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ORIGINAL ARTICLE

Biophysical Aspects of Lipid Digestion in Human Breast Milk and Similac™ Infant Formulas

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Abstract Physico-chemical properties of human breast milk were compared to four Similac™ infant formulas, and correlated with in vitro free fatty acid bioaccessibility using a simulated gastrointestinal system (TIM-1). Viscoelastic measurements, as a function of pH (pH 6.5 to 3.0) and shear rate, showed lower viscosities in breast milk compared to infant formulas. Droplet size and distribution measurements showed distinct differences between the tested formulas and breast milk. During lipid digestion, a lag period was observed for only breast milk. The rate of lipolysis was found to be higher in breast milk compared to Similac™ formulas. The total bioaccessible free fatty acids for Advance infant formula and breast milk were not statistically different for the in vitro TIM-1 model and the shifted-logistical model using one-way ANOVA ($p<0.05$) with a Tukey's Multiple Comparison Test. All other infant formulas had significantly lower free fatty acid bioaccessibilities at the end of the simulated digestion. A positive correlation between rate of lipolysis and droplet surface area per gram for the Similac™ infant formulas was found. However, breast milk did not follow that trend, suggesting the possible involvement of other factors in rate of lipolysis for breast milk.

Keywords Lipid bioaccessibility · Infant formula · Breast milk · Digestibility

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Introduction

Childhood obesity is an alarming universal epidemic where the rate of incidence has precipitously increased over the last 25 years [1–7]. It was reported in the 1980s, that 7.2 % of 6 to 23 months old children in the United States were classified as obese and this has increased to 11.6 % by 2000 [8–10]. Amongst the numerous adverse health effects associated with childhood obesity is Type 2 diabetes; once a disease explicit to adults, now half of recently diagnosed incidences of diabetes are adolescents [11]. It has also been noted that the prevalence of childhood obesity is lower in breast-fed infants compared to formula fed infants [12–14]. Regrettably, the mechanism responsible for this correlation is not understood [15]. Numerous hypothetical links between obesity and breast feeding pertain to the passive nature of bottle-feeding, regardless of the type of milk, versus active suckling when infants are fed directly at the breast [15, 16], the hormones and adipokines in breast milk [17–23], the rates of gastric emptying [24, 25], and the rate of the ileal break in emulsions [26]. Accordingly, supplemental in-depth studies comparing the physico-chemical properties and digestibility of breast milk and infant formula are imperative.

The high caloric density and functionality of the lipid composition in both breast milk and infant formulas may be considered an important marker of their nutritional quality. At birth, there is a switch from a glucose-dominated to a lipid-dominated energy supply since fat constitutes half of the energy content in breast milk and infant formulas [27]. This presents major challenges to the digestive system, because of the poor solubility of lipids, which must first be converted to structures that are less insoluble and available for uptake. Despite these limitations, the human digestive system is effectively designed at digesting and absorbing most lipids [28]. Key reactions, such as adsorption of digestive lipases and emulsification of fatty acid and monoglyceride during

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digestion, must take place at the oil–water interface to solubilize lipids and lipid soluble nutrients [29].

Lipid hydrolysis depends on the ability of lingual, gastric and pancreatic lipases, to access the interface, which depends on both the action of bile salts and co-lipase. It is believed during infant digestion, lingual lipase, which does not require co-factors, may play a more significant role in digestion due to the immaturity of the pancreatic lipase system [30]. von Ebner's glands of the human tongue secrete lingual lipase [31]; which facilitates initiation of digestion of triglyceride components of the milk when it reaches the stomach [32]. It is reported that milk fat globular exposure to lingual lipase prior to exposure to pancreatic lipase results in increased rate of lipolysis [33]. Another lipase that is considered to be of interest with regards to infant digestion of milk fats is bile salt-stimulated lipase (BSSL); [34] which is understood to be of maternal origin, produced in the human mammary glands [35, 36]. BSSL is unique, in its ability to hydrolyze the 3 *sn*-positions of a triglyceride, thus generating free fatty acids and glycerol [37, 38]. BSSL is reported to withstand (dormant) the infant stomach pH, while resuming its activity in gastrointestinal areas where elevated levels of bile salt are available [39–41]. [42–44] Bile salts not only facilitate the adsorption of co-lipase and lipase, but also aid in the solubilization of the lipolysis products as they accumulate at the interface, into mixed micelles, allowing the transport to and through the gut mucosal surface [28]. The gut cell lining is then able to sense these nutrients and in response, secrete hormones and peptides that slow digestion and send signals to the brain that reduce appetite [45].

Michalski et al. [46] found infant formula fat droplets to be smaller than human milk fat droplets and that the milk fat globule surface area, required for lipolytic activity, is relevant to lipid digestion [47]. In addition it has been shown that human milk has a greater lipid bioaccessibility during the gastric digestion than infant formula [48]. We hypothesize that a correlation lies between the physico-chemical properties of these different infant formulas and breast milk and the bioaccessibility/digestion of their lipid composition. Digestion refers to the process of disassembling food macronutrients into absorbable units [49], while bioaccessibility of a nutrient refers to its potential to be absorbed.

In the present work, we investigate the physico-chemical properties of human breast milk and infant formula by light scattering, to illustrate droplet size and distribution, as well as rheological viscosity measurements. The TNO Intestinal model (TIM-1), an in vitro model that simulates the upper gastrointestinal tract, was used to monitor the bioaccessible free fatty acids generated throughout a 5-h simulated digestion. A study on the effect of partially hydrolysed guar gum on fat bioaccessibility showed good correlations between the TIM-1 and in vivo bioaccessibility data [50]. Free fatty acid release profiles are described for each of the tested infant milks and a shifted logistical model, developed by Troncoso et al. [51], was used to elucidate the rate of lipolytic generation of free fatty acids as a function of time.

Method

Materials and Sample Preparation

Similac ™ (Abbott Nutrition, OH, USA) infant formulas (Total Comfort, Sensitive, Soy, and Advance) were prepared, immediately before analysis, as per the label instructions. ~8.8 g of dry powered formula was added to 2 oz of distilled tap water and mixed with a magnetic stirrer until homogenous. Human breast milk was expressed (as per IRB Protocol 13–824 M) on the same day of the corresponding experiment and stored at 4 °C while being mixed thoroughly to prevent creaming.

Light Scattering

Droplet size in the infant formulas and breast milk were studied using light scattering (Masterisizer 2000, Ver 5.54, Malvern Instruments Ltd., Malvern UK). The ultrasound was not used to ensure that particle size reduction did not occur. Instead, constant stirring at 2700 rpm was used to prevent creaming. A refractive index (RI) of 1.42 (approximate RI of oils) was used for particle size determination [52]. Five replicates were conducted for each sample and the average droplet size distribution (in μm) was used for data analyses. Standard deviation of the maximum, minimum, and mean diameters, as well as the surface area and Sauter mean diameter (D[3,2]) values were determined for each of the five formulas and averaged.

Viscosity at Different pH

The initial pH of the infant formulas and breast milk all fell between 6.5 and 7.0. Using 1 M hydrochloric acid, each sample was acidified from pH 6.5 to 3.0 in 0.5 increments. For each pH, six replicates were run on a Discovery Hybrid Rheometer (TA Instruments, DE, USA) to measure viscosity as a function of shear rate. A temperature controlled peltier plate and a 6 cm stainless steal cone (cone angle (3 °: 59 min: 20 s) and truncation (105 μm)) was set at 37 °C, and a logarithmic shear rate sweep from 0.5 to 500 s^{-1} was used to determine the viscoelastic properties. The points per decade of data collection were limited to ensure that the run did not exceed 5 min, this was in part to prevent phase separation from occurring, no macroscopic separation was observed during the viscosity measurements.

TIM-1 Simulated Digestion

The TIM-1 (TNO, Zeist, The Netherlands) simulated gastrointestinal tract was used to mimic the gastrointestinal tract of infants and the lipid digestion of breast milk and Similac™ formulas. TIM-1 consists of four compartments representing the stomach, duodenum, jejunum and ileum. Duodenal start residue (60 g; 15 g SIES, 30 g fresh porcine bile, 2 mg trypsin solution (bovine pancreas (7500 N-α-benzoyl-L-arginine ethyl ester (BAEE) units/mg, T9201) was obtained from Sigma Aldrich), 15 g pancreatin solution), jejunal start residue (160 g: 40 g SIES, 80 g fresh porcine bile, 40 g pancreatin solution), and ileal start residue (160 g SIES) were injected into their respective compartments prior to heating the system to physiological temperature (37 °C) in preparation for feeding. Also, prepared solutions simulating bile, gastric and pancreatic secretions were attached to the TIM-1. Pancreatin was obtained from Sigma Aldrich, USA. Fresh pig bile (Farm-to-Pharm (Warren, NJ, USA)) was collected from a slaughterhouse, standardized by pooling numerous collections, aliquoted into single use amounts for individual TIM experiments, and then stored at −20 °C until use. Enzyme solutions were placed in ice, until they were used to fill the TIM-1. Although the same lot of each enzyme was consistently used for each run, it is important to note that biological differences exist between commercially available enzymes and enzymes present in vivo. Therefore, the absolute values obtained from the simulated digestions may not be biologically relevant, however, the changes observed between each sample are relevant.

Also, Modifications to the solutions (a), software program (b), fed sample(c), and total digestion period/run (d) were implemented to mimic the human infant gastrointestinal conditions.

(a) Modified solutions attached to the TIM-1 to simulate infant conditions: only 75 % of the 7 % pancreatin solution (Pancrex V powder, Paines & Byrne, UK) was utilized; the small intestinal electrolyte solution constituted of NaCl 5 g/L, KCl 0.6 g/L, CaCl2 0.25 g/L. the initial gastric enzyme solution included 150 g gastric electrolyte solution, 28.1 mg of lipase (Rhizopus lipase (150,000 units/mg F-AP-15), obtained from Amano Enzyme Inc. (Nagoya, Japan)), and 22.5 mg pepsin (from porcine gastric mucosa, lyophilized powder, >2500 units/mg protein, Sigma Aldrich) (pepsin and lipase quantities used are 75 % of the quantity typically used in an adult-digestion simulated TIM-1 experiment); the solution was mixed for 10 min at room temperature.

(b) In addition, the TIM-1 system protocol used was that of the 'infant fed-state'; thus controlling peristaltic movements, nutrient and water absorption, gastric emptying, pH, enzyme secretion rates and transit times to be similar to the in vivo gastrointestinal environment of the human infant [53]. The software provides the following conditions in the respective TIM-1 compartments (Table 1).

(c) The total sample fed into TIM-1 was reduced to 200 g (67 g of gastric electrolyte solution and 133 g meal), while an adult run would involve feeding 300 g of total fed sample.

(d) Five hour simulated digestions were performed to mimic the suggested transit time to the cecum in infants [54]. Samples were collected from the jejunal and ileal filtrates and ileal efflux at 30, 60, 90, 120, 180, 240, and 300 min and then frozen (~ −40 °C) until extraction.

Experimental Meals

The gastric electrolyte solution (8.25 mg of amylase and 5 g of the above mentioned gastric electrolyte solution) was mixed with 133 g of the sample, and fed into TIM-1 after the four GI compartments were heated to 37 °C. 133 g of the mixed sample was then fed to the TIM-1 standardizing the amount of fat (~5 g fat in each meal) fed to the TIM-1 allowing the bioaccessibility to be approximated. To fill the gastric compartment a 200 g meal was fed for the infant model, therefore 67 g of gastric electrolyte solution was added to the 133 g meal.

Free Fatty Acid Extraction

The fatty acid concentrations from the jejunum, ileum and ileum efflux, at each of the aforementioned time intervals, were thawed and extracted in duplicate. Fatty acids were extracted by first adjusting the pH of 5 mL of filtrate to a pH value between 10 and 12 using 10 N NaOH. 200 μl of 5 mg/ml nonanoic acid was added to serve as an internal standard and 15 ml dichloromethane was added. After 24 h, the organic and aqueous layers were separated and the bottom dichloromethane layer was discarded. 1 N HCl was added reducing the pH to between 1–2 and 5 mL dichloromethane was added. After 24 h the layers were separated and the bottom layer was collected and frozen at −20 °C until further analyses.

Table 1 Software pH values of TIM-1 stomach throughout 5-h digestion period to mimic human infant gastrointestinal conditions

Digestion Time (Min.)	pH value
0	6.5
30	6.5
150	4.5
240	3.5
300	3.5

High-Performance Liquid Chromatography (HPLC) analysis of Free Fatty Acids

The extracted free fatty acids, from the TIM-1 runs (completed in duplicated) were analyzed using High-Performance Liquid Chromatography (HPLC) (Alliance, Waters e2695) with 2424 Evaporative Light Scattering (ELS) Detector (Waters). A reverse phase free fatty acid HP 4 μm (3.9×150 mm) column (Waters, Milford, MA, USA) was used. The stroke volume for the system was 50 μL; and a gradient pump mode was used. Ideal separation occurred when the solvents selected were: 35 % Water HPLC Grade 0.22 μm filtered (Pharmco-Aaaper, Brookfield, CT, USA), 20 % Tetrahydrofuran (THF) stabilized with 250 ppm BHT (OmniSolv, Bellerica, MA), and 45 % Acetonitrile HPLC Grade (EMD Chemicals, Inc., Gibbstown, NJ, USA). The column temperature was set at 30±5 °C. Each of the extractions was run in the HPLC and two injections were taken for each replicate. Accordingly, 12 chromatograms were attained for each sample at every collection interval (30, 60, 90, 120, 180, 240, and 300 min) at each of the small intestine locations (i.e., jejunum, ileum, and ileal efflux).

Determination of Bioaccessibility

Samples fed to the TIM-1 were controlled to contain 5 g of fat allowing the bioaccessibility to be determined. The % dispersed phase volume, determined with light scattering, for the infant formulas and breast milk were not statistically different and assuming that the fats have the same density it was assumed that they had similar fat contents (albeit it should be mentioned that differences in density may be a potential source of error). From a computational standpoint, the triglyceride composition in the samples was assumed to have the molecular weight of triolein and that 2 mols of oleic acid are released per mol of triolein (i.e., of the 5 g of fat fed 3 g of fatty acids may be released) (eq. 1):

$$Maximum\,Bioaccessibility = \frac{feed\,fat}{MW_{TO}} \times 2\,MW_{OA} \qquad (1)$$

where MW_{TO} is the molecular weight of triolein, MW_{OA} is the molecular weight of oleic acid.

Statistical Analysis

A one-way ANOVA ($p<0.05$) with a Tukey's Multiple Comparison Test (Graph Prism 5.0 (La Jolla, CA) was used to determine the statistical significance in the means.

Results and Discussion

Lipid droplet size distributions of Sensitive, Total Comfort, Advance, and Soy formulas were compared to human breast milk (Fig. 1). Breast milk has three primary distributions of particle sizes (~0.1, 1 and 7 μm), which have been previously reported [46], while the Total Comfort infant formula has a single distribution of particles at~0.4 μm and Advance, Soy, and Sensitive formulas have two distinct peaks, one at 0.4 μm and the other at 3 μm. Variations in the individual particle size volumes are shown in Fig. 1. Based on these results, it is evident that the particle size distribution varies distinctly between infant formulas and breast milk.

The effects of pH on viscosity (Pa s^{-1}) were determined, for the formulas and breast milk, using strain rate sweeps from 5 to 500 s^{-1} (data not shown). Initially, the pH for each sample was between 6.5 and 7, following they were incrementally (every 0.5) acidified using HCl to the pH of an infant's stomach pH~3 [55]. Upon feeding, the pH of the gastric compartment is buffered to near the initial pH of the formula/milk, after which the pH decreases until it returns to the initial pH of the stomach [55]. It was apparent that the non-acidified formulas behave as Newtonian fluids and the viscosity is independent of pH. However, as the meals are acidified the viscosity not only increases but the solution behavior changes to a non-Newtonian shear-thinning fluid (data not shown). Since non-Newtonian fluid behavior is observed, a single strain rate of 20 s^{-1} was arbitrarily selected and was plotted against each pH value to allow statistical comparison (Fig. 2). Enzymatic reactions are dependent on the solution properties, including the viscosity, and can possibly play a role in digestion. Viscosity alters the rate of gastric emptying, postprandial glucose concentrations, and satiety [56]. Various researchers agree upon the presence of a positive correlation between the viscosity of the ingested meal and both gastric emptying and satiety sensation [57–60]. However, the role of

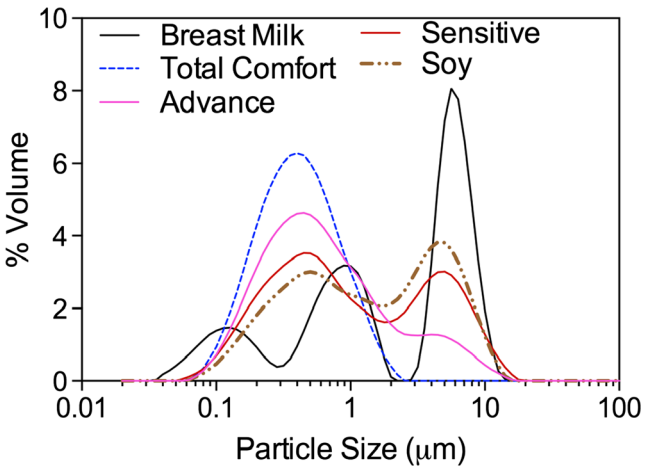

Fig. 1 Particle size distribution for Similac ™ brand types: Sensitive, Total Comfort, Advance, and Soy compared to human breast milk

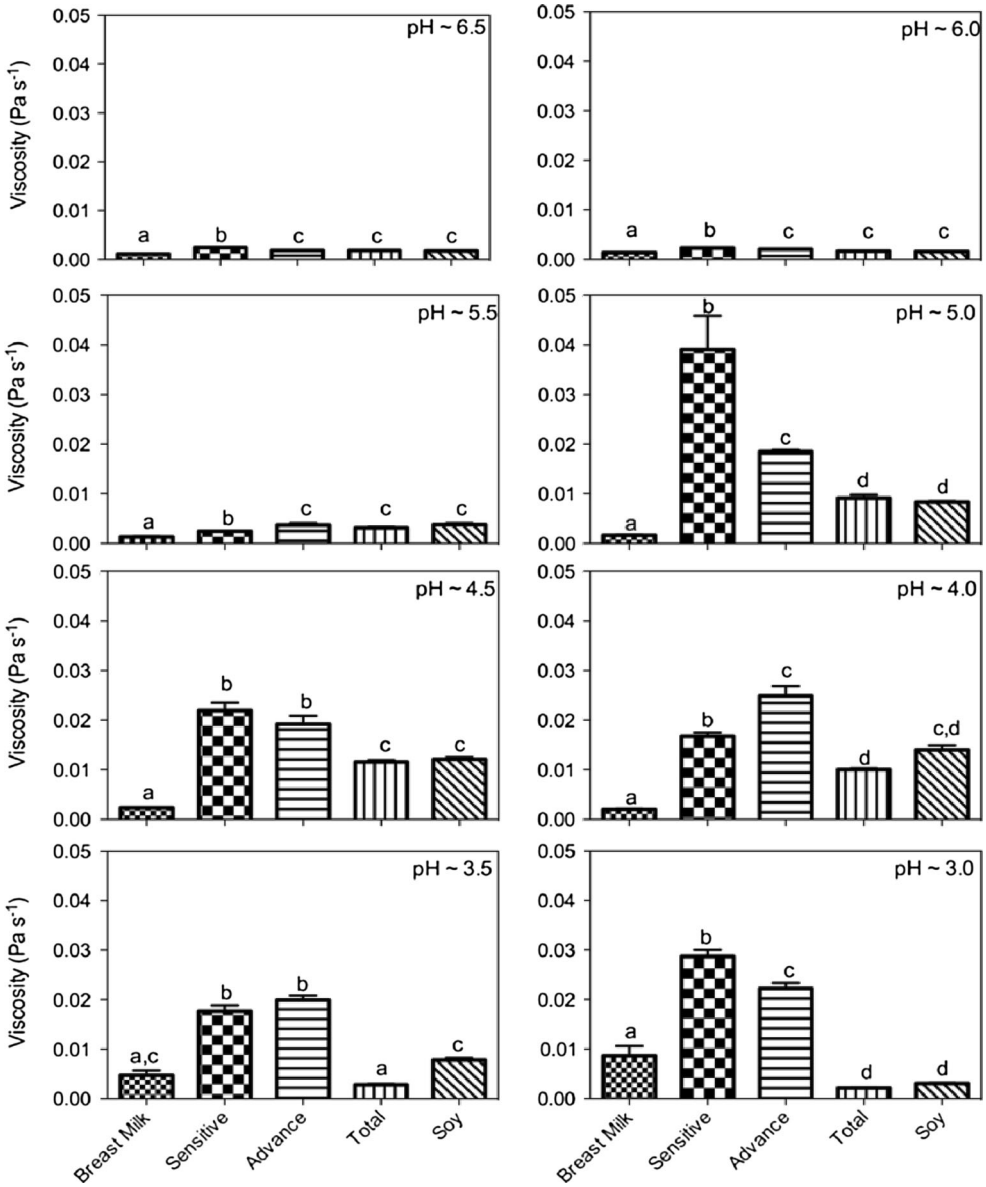

Fig. 2 Viscosity determined at 20 s^{-1} for Sensitive, Total Comfort, Advance, and Soy based infant formulas compared to human breast milk at pH values between 3.0 and 6.5. Different letters represent significant differences determined using a one-way ANOVA ($P<0.05$) and a Tukey's Multiple Comparison Test

viscosity, throughout its transit time in the gastrointestinal tract, is not understood. The question remains, if it is the initial viscosity of the meal or its changing viscosity in the gastrointestinal tract that plays a role in satiety. The viscosity of both Sensitive (Fig. 2a) and Advance (Fig. 2c) remain unchanged from their initial pH value to pH 5.5. Upon further acidification there is a dramatic increase in the viscosity after followed by decrease in viscosity. Similar patterns are seen in Total Comfort (Fig. 2b) and Soy (Fig. 2d); however, these samples have the same viscosity at the extreme pH values tested. Breast milk does not follow similar trends to the infant formulas; instead, the viscosity of breast milk does not drastically change between pH values of 6.5 and 4.0, and it is not until a pH of 3.5 that an increase, albeit not dramatic, in the viscosity is observed.

The maximum viscosity for Sensitive is pH 5.0 (Fig. 2a), for Total Comfort 4.5 (Fig. 2b), Advance and Soy are 4.0 (Fig. 2c and d), and breast milk is 3.0. Advance, Total Comfort, and Soy had statistically similar viscosities between pH 5.5 and 6.5; while Sensitive was significantly higher at pH 6.5 and 6 and lower at 5.5. Similar pattern are seen at pH 4.5; however, there is no significant difference between Sensitive (0.023 Pa s^{-1}) and Advance at this pH. At all pH values tested, the viscosity of breast milk is statistically lower than the infant formulas with the exception of at pH 3.0 where both Total Comfort and Soy had lower viscosities. In addition, breast milk had the lowest viscosity between 4.0 and 6.5. It is clear that significant differences exist in both the droplet sizes and the viscosity profiles between infant formulas and breast milk; however, it is unclear if these physical differences correlate to differences in lipid bioaccessibility.

The TIM-1 (TNO, Zeist, The Netherlands) simulated gastrointestinal tract was used to quantify the absolute, non-cumulative bioaccessible fatty acids in the jejunum (Fig. 3), ileum (data not shown) compartments of the small intestine, as well as the ileal efflux (data not shown). In the Sensitive, Total Comfort and Soy, there is an initial rapid release of fatty acids at the first time point measured and as time progressed the concentration of free fatty acids decreased in the jejunum (Fig. 3a, b, and d). Fatty acids bioaccessibility increases with time and plateaus at ~200 min for the Advance formula (Fig. 3c). In all infant formulas, no lag time for lipolysis is observed. Interestingly, in only the breast milk, there is a significant lag (~90 min) before appreciable amounts of fatty acids are released (Fig. 3e) in the jejunum.

The cumulative free fatty acid, in the jejunum (Fig. 4a) and ileum (Fig. 4b), have similar release profiles for Sensitive, Soy and Total Comfort; while the Advance has a greater free fatty acid bioaccessibility. After 300 min of digestion, approximately 1850 mg and 500 mg of fatty acids were bioaccessible in the jejunum and the ileum for Advance. On the other hand, in Sensitive, Soy, and Total Comfort ~250 mg and ~150 mg fatty acids are bioaccessible in the jejunum and ileum. Unlike the infant formulas, breast milk has a significant lag period and even with this delay in lipid digestion, breast milk still provides a total of ~1000 mg and ~400 mg of bioaccessible free fatty acids in jejunum and ileum. Given that breast milk contains maternal BSSL, endogenously, this could lead to the conclusion that the initial interfacial layer is extremely apt at preventing pancreatic lipase from accessing the lipid droplet interface.

This lag phase was also reported by Bernback et al. [38] in human breast milk triglycerides upon in vitro hydrolysis with gastric lipase, colipase-dependent lipase, and BSSL. Similarly, Berton et al. reported a similar lag phase for hydrolysis of triglycerides in raw, non-homogenised cow's milk by human pancreatic lipase [61]. Structurally, breast milk fat globules differ from those of infant formula. Breast milk fat globules consist of a triglycerides core, enveloped by a membrane of phospholipids, cholesterol and protein; [62] whilst infant formula fat globules consist of a triglycerides core, surrounded by phospholipids [48]. Lindstrom et al. [63, 64], found barrier properties of phospholipids against BSSL hydrolysis and that pancreatic colipase-dependent lipase hydrolysis activity was found to be constrained by the presence of phospholipids and proteins [65]. Accordingly, the constituents of the human breast milk fat globule membrane could play a role in delaying the initiation of the triglyceride hydrolysis process, thus resulting in the lag period evident in lipid digestion. It is

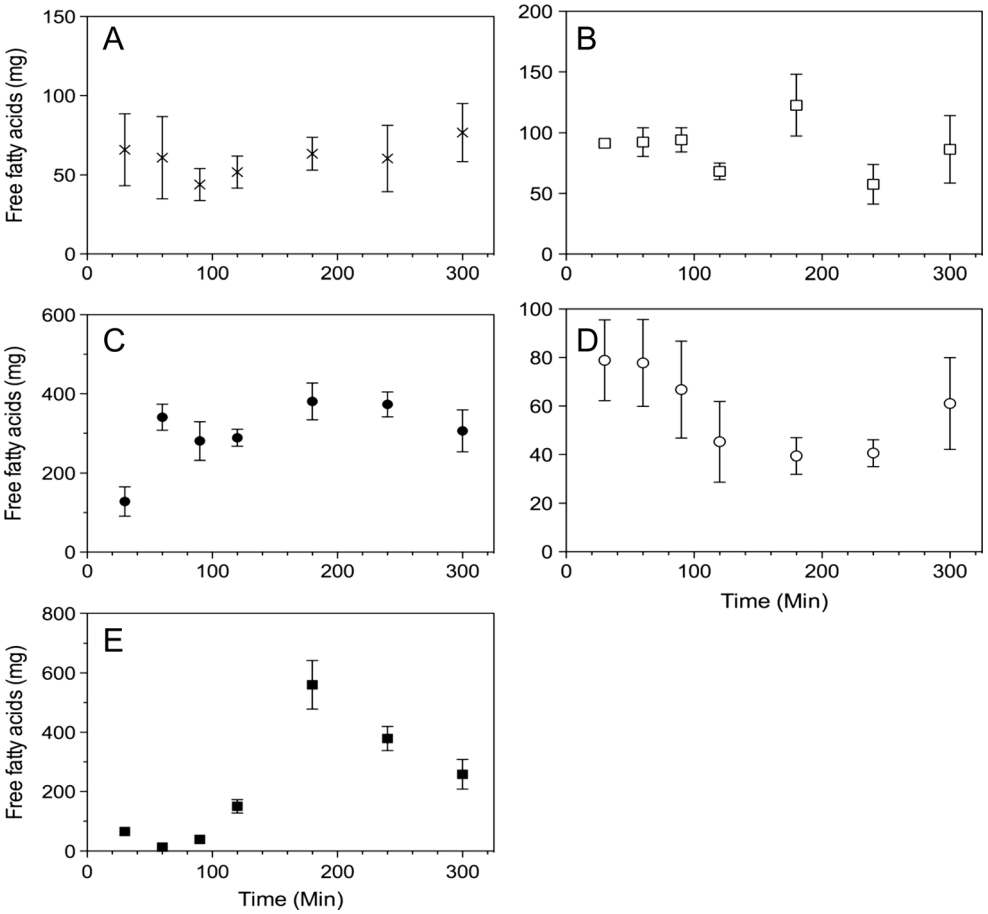

Fig. 3 Total fatty acids per given time point in the jejunum and ileum (TIM) for (**a**) Sensitive, (**b**) Total Comfort, (**c**) Advance, and (**d**) Soy based infant formulas compared to (**e**) human breast milk

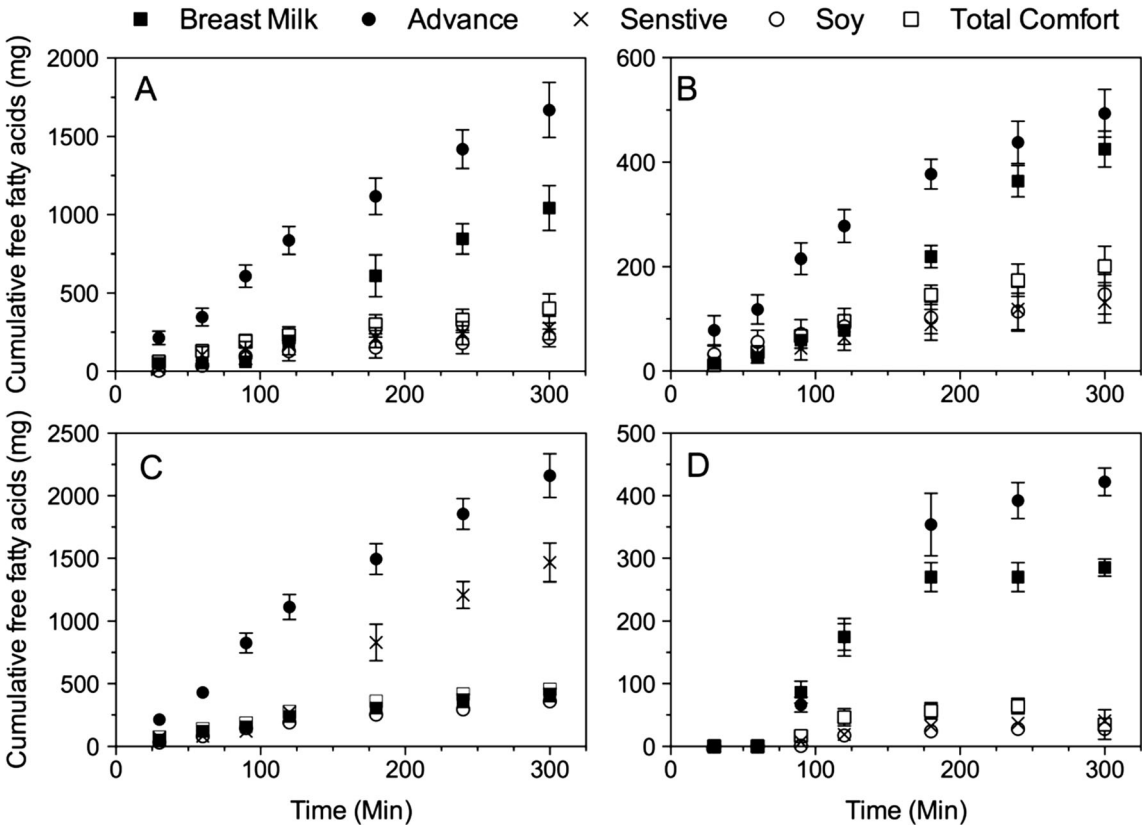

Fig. 4 Total fatty acids bioaccessibility for Sensitive, Total Comfort, Advance, and Soy based infant formulas as well as human breast milk in various parts of the simulated in vitro (TIM) gastrointestinal tract; **a** jejunum, **b** ileum, **c** combined jejunum and ileum, and **d** efflux

reported that minimal hydrolysis does occur during this lag phase; [66] hence possibly modifying the fat globule membrane composition by increasing its fatty acid and diacylglycerol composition [61]. As a result, the lipases' access to the human breast milk fat globules' triglyceride core is facilitated following the lag phase, allowing for hydrolysis to take place [61].

The absence of the lag phase in infant formulas could be explained by referring to Fig. 1, which illustrates that the bulk of the droplet size distribution in the various infant formulas is ~0.4 μm. Thus, the lipase will be more capable of accessing the triglyceride core when compared to the breast milk fat globules, whose particle size distribution contains mostly larger size droplets (i.e., 1 and 7 μm diameters). These differences in the rate of fatty acid release and the presence of a lag period in breast milk, in both the jejunum and ileum may play a role in satiety. It is extremely important to note however, there is not scientific evidence as of yet to support this.

The overall fatty acids bioaccessibility (Fig. 4c) is a combination of the fatty acids released from the jejunum and ileum. Fatty acids that were not removed via the jejunal and ileal filtrates were measured at the outlet of the ileal compartment (Fig. 4d). Similar trends, albeit at much lower concentrations, were observed between the jejunum and at the ileal efflux.

The total fatty acid bioaccessibility, in Fig. 4c at 300 min, was the sum of the fatty acids measured in the ileum and jejunum filtrates. The bioaccessibility from highest to lowest are: Advance (~74.5±6.1 %), breast milk (~50.6±5.3 %), Total Comfort (~24.5±5.56 %), Sensitive (~14.1±3.9 %) and Soy (~12.4±2.7 %). Using a one-way ANOVA, no significant differences between fatty acid bioaccessibility in breast milk and Advance was found and Total Comfort, Sensitive, and Soy are not statistically different from one and other. Breast milk and Advance fatty acid bioaccessibility are significantly higher than the remaining Similac™ infant formulas tested.

A three-parameter shifted logistic model (eq. 2) [51, 53, 67] was used to characterize the free fatty acids generated as a result of lipolytic activity in each digestive compartment as a function of time, *t*:

$$C(t) = \frac{C_{asymp}}{1 + e[k(t_C - t)]} - \frac{C_{asymp}}{1 + e[kt_C]} \quad (2)$$

Where C_{asymp} is the total amount of fatty acids released, k is the rate of release of fatty acids per unit time, and t_c is the critical time at which half of the total amount of fatty acids is released [53]. Through the use of nonlinear analysis in

Table 2 Fitted parameters from eq. 2 including the total fatty acids released, induction time, and rate of release in jejunum and ileum in the TIM-1

	Sensitive	Total Comfort	Advance	Soy	Breast Milk
FFAs released (C_{asymp}) (mg)	725	796	3000	739	1534
Induction time (T_c) (min)	48	41	79	38	175
Rate constant (K) (mg/min)	0.0106	0.012	0.0099	0.0083	0.0245

Graphpad Prism (La Jolla, CA), the three bioaccessibility parameters determined from eq. 2 (C_{asymp}, k, and t_c) were analysed.

Upon fitting the shifted logistic model to the free fatty acid bioaccessibility curves (Fig. 4c), the theoretical bioaccessibility (C_{asymp}) (Table 2) showed similar trends compared to what was observed at 300 min of simulated digestion from the in vitro digestion. The critical time, t_c, is a parameter which combines the initial lag time, the rate of fatty acid release and the duration of lipolysis. It is evident that breast milk had a much longer T_c compared to the infant formulas, coinciding with our in vitro observation of a lag time presented earlier. It has been well established that there is an inhibitory effect of the native milk fat globular membrane on pancreatic lipase activity [68]. No major differences were found between the studied infant formulas. The rate constants, K, for the infant formulas were very similar while breast milk had a much higher rate of release.

Two different phenomena can account for the differences observed in the rate constants. First, interfacial lipase activity is dependent on the amount of surface area available for the reaction to progress. Therefore, the particle size and particle size distribution may account for differences in the reaction rates, as discussed earlier. As well, breast milk has endogenous maternal BSSL, which could account for the elevated reaction rate in breast milk. Bernback et al. [38], show that the addition of only gastric lipase and colipase-dependent lipase to human milk, in vitro, results in an increase in the release of the free fatty acids up to 60 min of digestion, beyond which, a stagnant release profile is evident. When they added BSSL to the same mixture, the stagnant free fatty acid release profile was replaced with a continuous increase in the rate of free fatty acids generation. This supports our hypothesis that the presence of BSSL in breast milk influences the rate of free fatty acid release (Table 2) and total free fatty acids released (Table 2 and Fig. 4c), despite the initial lag phase. This further explains

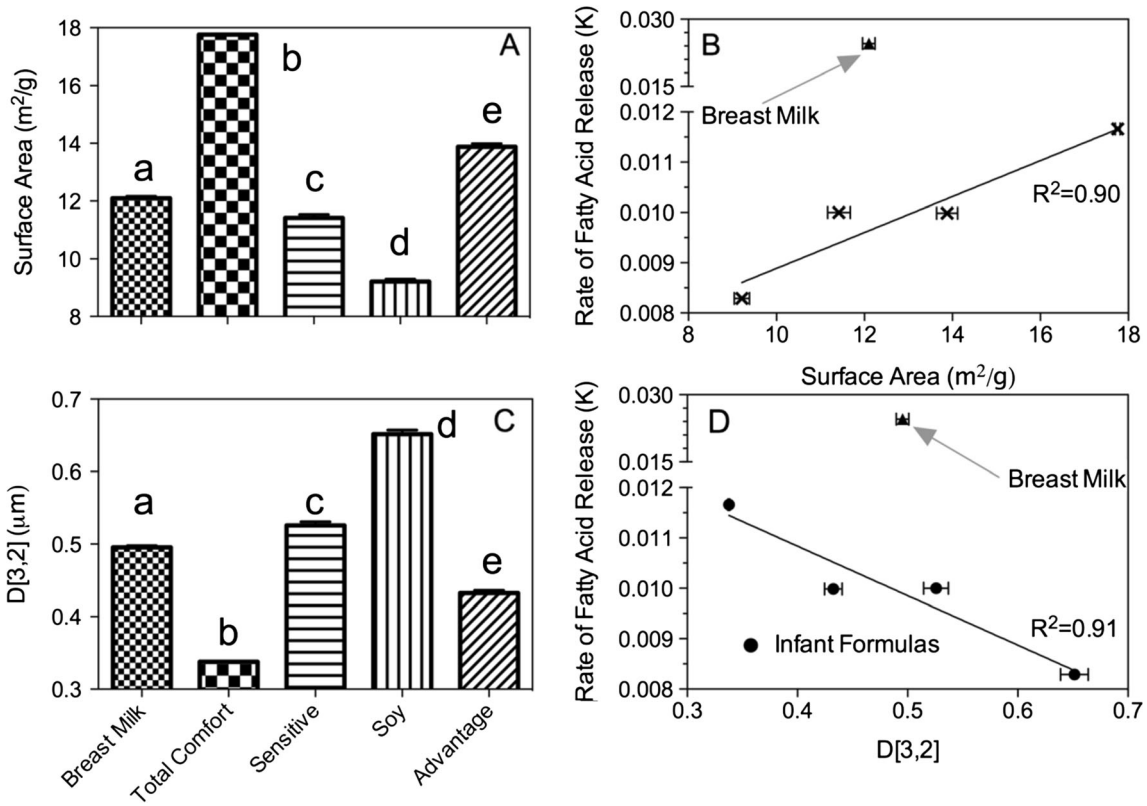

Fig. 5 Surface area (**a**), and D[3,2] (**c**) for human breast milk and Similac™ infant formulas and correlations between surface area (**b**) and D[3,2] (**d**) against bioaccessibility

the stagnant pattern observed in all the infant formulas over the entire digestion period in both the jejunum and ileum compartments (Fig. 3a-d). To our knowledge, no data exists pertaining to the quantitative rate of free fatty acid release in both infant formula and breast milk in the small intestinal compartments. However, Armand et al. [48] compared the rate of gastric free fatty acid release in vivo, and found, similar to our findings, a significantly higher rate of gastric lipolysis in breast milk than in infant formulas. This suggests consistency in the hydrolysis pattern by varying enzymes, since gastric lipase is the predominant hydrolysis performer in the gastric compartment of the gastrointestinal tract; while pancreatic lipase and BSSL (in case of human breast milk) are the predominant hydrolysers in the small intestinal compartments.

Using particle size distributions (Fig. 1), the surface area per gram (Fig. 5a), Sauter Mean Diameter (surface weighted mean - D[3,2]) (Fig. 5c) and the De Brouckere Mean Diameter (volume weighted mean - D[4,3]) (Fig. 5e) were determined. Total Comfort infant formula has the highest surface area followed by Advance, breast milk, Sensitive and Soy. Correlations between the rate of lipolysis and surface area (Fig. 5b) show a very strong positive correlation (R^2=0.90) for the infant formulas, which did not include breast milk. This highlights that the rate of lipolysis, in the infant formulas, varied based on the surface area available for lipase access. However, the difference in the rate of the reaction for breast milk suggests that it is not dictated by surface area alone; and other factors such as endogenous maternal BSSL and/or the unique interfacial properties of the milk fat globular membrane could be playing a role in the rate of lipolysis seen in breast milk.

The Sauter mean diameter (surface weighted mean - D[3,2]) (Fig. 5c) provides information about the central point around which the surface area for each sample resides. This type of particle size measurement does not require measurement of the number of particles involved, and takes into consideration the individual value and the frequency of its presence in the distribution. D[3,2] (Fig. 5c) values, in descending order, are Soy (~0.7 μm), Sensitive (~0.55 μm), breast milk (~0.5 μm), Advance (~0.45 μm), and Total Comfort (~0.3 μm) (Fig. 2a, b). A strong, negative correlation (R^2=0.91) is seen between rate of fatty acid release and D[3,2] for the infant formulas while breast milk does not follow the trend (Fig. 5d).

Conclusion

Comparing breast milk to different Similac™ infant formulas highlighted numerous differences that could result in important alterations in the biophysics of digestion. Breast milk had lower viscosities, at all pHs, than infant formulas and the highest viscosity of the breast milk was at a pH of 3 (the pH of an infants' stomach), while the highest viscosity for infant formulas were observed between 4 and 5. These changes in the viscous properties could influence gastric emptying rates and potentially satiety; however, we found no correlations between viscosity and lipid bioaccessibility. Free fatty acid release profiles showed a lag phase during lipid digestion, exclusive to human breast milk. The droplets size and distribution varied for the different milk samples tested; similarly the calculated surface area and D[3,2] varied between different samples. It was found, for the infant formulas, that the rate of lipolysis was positively correlated with the surface area per gram. However, the rate of lipolysis, in breast milk, did not follow that trend. These biophysical aspects of breast milk versus infant formulas may alter different facets of their respective digestibility which may have unintended consequences.

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