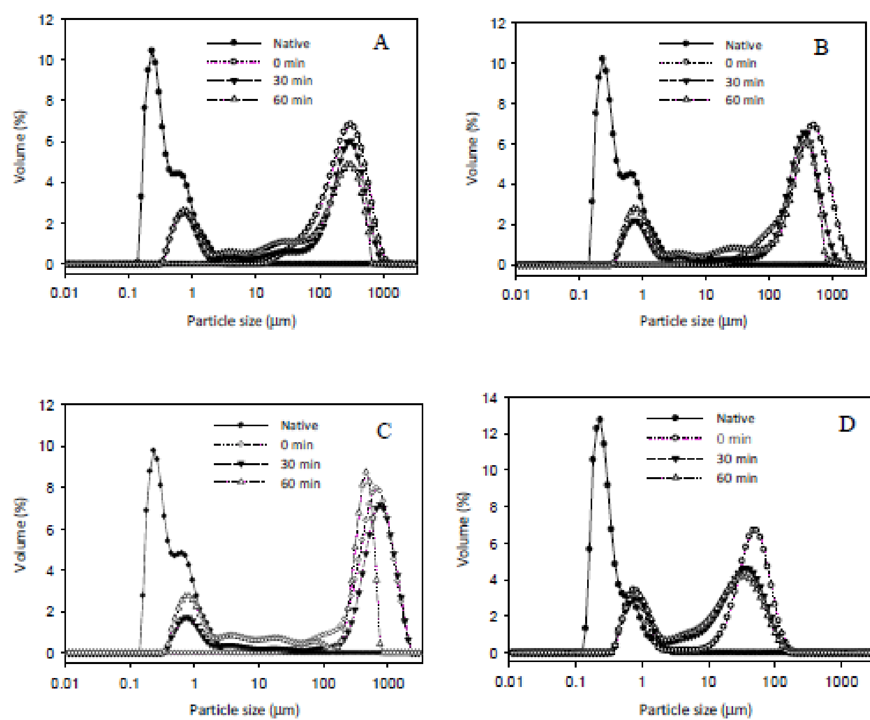

Figure 6

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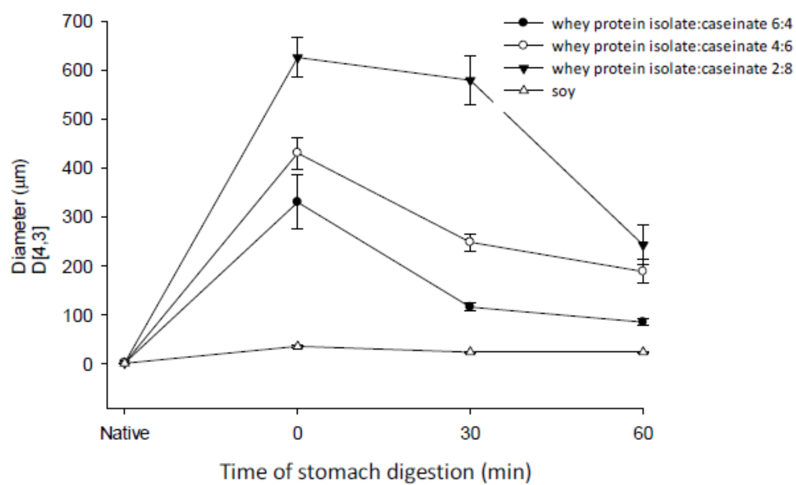

Figure 7

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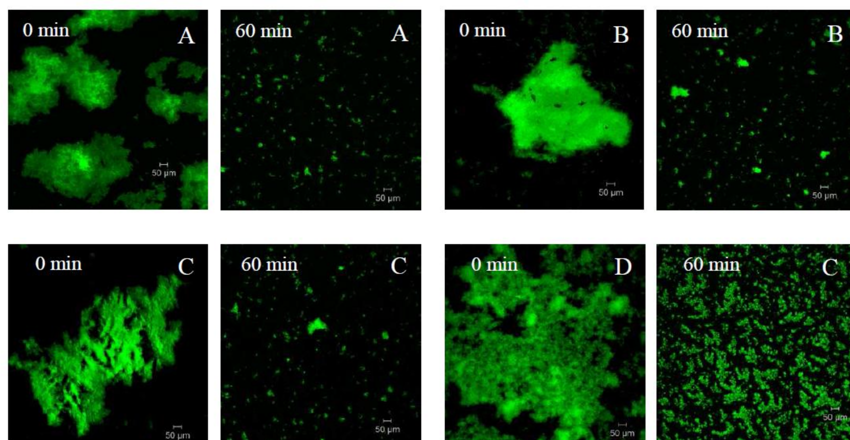

Figure 8

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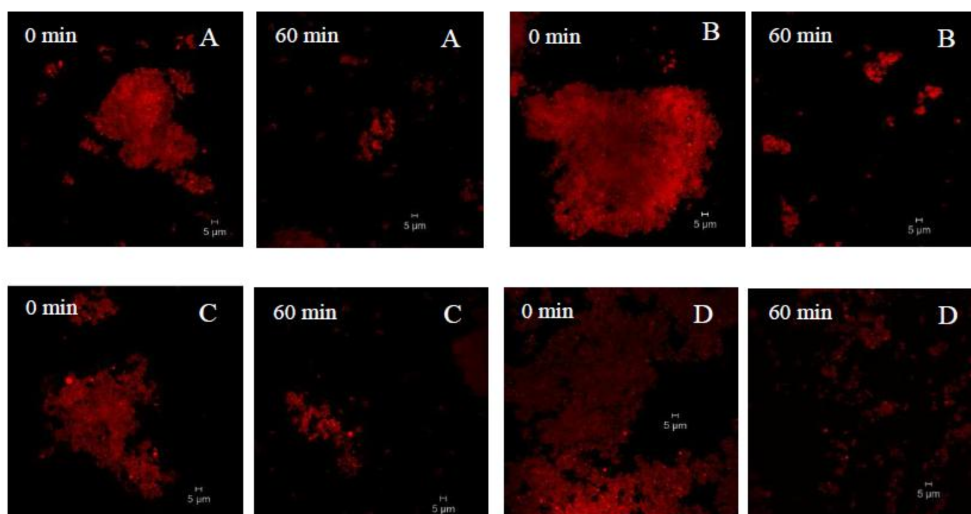

Figure 9

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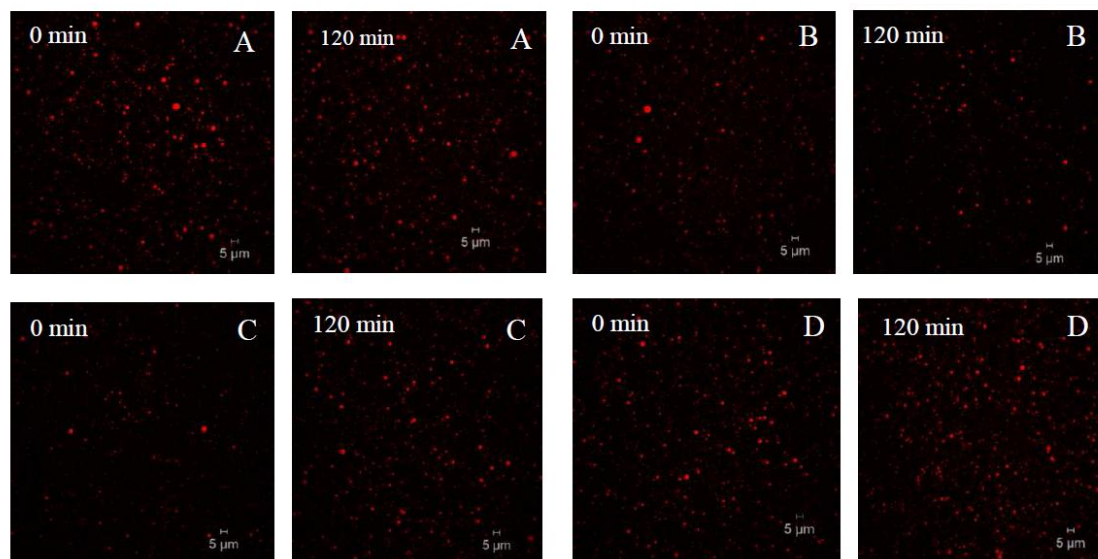

Figure 10

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Figure captions:

Figure 1. Flow diagram of the bench-top *in vitro* digestion unit

Figure 2. Schematic diagram of making infant formulations

Figure 3. Flow diagram of *in vitro* protein digestion in infants

Figure 4. Reducing SDS-PAGE analysis of *in vitro* digested samples of the four infant formulations: whey protein isolate:caseinate 6:4 (A), whey protein isolate:caseinate 4:6 (B), whey protein isolate:caseinate 2:8 (C), and soy (D) during gastric phase from 0 min (S0) to 60 min (S60) and duodenal phase from 0min (D0) to 120 min (D120).

Figure 5. Reduction in pH during *in vitro* duodenal digestion of the four infant formulations: whey protein isolate:caseinate 6:4, whey protein isolate:caseinate 4:6, whey protein isolate:caseinate 2:8, and soy.

Figure 6. Size distribution of native and digested samples under *in vitro* gastric digestion of the four infant formulations: whey protein isolate:caseinate 6:4 (A), whey protein isolate:caseinate 4:6 (B), whey protein isolate:caseinate 2:8 (C), and soy (D).

Figure 7. Volume mean D[4,3] diameter of native and gastric digested samples under *in vitro* gastric digestion of the four infant formulations: whey protein isolate:caseinate 6:4, whey protein isolate:caseinate 4:6, whey protein isolate:caseinate 2:8, and soy.

Figure 8. CLSM of protein agglomerates in gastric digested samples at 0 min and 60 min of the four infant formulations: whey protein isolate:caseinate 6:4 (A), whey protein isolate:caseinate 4:6 (B), whey protein isolate:caseinate 2:8 (C), and soy (D).

Figure 9. CLSM of fat globules in gastric digested samples at 0 min and 60 min of the four infant formulations: whey protein isolate:caseinate 6:4 (A), whey protein isolate:caseinate 4:6 (B) , whey protein isolate:caseinate 2:8 (C), and soy (D).

Figure 10. CLSM of fat globules in duodenal digested samples at 0 min and 120 min of the four infant formulations: whey protein isolate:calcium caseinate 6:4 (A), whey protein isolate:calcium caseinate 4:6 (B), whey protein isolate:calcium caseinate 2:8 (C), and soy (D).

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Table 1. *In vitro* digestibility of the four infant formulations: whey protein isolate:caseinate 6:4, whey protein isolate:caseinate 4:6, whey protein isolate:caseinate 2:8, and soy.

Sample	***In vitro* digestibility**
Soy	76.384±0.039[d]
Whey protein isolate:casein 6:4	81.542±0.039[c]
Whey protein isolate:casein 4:6	84.258±0.173[b]
Whey protein isolate:casein 2:8	86.358±0.105[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.

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Table 2. Particle size distribution of native and gastric digested samples of the four formulations: whey protein isolate:caseinate 6:4, whey protein isolate:caseinate 4:6, whey protein isolate:caseinate 2:8, and soy.

Formulations	**Samples**	**d(0.1) µm**	**d(0.5) µm**	**d(0.9) µm**
	Native	0.18±0.01	0.31±0.01	0.92±0.02
Whey protein isolate:casein 6:4	S0	70.96±15.56	237.31±49.24	521.57±91.79
	S30	0.67±0.03	101.52±9.72	265.76±16.70
	S60	0.65±0.02	42.19±4.47	234.23±20.73
	Native	0.18±0.01	0.30±0.01	0.90±0.01
Whey protein isolate:casein 4:6	S0	138.60±7.57	341.01±16.22	660.12±37.91
	S30	0.76±0.01	243.78±16.35	570.94±41.64
	S60	0.68±0.02	187.30±26.63	491.54±73.88
	Native	0.18±0.01	0.33±0.01	0.96±0.01
Whey protein isolate:casein 2:8	S0	179.33±25.10	566.23±50.30	1168.56±82.40
	S30	0.93±0.10	552.28±55.70	1188.00±79.70
	S60	0.70±0.01	272.18±72.65	511.30±50.06
	Native	0.17±0.01	0.26±0.01	0.71±0.01
Soy	S0	0.67±0.01	31.68±1.25	74.80±4.96
	S30	0.68±0.01	18.49±2.92	61.30±5.71
	S60	0.67±0.01	14.60±1.24	55.97±2.46

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Highlights

- α–lactalbumin and β–lactoglobulin resist *in vitro* infant gastric proteolysis
- β–lactoglobulin and caseins completely hydrolyse in the *in vitro* infant duodenal phase
- Degradation of proteins is highest for formulations with the highest casein fraction
- Glycinin and β-conglycinin partially hydrolyse in the infant *in vitro* digestion
- Pepsinolysis decreases the particle size distribution of the protein aggregates

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