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Development of a reliable pH-STAT *in-vitro* model for gastrointestinal digestion of lipids and application for infant formula

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Abstract

Present study aims to establish a reliable infant digestion model using pH-STAT method and the model is also applied to static *in-vitro* digestion of different infant formulas. Model-3 which consisted of NaCl, KCl and $CaCl_2$ as gastrointestinal digestive fluids demonstrated better repeatability than model-1 was selected for the evaluation of static *in-vitro* digestion of different infant formulas (different fatty acids, protein composition and with/without milk fat globule membrane) using pH-STAT method. Three infant formulas (IF) with different fatty acids composition were evaluated and IF2 which contained the highest amounts of medium-chain saturated fatty acids (8.02 ± 0.47%) demonstrated the highest *in-vitro* gastrointestinal lipolysis rate (65.88 ± 0.24%). Infant formulas containing peptides and amino acids were more easily hydrolyzed by protease, which probably enhanced the release of FFA (IF1, 50.60 ± 0.87%; IF4, 60.17 ± 2.97%; IF5, 62.21 ± 2.27%; IF6, 66.51 ± 2.38%). In addition, the presence of milk fat globule membrane (MFGM, IF7) was found to accelerate *in-vitro* gastrointestinal lipolysis (IF1, 50.60 ± 0.87%; IF7, 58.05 ± 1.77%). In summary, the new developed model is suitable for investigating *in-vitro* digestion of different infant formulas.

Keywords: pH-STAT; simulated digestive fluids; *in-vitro* gastrointestinal lipolysis; fatty acid composition; protein composition; MFGM.

Practical Application: The study establishes a reliable infant *in-vitro* model for lipid digestion. The model has been also applied and analyzed for lipid digestion of various infant formulas in this research. Several factors including particle size, TAG type and interfacial compositions possibly have a great impact on the process of lipid digestion. Findings from present study will be useful in guiding development of a consistent infant digestion model for evaluation of static *in-vitro* lipolysis rate and providing suggestions for designing infant formulas with good lipolysis rate.

1 Introduction

Human milk is very important for the growth and development of infant (Gallier et al., 2015) and provides both energy and nutrients. World Health Organization (WHO) recommends exclusive breastfeeding for the first 6 months of life, followed by continued breastfeeding with appropriate complementary foods for up to 2 years (World Health Organization, 2003). Besides, infant formula is also the alternative feeding strategy when breastfeeding is not possible, such as presence of infectious and metabolic diseases in the mother (Lawrence, 2013). Moreover, moderate process such as ohmic heating would possibly improve the quality of infant formulas (Pires et al., 2020, 2021). The bioactive compounds in infant formulas were enhanced and partial and total hydroxymethylfurfural (HMF) levels were decreased. In addition, the higher whiteness index and lower viscosity indicated better reservation of infant formulas. Although infant formula is not comparable to breast milk, it is still a better substitute to meet the body development of infant.

Lipid is one of the important macronutrients in human milk. It provides 50-60% of energy for infants. Besides that, it is also involved in cell functions and brain development of infant (Cheong et al., 2018; Yuan et al., 2020a). Lipids are mainly digested in gastrointestinal tract by gastric and pancreatic lipases. As the infant digestive systems are still immature, the activities of infant gastric and pancreatic lipases are low. The activity of pancreatic lipase in infant is only about 90 U/mL, which is far more lower than that in adult about 2000 U/mL (Ménard et al., 2018). In infant, about 10-30% of triacylglycerols (TAGs) are hydrolyzed into *sn*-1, 2-diglyceride (DAG) or sn-2,3-DAG and free fatty acid (FFA) by gastric lipase (Poquet & Wooster, 2016), which enhance the solubility and digestibility of the remaining TAG in the intestinal stage (Go, 1973). The lipolysis products mainly short and medium-chain saturated fatty acids are directly and easily absorbed in the portal vein. Meanwhile, *sn*-2-monoglyceride (MAG) and long chain fatty acid (LCFA) are esterified into TAG again forming chylomicron (Bourlieu et al., 2014).

In-vitro digestion models for simulating full-term infant digestion can be either static or dynamic. The static infant digestion model simulates mainly the infant digestive enzymes

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activity (lipases and proteases), digestive fluids and digestion times. Meanwhile, dynamic model takes gastric emptying pump, digestive fluids secretions, dynamic pH changes and intestinal peristalsis into consideration. At present, *in-vitro* static infant digestion has been conducted using different digestion models and a consensus has not yet been reached (Mat et al., 2016; Ménard et al., 2018; De Oliveira et al., 2016; Cheong et al., 2018; Sassene et al., 2016). In a study by Ménard et al. (2018), NaCl, KCl, $NaHCO_3$ and $CaCl_2$ were used as simulated digestive fluids. The ratios of volume of digestive fluids were respectively 39% of meal, 23% of gastric secretions and 38% of intestinal secretions, respectively. In another study by Sassene's et al. (2016) model, NaTC, NaCl, Tris, maleic acid and phospholipid were used as simulated digestive fluids. In this model, the ratios of volume of digestive fluids were respectively 12% of meal, 28% of gastric secretions and 60% of intestinal secretions, respectively. Different model has resulted in inconsistency in the results of *in-vitro* static infant digestion.

In-vitro static digestion conducted using pH-STAT is becoming increasingly popular due to its easy operation and rapidity. Digestion process modifies the pH of a digestive fluid; pH-STAT method maintains a constant pH of the digestive fluid through addition of a titrant. The quantity of titrant added during the experiment versus time is directly related to the production of hydrolyzed species (Calvo-Lerma et al., 2019). Digestion model particularly electrolytes used for simulating the infant digestive fluids must be carefully designed and considered as study has shown improper designed models resulted in increased of pH of the intestinal phase, which could hampered the titration process (Mat et al., 2016). Considering the convenience of pH-STAT model, it's necessary to establish a reliable infant static digestion model to analyze *in-vitro* lipid digestion of infant formulas.

Present study aims to establish a reliable infant digestion model using pH-STAT method and the model is also applied to static *in-vitro* digestion of different infant formulas. The mechanism of lipid digestion of different infant formulas was further elucidated. Findings from present study will be useful in guiding development of a consistent infant digestion model for evaluation of static *in-vitro* lipolysis rate and providing suggestions for designing infant formulas with good lipolysis rate.

2 Materials and methods

2.1 Materials

Amano lipase DF-15 was obtained from Amano Enzyme (specific lipolytic activity: 175 U/mg); Pepsin from porcine gastric mucosa (≥250U/mg, P6887), pancreatic lipase from porcine pancreas (8×USP specification, P7545) and bovine bile were purchased from sigma; sodium chloride and potassium chloride were standard analytical grade; 5×SDS-loading buffer, 4-20% precast gels and protein marker (10-170kD) were from Beyotime Biotechnology. (A) Liquid, dry-mixed milk powder and wet-mixed milk powder infant formulas are all belonged to the stage 1. The other seven types of stage 1 infant formulas were purchased from a local market (Table 1 and 2). IF1, IF2 and IF3 have different fatty acids composition (IF1 is cow-based infant formula; IF2 is goat-based infant formula; IF3 is soybean-based infant formula). IF1, IF4, IF5 and IF6 have different protein composition (IF1 is non-hydrolyzed protein infant formula; IF4 is partially-hydrolyzed protein infant formula; IF5 is exhaustively-hydrolyzed protein infant formula; IF6 is amino acid infant formula). IF7 contains the addition of milk fat globular membrane (MFGM).

Table 1. Seven commercial stage 1 infant formulas.

Infant Formulas	FAC[1]	Protein composition	MFGM protein
IF1	**Cow**	**Standard**	**None**
IF2	**Goat**	Standard	None
IF3	**Soybean**	Standard	None
IF4	Cow	**Partially**	None
IF5	Cow	**Extensively**	None
IF6	Cow	**Amino acid**	None
IF7	Cow	Standard	**MFGM**

[1]FAC: Fatty acid composition; MFGM: Milk fat globular membrane.

Table 2. Macronutrients Composition of Infant formulas (per 100 mL).

	IF1	IF2	IF3	IF4	IF5	IF6	IF7
Energy (KJ)	269	268	277	284	250	283	284
Fat (g)	3.25	3.13	3.5	3.7	3.18	3.51	3.65
Carbohydrate (g)	7.25	7.43	6.70	7.10	6.18	7.16	7.29
Protein (g)	1.47	1.50	1.7	1.54	1.68	1.88	1.43

2.2 In-vitro *lipolysis of infant formulas: effects of different electrolytes composition in simulated digestive fluids*

Different digestion models were used in static *in-vitro* digestion of infant formulas including commercial liquid, milk powder and labatory-made milk powder (Table 3 and 4). The *in-vitro* gastrointestinal lipolysis rate was evaluated by pH-STAT and cross-checked with thin layer chromatography coupled with flame ionization detector (TLC-FID). *In-vitro* digestion experiments were carried out in a jacketed vessel kept at 37 °C. Firstly, 11.25 g of milk powder was dissolved in 150 mL of lukewarm water and liquid infant formula was diluted to attain the same as fat concentrations with milk powder. The samples were pre-heated to 37 °C and pH adjusted to 5.3 using either HCl (0.1M) or NaOH (0.1M). Gastric lipolysis was initiated by adding Amano DF-15 lipase and pepsin to the mixture in a thermal vessel. It was kept at 5.3 by the automatic titration of NaOH (0.25M) using pH-STAT method. After 60 mins, gastric digestion was terminated. Subsequently, pH was adjusted to 6.6 by NaOH (1M) and subjected to intestinal digestion. The intestinal digestion was initiated with addition of pancreatic lipase and maintained for 120 mins.

In-vitro gastrointestinal lipolysis degree was calculated by the percentage of FFA released from total acyl moieties initially esterified in TAG in emulsion as follows (Bourlieu et al., 2015; Cheong et al., 2018) (Equation 1):

$$LD = \frac{FFA \times \mathrm{MMeq}}{FC \times \mathrm{V} \times 3} \tag{1}$$

LD: the lipolysis degree%; FFA: free fatty acid in mole by titration of NaOH; MMeq: average TAG molar mass of 798 g/mol, deduced

Table 3. Simulated gastrointestinal digestion fluids of three *in-vitro* digestion models.

Model-1 (Cheong et al., 2018)	Simulated gastric fluid	NaTC	NaCl	Tris	Maleic acid	phospholipid
		80 μM	68 mM	2 mM	2 mM	2 μM
	Simulated intestinal fluid	NaTC	NaCl	Tris	Maleic acid	phospholipid
		2 mM	150 mM	2 mM	2 mM	0.18 mM
Model-2 (Ménard et al., 2018)	Simulated gastric fluid	NaCl	KCl			
		94 mM	13 mM			
	Simulated intestinal fluid	NaCl	KCl	$NaHCO_3$	$CaCl_2$	
		164 mM	10 mM	85 mM	3.1 mM	
Model-3 (Mat et al., 2016; Ménard et al., 2018)	Simulated gastric fluid	NaCl	KCl			
		94 mM	13 mM			
	Simulated intestinal fluid	NaCl	KCl	NaCl	$CaCl_2$	
		164 mM	10 mM	85 mM	3.1 mM	

Table 4. The meal ratios and enzyme concentrations of different infant *in-vitro* digestion models.

Model	The Volume of Digestive fluids	Enzyme concentrations
Model-1	12% of meal, 28% of gastric secretion and 60% of intestinal secretion	Gastric lipase: 60U/mL Pepsin: 450 U/mL
		Pancreatic: 300 USP/mL
Model-2	39% of meal, 23% of gastric secretion and 38% of intestinal secretion	Gastric lipase: 19U/mL Pepsin: 268 U/mL
Model-3		Pancreatic lipase: 90/mL

from the fatty acids by GC; FC: the average concentration of fat in g/mL; V: volume of dissolved infant formulas in the pH-STAT vessel.

2.3 Lipid extraction from infant formulas and gastrointestinal digestion fluids

Lipid was extracted from infant formulas and gastrointestinal digestion fluids. According to the method provided by Barbano (Barbano et al., 1988), five milliliter of infant formula was mixed with 1 mL ammonium hydroxide at 65 °C using a water bath. After 15 mins, 5 mL of ethanol, a mixture of 12.5 mL of diethyl ether and 12.5 mL petroleum ether were added to the above mixed solution. The mixtures were then centrifuged at 5000 rpm for 5 min and the supernatant was removed. Extractions were conducted using the same solvents for two times. Supernatant were collected and evaporated dry at 40 °C. Lipid was extracted from gastrointestinal digestion fluids using chloroform/methanol (2:1) and sodium chloride (0.73%) as described by Bourleiu (Bourlieu et al., 2015; Cheong et al., 2018). Five milliliter of digestion fluid was mixed with 20 mL chloroform/methanol (2:1) and 5mL sodium chloroform (0.73%). The mixtures were centrifuged at 5000 rpm/min for 10 min, and the organic phase of the lower layer was collected. The upper was extracted using the same method again. All organic phase was collected and evaporated dry at 60 °C. The extracted lipid was stored at -20 °C until further analysis.

2.4 Lipids composition of the infant formulas and **in-vitro** *lipolysis milk*

Lipids composition (triacylglycerol, diacylglycerol, monoacylglycerol and free fatty acids) were analyzed using thin-layer chromatography coupled with a flame ionization detector (TLC-FID, IATROSCAN MK5, Iatron Laboratories, Tokyo, Japan) (Carrière et al., 2005). The detection were carried out at the following conditions: air flow rate of 200 mL/min, hydrogen flow rate of 160 mL/min, and scan speed of 30s/scan (Cheong et al., 2018). One milliliter of n-hexane was added to the extracted lipid (15-20 mg). One microliter of the lipid in hexane was spotted onto TLC bars. TLC bars were developed in the solvent tank containing n-hexane: diethyl ether: acetic acid (80: 20: 1). *In-vitro* gastrointestinal lipolysis degree during digestion was expressed as the percentage of FFA versus the total acyl chains present in residual glycerides as the following Equation 2 (De Oliveira et al., 2016):

$$LD = \frac{[FFA] \times 100}{[TAG] \times 3 + [DAG] \times 2 + [MAG] + [FFA]} \quad (2)$$

with LD lipolysis degree in%, TAG, FFA, DAG and MAG represent triglycerides, free fatty acids, diglycerides and monoglyceride molar concentration (mole/L)

Particles size and ζ-potential measurements of infant formulas and in-vitro *lipolysis milk*

Particles size and ζ-Potential were measured by Zetasizer Nano ZS90 (Malvern Instruments, Malvern, U.K.). Digestion samples in different time were collected. Samples were prepared that 15μL digestive fluids were diluted in 10mL deionized water in different digestion phases.

Protein composition of the infant formulas and in-vitro *lipolysis milk*

The electrophoretic analysis was used to analyze the protein composition using 4-20% polyacrylamide precast gels 10 wells

(Beyotime biotechnology) according to methods previously described by Ménard et al. (2018) and De Oliveira et al. (2016). All infant formulas (IF) protein samples were diluted in the 5×SDS-loading buffer before analysis and 5×tris-glycine was used as the electrophoretic buffer equipped with 130 V, running for 40-60min. Marker protein was used as the different molecular, as a reference of the position of the protein band. Each loading position was prepared about 15-20ug protein in the gel. The gel was stopped when the loading sample reached the bottom of the gel (1-2cm). Finally, gel was rinsed by the solution containing acetic acid (10%) /ethyl alcohol (5%)/deionized water (85%) after staining with Coomassie blueR-250.

Statistical analysis

Results were expressed in Means ± SD from triplicates. The significance of experimental results was analyzed by one-way of variance (ANOVA) and Duncan using Spss24.0 (p-value < 0.05).

3 Results and discussion

3.1 Repeatability improved pH-STAT model for in-vitro *gastrointestinal lipolysis of infant formulas*

In this study, *in-vitro* lipolysis digestion of liquid, dry-mixed milk powder and wet-mixed milk powder infant formulas were analyzed by different digestion models using pH-STAT. Model-1 showed relatively poor repeatability ($SD_{Model-1}$: 6.59%/ 7.44%/ 8.10%). With regard to the *in-vitro* lipolysis rate of model-2, there is an increased in pH of the intestinal phase which hampered the titration process. This is mainly due to presence of high concentrations of $NaHCO_3$ (85 mM, 7.14g/L) in the digestive fluids of model-2, which is higher than the solubility limit of CO_2 (1.6 g/L, 20 °C). Model-2 with $NaHCO_3$ had caused basification of the simulated digestion environment (increased in pH). This observation has also been reported in a work previously described by Mat et al. (2016). Thus, we have used NaCl at an equimolar to replace $NaHCO_3$ in model-3. As the standard deviation of model-3 was lower than model-1 by analysis of the aforementioned three kinds of infant formulas for the *in-vitro* lipid digestion (SD-Model-3: 1.87%/ 1.77%/ 1.24%). Besides, for the simulated gastric fluides (SGF) compositions of model-3, which are based on a study for 30 full-term infants reported by Hyde (1968); for the simulated intestinal fluides (SIF) compositions of model-3, which are based on 1-week-old full-term infants reported by Zoppi et al. (1973). Therefore, model-3 is applied to the following experiments to analyze the lipid digestion of the other different kinds of infant formulas (Figure 1).

3.2 Effects of fatty acid composition on in-vitro *gastrointestinal lipolysis of infant formulas*

Table 5 shows the fatty acid composition of infant formulas. All infant formulas have high amounts of saturated (ranging from 36.92 ± 0.35 to 41.08 ± 1.45%), monounsaturated (ranging from 36.5 ± 1.05 to 40.87 ± 0.28%) and polyunsaturated fatty acids (ranging from 20.76 ± 0.08 to 25.3 ± 0.17%). The major saturated and unsaturated fatty acids were palmitic (ranging from 17.21 ± 0.09 to 19.75 ± 0.05%), oleic (ranging from 32.33 ± 0.96 to 40.61 ± 0.28%) and linoleic (ranging from 18.38 ± 0.12 to 22.10 ± 0.13%) acids. All infant formulas have less than 10% of medium-chain saturated fatty acids (MC-SFA).

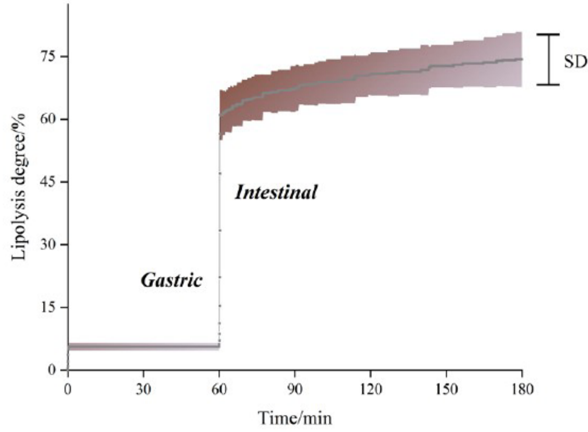
Table 5. Fatty acid compositions of the infant formulas (IF1, IF2, and IF3).

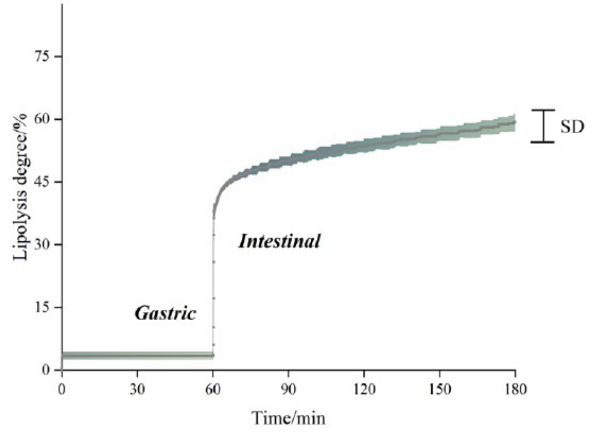
	IF1	IF2	IF3
C8:0	0.65 ± 0.18[a]	5.48 ± 0.37[b]	1.44 ± 0.16[a]
C10:0	1.20 ± 0.06[a]	2.55 ± 0.09[b]	1.21 ± 0.01[a]
C12:0	5.06 ± 0.19[a]	4.74 ± 0.13[a]	10.5 ± 0.41[b]
C14:0	4.93 ± 0.01[a]	4.39 ± 0.12[b]	4.31 ± 0.01[b]
C14:1	0.40 ± 0.02[a]	0.16 ± 0.01[b]	ND
C15:0	0.35 ± 0.01[a]	0.25 ± 0.01[b]	ND
C16:0	19.75 ± 0.05[a]	18.40 ± 0.53[b]	17.21 ± 0.09[c]
C16:1	0.46 ± 0.01[a]	0.31 ± 0.01[b]	0.12 ± 0.01[c]
C17:0	0.21 ± 0.01[a]	0.21 ± 0.01[a]	ND
C17:1	0.12 ± 0.01[a]	0.12 ± 0.01[a]	ND
C18:0	4.79 ± 0.05[a]	5.06 ± 0.16[a]	3.66 ± 0.04[b]
C18:1	32.63 ± 0.07[a]	32.33 ± 0.96[a]	40.61 ± 0.28[b]
C18:1t	3.66 ± 0.18[a]	2.98 ± 0.05[b]	ND
C18:2	22.10 ± 0.13[a]	21.07 ± 0.65[a]	18.38 ± 0.12[b]
C18:2t	ND	0.13 ± 0.01	ND
C18:3t	3.02 ± 0.04[a]	2.80 ± 0.09[b]	1.61 ± 0.06[c]
C20:1	0.58 ± 0.02[a]	0.60 ± 0.04[a]	0.15 ± 0.01[b]
C20:3n3	ND	0.59 ± 0.02[a]	0.53 ± 0.1[a]
C22:6n3	0.19 ± 0.01[a]	0.17 ± 0.01[b]	0.24 ± 0.01[c]
MC-SFA(C6-10)	1.84 ± 0.24[a]	8.02 ± 0.47[b]	2.65 ± 0.16[a]
SFA	36.92 ± 0.35[a]	41.08 ± 1.45[b]	38.32 ± 0.44[a]
MUFA	37.83 ± 0.25[a]	36.5 ± 1.05[a]	40.87 ± 0.28[b]
PUFA	25.3 ± 0.17[a]	24.68 ± 0.69[a]	20.76 ± 0.08[b]
ratio of saturated to unsaturated fatty acids	0.59 ± 0.007	0.67 ± 0.05	0.62 ± 0.01

Values are mean ± standard deviation. Abbreviations: IF, infant formula; SFA, saturated fatty acid; MC-SFA, medium-chain saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; ND, no detected.

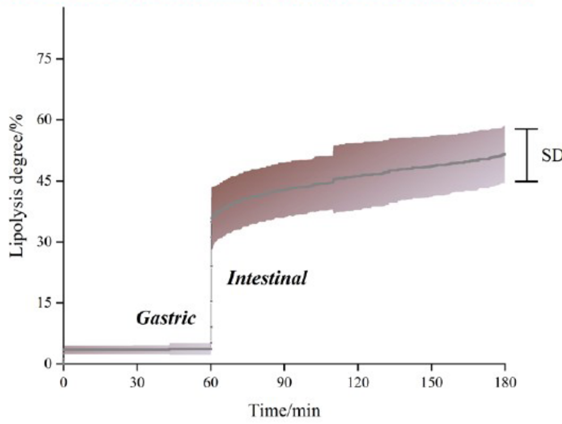
In-vitro lipolysis rate of IF1, IF2 and IF3 monitored using pH-STAT were respectively 50.60 ± 0.87%, 65.88 ± 0.24% and 66.01 ± 1.66% (Figure 2A). IF2 which contained highest amounts of medium-chain saturated fatty acids (8.02 ± 0.47%) have the highest *in-vitro* lipolysis rate (65.88 ± 0.24%). This is in agreement with previously reported findings that demonstrated medium-chain saturated fatty acids (MC-SFA, C8:0, C10:0) are more efficiently digested and absorbed than long-chain saturated fatty acids (LC-SFA, C14:0, C16:0 and C18:0) (Holt et al., 1935; Lien, 1994; Yuan et al., 2020a). Thus, medium-chain triacylglycerol (MCT) is widely used as lipid source in infant formulas to enhance lipid digestion. Besides, the release of FFA in IF3 was also significantly (P<0.05) larger than IF1, which is in agreement with the studies of Nguyen et al. (2018) and Nik et al. (2011). IF3 that has the smallest particles size in the intestinal stage probably enlarged the contact area between lipases and particles, which might lead to higher *in-vitro* lipolysis rate than IF1 (Yuan et al., 2020b).

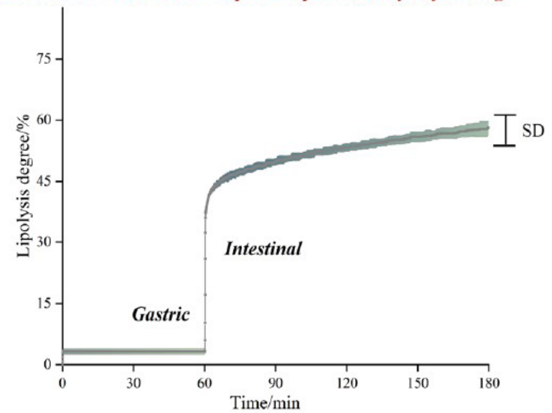
Figure 2B and Figure 2C shows the changes in particles size and ζ-potential of the different infant formulas during the *in-vitro* gastrointestinal digestion. It is important to note that the particles size of the three infant formulas were increasingly aggregated in



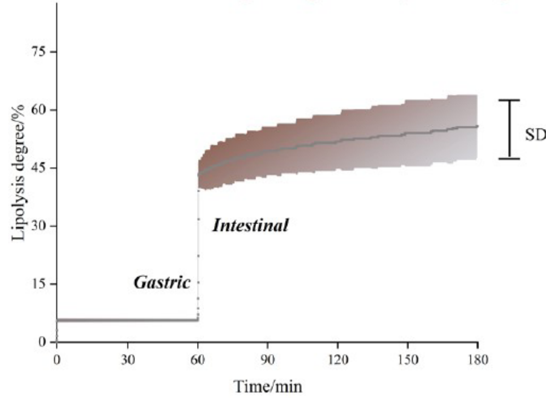


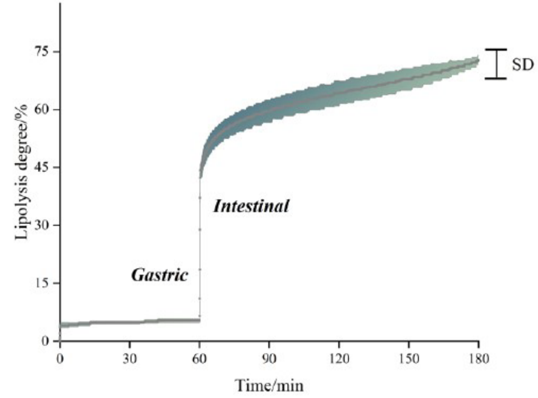
(A)





(B)





(C)

Figure 1. Comparison of *in-vitro* lipolysis rate of infant formulas using different gastrointestinal fluids (A) Liquid infant formula; (B) Infant formula milk powder produced by dry mixing; (C) Infant formula milk powder produced by wet mixing.

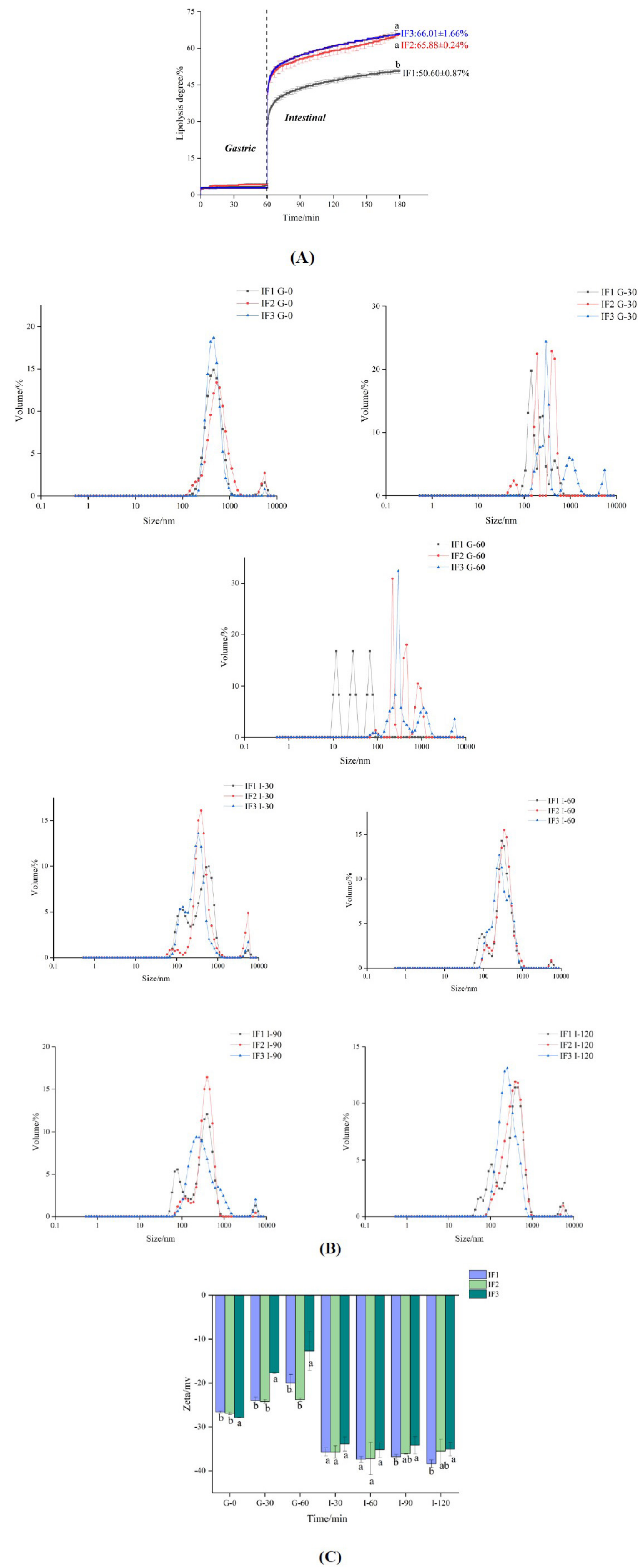


Figure 2. (A) Time course curve of *in-vitro* gastrointestinal lipolysis and changes in the (B) particles size and (C) zeta-potential during the *in-vitro* gastrointestinal lipolysis of IF1, IF2 and IF3.

gastric stage. Following that, particle sizes of the infant formulas were significantly reduced ($P<0.05$) in the intestine stage. During the gastric digestion, TAG were hydrolyzed into MAG and DAG forming large fat globular droplets which were then subsequently digested by pancreatic lipase forming hydrophilic micelles during the intestinal phase. In addition, studies have shown that the large fat globular droplets can dissociated into smaller micelles at a higher pH and the interface was displaced by bile salts in intestinal stage (Gallier et al., 2016).

ζ-potential reflects the changes of interface composition during *in-vitro* gastrointestinal digestion (Yuan et al., 2020b). The whole system is regarded as relatively stable when ζ-potential is less than -30mv or larger than +30mv (Deluca et al., 2006; Heurtault et al., 2003). All the infant formula has a ζ-potential of (-12.7 ± 4.44~-27.9 ± 0.06) in the gastric phase indicating that interface protein is hydrolyzed by pepsin. In the intestinal stage, ζ-potential of IF1, IF2 and IF3 were about -35mv. There were no significant differences in the ζ-potential of three kinds of infant formulas in intestinal phase ($P>0.05$). Hydrolysates that were formed before fuse into bile salts, phospholipid micelles and vesicles of the phospholipids (Yao, 2017; Zhang, 2019).

3.3 Effects of protein composition on in-vitro *simulated gastrointestinal lipolysis of infant formulas*

The protein composition of infant formulas (IF1, IF4, IF5 and IF6) is shown in Figure 3. IF1 had larger protein molecular weight than IF4. As for the IF5 and IF6, no bands could be seen in the gel indicating the molecular weight of protein was less than 3.5kD. Previous study has shown that extensively hydrolyzed infant formula was mainly composed of peptides of less than 3kD (Lowe et al., 2013).

In-vitro gastrointestinal lipolysis of IF1, IF4, IF5 and IF6 were as follows: IF6 >IF5≈IF4>IF1 (Figure 4A). The aforementioned *in-vitro* lipolysis results were in agreement with studies by Zhang (2019). IF4 and IF5 which contained smaller peptides might accelerate the hydrolysis of lipids as compared to the intact protein of IF1. Higher content of amino acids and small peptides at the interface of the lipid droplets can be easily broken down by pepsin, which increased the interface reaction between pancreatic lipase and lipid droplet and hence accelerated the *in-vitro* lipolysis rate (Cheong et al., 2018).

Figure 4B and Figure 4C show the particles size distribution (PSD) and ζ-potential of during *in-vitro* digestion of the infant formulas with different protein composition. Unlike IF 1 which had large aggregated particles in the gastric phase, IF4, IF5 and IF6 had significantly ($P<0.05$) decreased particles size in the G-30 and G-60. This is in agreement with previously reported findings by Nguyen et al. (2016). In the intestine, large aggregated particles of IF1 were found, which was in consistent with the results of Zhang (2019) that large fat droplets still existed by **CLSM** (Confocal Laser Scanning Microscopy) in the end (shown in Figure 4C). The reason was possibly that IF1 has the larger molecular weights peptides as compared to IF4, IF5 and IF6 that could not be easily digested in the intestine.

For the ζ-potential of IF1, IF4 and IF5 in the process of digestion, the higher the protein hydrolysis is, the lower

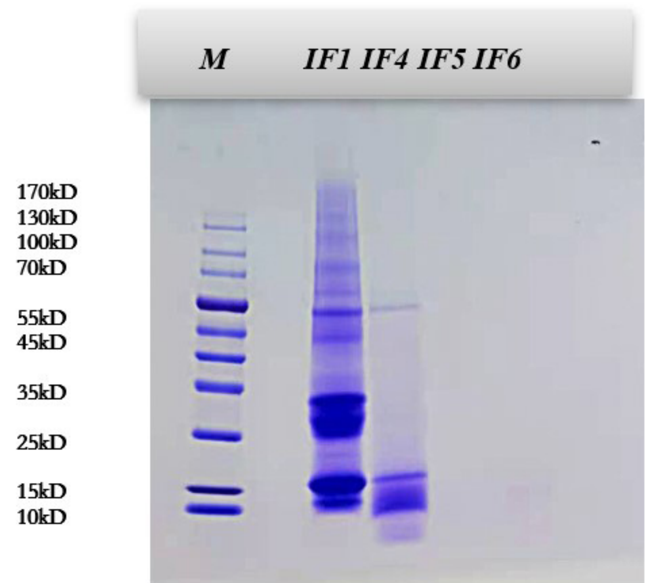


Figure 3. Protein molecular weights of IF1, IF4, IF5 and IF6 (M: Molecular).

the ζ-potential is. The negative charge decreased (from -33.3 ± 1.66mv to-25.5 ± 1.01mv) in the intestine due to contact area between fat globule and lipase was larger and the interface reaction was stronger for IF5. This is in agreement with that the IF5 and IF4 had higher *in-vitro* gastrointestinal lipolysis than none-hydrolyzed infant formulas.

As for the IF1, IF4 and IF6 in the digestion process of intestine; all ζ-potentials were larger than about -30mv indicating that the whole system had higher electrostatic stability. Negative zeta potential of IF6 in different digestion time (-23.5 ± 2.15mv~-25.8 ± 1.44mv) were significantly ($P<0.05$) lower than the other three kinds of infant formulas (ζ-potential<-30mv), which may reduce the electrostatic repulsion among particles (Liu et al., 2021).

3.4 Effects of MFGM protein on in-vitro *simulated gastrointestinal lipolysis of infant formulas.*

Milk fat globule is covered with milk fat globular membrane which is mainly composed of phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and sphingomyelin), glycoproteins, enzymes and cholesterol (Bitman et al., 1984). Figure 5A shows *in-vitro* gastrointestinal lipolysis rate of infant formula with MFGM was significantly ($P<0.05$) larger than that without MFGM. Unlike IF7 which was supplemented with MFGM/ phospholipids-enriched, the lipid droplets in IF1 were coated mainly whey and casein protein that unfolded during processing and it would result in the formation of thick protein interfacial layer, which might hamper the contact area between lipase and milk fat globular and probably further inhibit *in-vitro* gastrointestinal lipolysis of IF1 (Garcia et al., 2014; Cheong et al., 2018; Gallier et al., 2015; Liu et al., 2021). Besides, the presence of MFGM also leads to the formation of large fat globules with thin interfacial layer which can be easily hydrolyzed by lipase (Cheong et al., 2018).

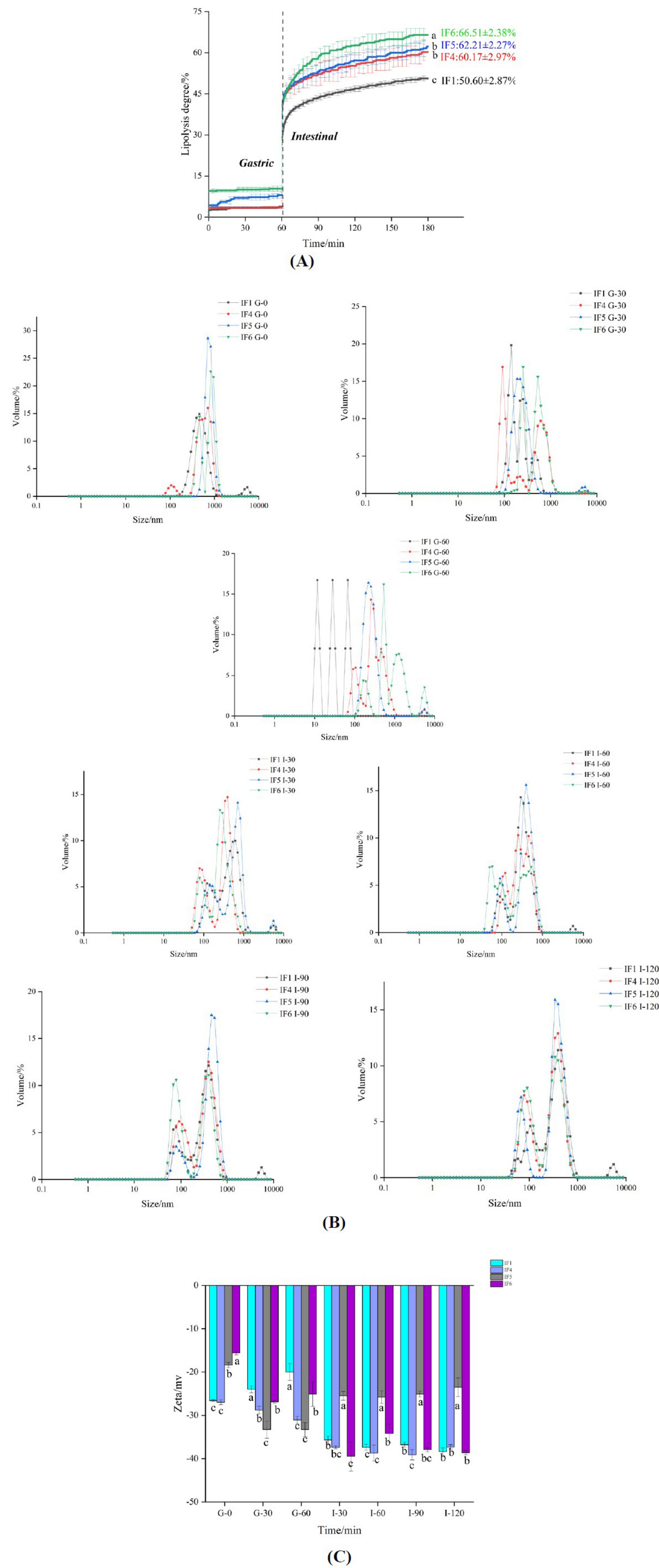


Figure 4. (A) Time course curve of *in-vitro* gastrointestinal lipolysis and changes in the (B) particle size and (C) zeta-potential during the *in-vitro* gastrointestinal lipolysis of IF1, IF4, IF5 and IF6.

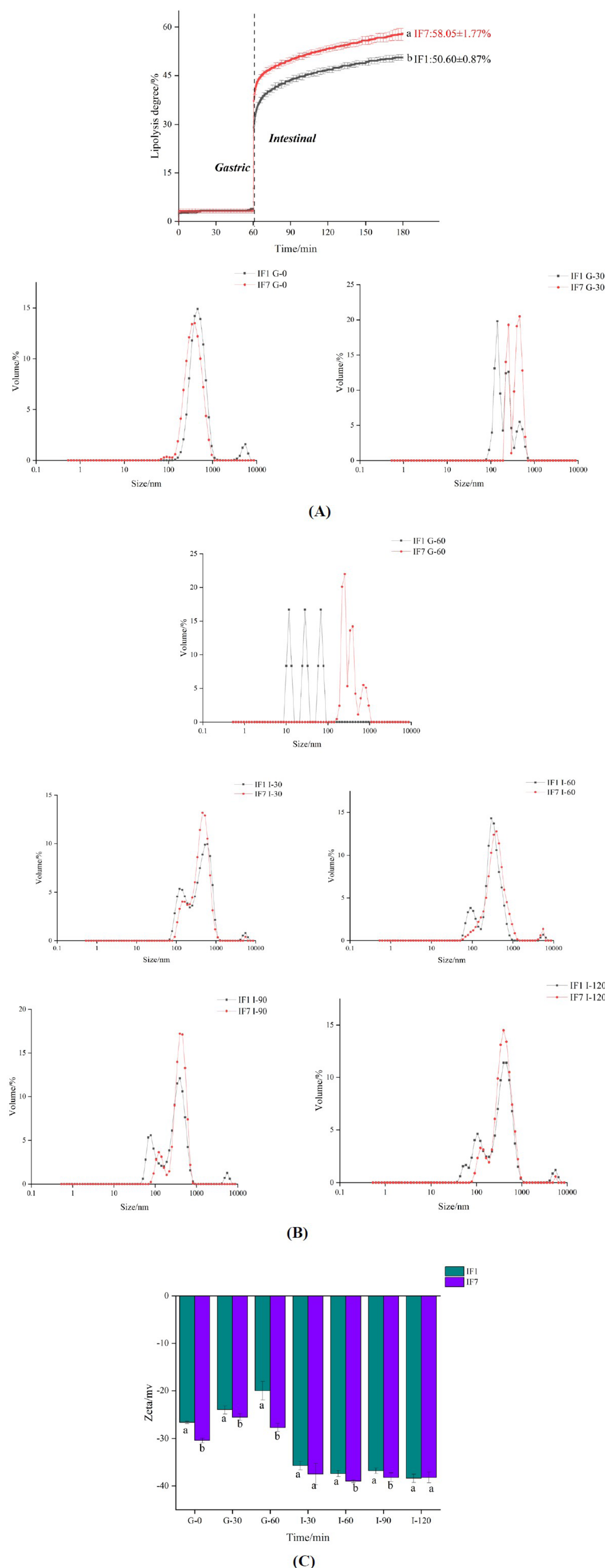


Figure 5. (A) Time course curve of *in-vitro* gastrointestinal lipolysis and changes in the (B) particle size and (C) zeta-potential during the *in-vitro* gastrointestinal lipolysis of IF1 and IF7.

The particle size distribution of different digestion time for IF1 and IF7 is Figure 5B. The result shows that particle sizes of IF7 were significantly ($P<0.05$) larger than IF1 in the intestine, which is in agreement with Jiang (2019) that fat globular droplets were aggregated that showed large particles in the intestine. The reason why the particles were aggregated is that the presence of MFGM was probably attributed to the formation of large fat globules. There existed some differences with the digestion of IF1, IF2 and IF3 that the effects of MFGM for lipid digestion were possibly greater than particle size distribution. Undoubtedly, human milk had the highest *in-vitro* lipolysis degree by pH-STAT despite having larger lipid droplets size that the thinner MFGM interfacial layer were mainly composed of phospholipids on the basis of previous study (Cheong et al., 2018).

The changes of ζ-potential are shown in Figure 5C, the negative charge of IF7 (-27.7 ± 0.87mv) was significantly larger than IF1 (-20.0 ± 1.96mv) in the G-60 ($P<0.05$). Furthermore, the negative charges still increased (-35.7 ± 0.91~-39.0 ± 0.31mv) in the intestine, which were may attributed to different anionic species, such as bile, free fatty acids (FFA) and peptides (Singh, 2011). The negative charge of IF7 was significantly ($P<0.05$) larger than IF1 in the I-60 (-39.0 ± 0.31mv and -37.4 ± 0.67mv) and I-90 (-38.2 ± 0.95mv and -36.8 ± 0.55mv), which could be possibly related to the MFGM/phospholipids-enriched materials that existed more negative charges in the interface reaction. Besides, the ζ-potential of IFs including without/with MFGM were about less than -30mv in the intestine and the particles were also relatively well-distributed, which show that the whole system had higher stability.

4 Conclusions

Three digestion models were used to simulate the infant digestive condition. Model-1 showed relatively poor repeatability ($SD_{Model-1}$: 6.59%/ 7.44%/ 8.10%). Model-2 couldn't be directly titrated because of its electrolytes composition that $NaHCO_3$ led to basification of the simulated digestion environment (increased in pH). Model-3 showed better repeatability ($SD_{Model-3}$: 1.87%/ 1.77%/ 1.24%) for *in-vitro* lipolysis than model-1and was used for *in-vitro* lipolysis digestion of infant formulas. The static *in-vitro* lipolysis digestion results of different infant formulas using pH-STAT method were as follows: IF2 and IF3 were significantly ($P<0.05$) larger than IF1, which could be possibly attributed to the amounts of MC-SFA (IF2) and smaller particles size (IF3) than IF1 after digestion; IF6>IF5≈IF4>IF1, which could be probably due to the differences of protein hydrolysis degree leading to the inconformity of interfacial contact area between fat globular droplets and lipases; IF7 was significantly ($P<0.05$) larger than IF1, which could be probably ascribed that MFGM/phospholipid-enriched materials changed the interface composition. In summary, the optimized model is suitable for studying the changes of food digestion kinetics by verification and application of digestion of different infant formulas. Besides, factors including protein composition, interfacial contact area and particles size distribution (PSD) which might affect *in-vitro* lipolysis of infant formulas in some degree still need to be further analyzed in the future study.

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