

In vitro gastrointestinal lipid handling and bioaccessibility rate of infant formula with large phospholipid-coated lipid droplets are different from those of standard formula and closer to human milk

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ABSTRACT

Background: Lipid droplet size and interfacial coating (protein or phospholipid) varies in early life nutrition and can impact gastrointestinal lipid handling, thereby affecting the rate at which free fatty acids and monoacylglycerides become potentially available for absorption (bioaccessible). We compared gastrointestinal handling and lipid bioaccessibility rates of infant formulas that varied in size and coating and human milk (HM). **Methods:** Infant dynamic digestion was simulated using tinyTIM with advanced gastric compartment. Gastrointestinal handling (lipid top layer formation, emptied lipid rates, bioaccessible lipid rates and enteroendocrine cholecystokinin secretions by Caco-2 cells) of standard IF (sIF), standard IF with added MFGM (sIFM) and concept IF (cIF, Nuturis®) with large, MFGM coated lipid droplets and HM were compared. **Results:** HM and cIF, both with large lipid droplets, formed an intragastric lipid top layer early during digestion, leading to delayed HM lipid gastric emptying compared to sIF. Gastric aggregate formation preceded lipid top layer formation for sIF, while sIFM remained homogeneous. sIF bioaccessible lipids elicited increased cholecystokinin secretion compared to HM and cIF. Both HM and cIF exhibited lower bioaccessible lipid rates than sIF, suggesting that lipolysis was slower with large lipid droplets. The addition of non-coating MFGM to the standard IF did not significantly impact gastrointestinal handling nor bioaccessibility rates. **Conclusion:** Gastrointestinal handling of cIF is different from sIF, resulting in a lower lipid bioaccessibility rate, which is closer to that of HM. A lower lipid bioaccessibility rate in early life may promote lipid oxidation over storage, benefiting metabolism, growth, and brain development.

1. Introduction

Early life is characterized by a high demand for dietary energy, with early life nutrition being of utmost importance to meet this energy requirement and influence short- and long-term health (Patel & Rouster, 2023; Rolland-et al., 2016). Human milk (HM) is the preferred nutrition in early life. It has a higher energetic efficiency and is associated with a leaner growth, and a reduced risk of later life metabolic disease and obesity compared to infant formula (IF) (Fleddermann et al., 2014; Gale et al., 2012). Furthermore, HM feeding is associated with improved brain development and cognitive outcomes compared to formula

feeding (Horta et al., 2015). As such, nutritional innovations that reduce the gap in developmental outcomes between breastfeeding and formula feeding have the potential to improve public health. Lipids in early life nutrition consist for approximately 98% of triglycerides, are the main source of energy, and their metabolites contribute to growth and development (George et al., 2022). The structural characteristics of lipid droplets differ between HM and standard IF. HM is a colloid with lipids existing as large lipid droplets (~4 µm mode diameter) with a core of triacylglycerol (TAG) coated with a tri-layer membrane consisting of phospholipids, sphingolipids, gangliosides, choline, sialic acid, cholesterol and membrane proteins, known as the milk fat globule membrane

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(MFGM) (Gallier et al., 2015). In contrast, lipid droplets in standard IF are small (0.3–1.0 μm mode diameter) and coated with milk protein (Yuan et al., 2020). A concept IF (Nuturis®) that mimics more closely the characteristics of human milk lipid droplets was developed, comprising large lipid droplets (mode diameter 3–5 μm) that are coated with MFGM components (Gallier et al., 2015). In a recent clinical study, exclusive consumption of this concept IF during the first 4 months of life has been shown to result in a growth trajectory and body mass index at 1–5 years of age that was lower than that of standard formula fed infants, and close to that of breastfed infants (Abrahamse-Berkeveld et al., 2023). The mechanism responsible for the beneficial growth outcomes of HM and concept IF compared to standard IF feeding is not well understood but may be a result of the milks' different lipid droplet characteristics that impact gastrointestinal lipid handling, which represents a sequence of interdependent physical and chemical processes (i.e., gastric lipid top layering, gastric lipid emptying rate, lipolysis rate, and intestinal enteroendocrine cholecystokinin response). Multiple studies in adults have investigated the effects of lipid droplet characteristics on gastrointestinal lipid handling. These studies showed that lipid droplet coating materials affects emulsion stability by their ability to maintain steric and electrostatic repulsion (Acevedo et al., 2022). Loss of this repulsion results in lipid droplet aggregates with increased size, facilitating their upward migration and accumulation in a gastric top layer (Krog, 2002). Lipid top layering, away from the pylorus, delays the overall lipid gastric emptying rate (Camps et al., 2021; Hussein et al., 2015). Lipid droplet size also impacts lipid digestion kinetics (Armand et al., 1999; Benzonana & Desnuelle, 1965). Furthermore, lipid droplet size impacts the intestinal cholecystokinin (CCK) response (Baumgartner et al., 2017; Maljaars et al., 2012), which initiates an inhibitory feedback mechanism that delays gastric emptying and increases satiety and intestinal secretions (Maljaars et al., 2007). Differences in gastrointestinal handling, such as described above, can influence the luminal accessibility of digestive enzymes to lipids, thereby affecting the rate at which lipids become potentially available for intestinal absorption, i.e., become bioaccessible (Grundy et al., 2024), which can subsequently impact the metabolic fate of lipids (Beek & Oosting, 2020; Michalski et al., 2005; Vors et al., 2013). Whether the effects of different lipid droplet characteristics on gastrointestinal handling are applicable to infants is presently unclear, since the infants' gastrointestinal conditions differs in many ways from that of adults (Abrahamse et al., 2012; Beek & Oosting, 2020). We hypothesize that the variations in size and coating of the infant formulas and human milk will lead to differences in their gastrointestinal lipid handling and bioaccessibility rates. To study the gastrointestinal lipid handling of human milk and infant formulas with different lipid droplet sizes and coatings, we employed *in vitro* models, namely: the dynamic TNO Intestinal model (tiny-TIM) and an intestinal epithelial cell model. Previously it has been shown that the TIM model is able to assess lipid digestion in early life nutrition, as well as lipid emulsion stability (Fondaco et al., 2015; Oosterveld et al., 2014).

2. Methods

2.1. Materials

Pancreatin from porcine pancreas (P7545, 8x USP), sodium taurocholate (TC, 85339), sodium taurodeoxycholate (TDC, T0875), phosphatidylcholine (PC, 61755), orlistat (O4139), and pepstatin (EI10) were purchased from Sigma-Aldrich (Amsterdam, the Netherlands). Rabbit gastric extract containing 70 U (tributyrin, pH 5.5 (Myriam et al., 2021))/mg rabbit gastric lipase and 300 U/mg pepsin was purchased from Lipolytech® (Marseille, France). PlasmaFlux P1 dry filters (pore size <0.05 μm) were purchased from the TIM company (Zeist, the Netherlands), Lacprodan® MFGM-10 was acquired from Arla Foods Ingredients (Engroslager, Denmark). Caco-2 cells were purchased from the European collection of authenticated cells cultures (Salisbury, UK). Gibco Dulbecco's modified Eagles medium (DMEM-61965) and

supplements: fetal calf serum (heat inactivated before use, FCS_{hi}, 10%), penicillin-streptomycin (1%), sodium pyruvate (1%), non-essential amino acids (NEAA, 1%) were purchased from Thermo Fischer Scientific (Bleiswijk, the Netherlands).

2.2. Infant formula preparation

A standard infant formula (sIF) with small milk protein coated lipid droplets was reconstituted in 37 °C demineralized water at 12.6 % (w/v). Aqueous MFGM phospholipids were added to the same standard formula to obtain standard IF with non-coating MFGM (sIFM). The aqueous MFGM phospholipids were obtained by pelleting a solution of 11.5 g Lacprodan® MFGM-10/L demineralized water at 60,000 x g for 1 h at 4 °C (Zanabria et al., 2013). The MFGM containing pellet was resuspended in demineralized water in which sIF was reconstituted (at 12.6% (w/v)) to reach a final phospholipid concentration of 0.54 g/L. Concept IF (cIF) with large MFGM coated lipid droplets was reconstituted in 37 °C demineralized water at 13.2% (w/v) and contained 0.54 g/L MFGM derived phospholipids. All IFs were manufactured by Danone, Nutricia Research and contained 52% (w/w) vegetable- and 48% (w/w) anhydrous milkfat. The powder-to-water ratio of the IF powders differed slightly from on-pack recommendations in order to reach the average HM lipid content (McManaman et al., 2021, pp. 91–102). The macronutrient composition of the IFs is shown in Table 1.

2.3. Human milk

HM was collected from 8 donors who indicated having surplus milk that was not needed to feed their infants and who signed written informed consent. The milk was expressed between 60 and 251 days (average 157 days, median 167 days) after term delivery and represented a composite of both foremilk and hindmilk. Per donation, 3 mL was used for macronutrient content determination by MIRIS human milk analyzer (Miris, Uppsala, Sweden), with the remaining volume stored at –80 °C. Prior to experiments, 17 donations were thawed at 4 °C and pooled. The HM macronutrient composition is shown in Table 1.

2.4. Characterization of the milks

The particle size distribution of the milks (Table 1) was measured using light scattering (Malvern 2000; Malvern Instruments, Malvern, UK) using a particle refractive index of 1.46. The theoretical creaming speed of lipid droplets in the milks was calculated according to Stoke's Law (Krog, 2002).

Table 1
Milk characteristics.

	sIF	sIFM	cIF	HM
Lipid (g/L)	34.0 ^b	34.0 ^b	34.0 ^d	34.0 ^a
Mode diameter (μm)	0.38 ± 0.00 ^e	0.38 ± 0.00 ^e	4.23 ± 0.14 ^e	5.37 ± 0.00 ^e
D [4,3] (μm)	0.74 ± 0.18 ^e	1.01 ± 0.06 ^e	6.10 ± 1.00 ^e	5.63 ± 0.03 ^e
D [3,2] (μm)	0.34 ± 0.00 ^e	0.34 ± 0.00 ^e	0.75 ± 0.15 ^e	3.12 ± 0.22 ^e
Protein (g/L)	13.1 ^b	14.7 ^c	12.6 ^d	10.6 ^a
Carbohydrates (g/L)	59.1 ^b	59.1 ^b	66.1 ^d	60.0 ^a
Energy (kcal/L)	623.0 ^b	623.0 ^b	641.0 ^d	620.5 ^a

^a Weighted means of data from donations by MIRIS human milk analyzer.

^b Recipe nutrient content reconstituted at 12.55%.

^c Recipe nutrient content reconstituted at 12.55% plus MFGM-10 Whey protein.

^d Recipe nutrient content reconstituted at 13.21%.

^e Mean ± SD particle size by Mastersizer 3000.

$$v = \frac{g * D^2 (d_p - d_s) * 3.6 * 10^6}{18\mu} \quad (\text{eq.1})$$

Where v is the creaming speed as a function of particle diameter (D), particle and serum densities (d_p and d_s respectively) and serum viscosity (μ). The factor 3.6×10^6 converts the unit of creaming speed from meters per second (m/s) to millimeters per hour (mm/h).

2.5. In vitro gastrointestinal model

Lipid digestion was performed using the dynamic *in vitro* gastrointestinal model tiny-TIM with advanced gastric compartment (agc). The tiny-TIM (agc) is an adaptation of the TIM-1 (Minekus Verhoeckx et al., 2015) wherein the small intestine is being simulated by one instead of three compartments and the gastric compartment has separate antrum and fundus functions, which accurately mimics the human stomach shape and motility (Bellmann et al., 2016). The gastric compartment is oriented in an 'upright' position, with the fundus positioned above the antrum. Ingested milk is mixed with digestive fluids, electrolytes, hydrochloric acid, bicarbonate and bile solution by peristaltic movements of a flexible inner wall (Fig. 1). To account for the presence of endogenous lipids, we conducted blank digestions using phosphate-buffered saline as a substitute for a meal. Gastric antral content was gradually emptied via a pyloric valve into the small intestine. Luminal digesta (200 μ L) was sampled from the bottom gastric-antrum and intestinal sample ports through a luer-lock valve. The fundic top layer was sampled from 30 min onwards by briefly opening the gastric compartment lid to remove luminal digesta from the surface of the meal (Fig. 1). After sampling, the lid was placed back and luminal pressure was restored. Intestinal compartment volume increased by inflow from gastric emptying and digestive fluid addition and decreased through filtration via a hollow-fiber filter cartridge, thereby maintaining a stable volume. The lipid fraction of the milk that passes the filter after gastrointestinal lipolysis and micellar solubilization, consists mainly of free fatty acids and monoglycerides and is considered bioaccessible.

2.6. Preparation of digestive fluids

Small intestinal electrolyte solution (SIES) consisted of 2.5 g NaCl, 0.3 g KCl, 0.15 g $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$ per liter demineralized water). Simulated gastric fluid (SGF, 2.9 g rabbit gastric extract 70, 6.2 g NaCl, 2.2 g KCl, 0.3 g $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$ and 0.82 g Na-acetate per liter demineralized water,

pH 5.0), pancreatin solution (17.5 g pancreatin/L in SIES:demineralized water (1:1 v/v)), centrifuged at $9000 \times g$, 20 min, 4°C) and bile solution were prepared on the day of the experiment. Bile solution was formulated to simulate the infant bile composition and was prepared by dissolving: 5.1 g TC/L, 2.1 g TDC/L, 2.2 g PC/L in 80 mL chloroform:MeOH (1:1 v/v) followed by rotary evaporation under N_2 (Andersson et al., 2011; Elsayed & Cevc, 2011). The resulting lipid film was rehydrated in 800 mL SIES which yielded micelles and vesicles with mode diameters of $7.2 \pm 0.8 \text{ nm}$ and $72.3 \pm 3.6 \text{ nm}$ respectively as measured with zetasizer Ultra (Malvern Panalytical, Malvern, UK).

2.7. Digestive conditions

Digestive conditions were selected to resemble those of a 0–6 month old infant. The meal consisted of 125 mL of milk. Before ingestion, 3 mL of SGF and 22 mL of demineralized water were added to the milk to simulate the fasting gastric contents. Gastric secretion consisted of SGF: Acid 0.5 M HCl/water (1:1 v/v) at 0.3 mL/min with the acid/water mixture regulated to follow a pre-set pH curve (pH 6.8–3.8) as described earlier (Abrahamse et al., 2022). Gastric emptying halftime was set at 60 min, as reported for HM in infants (Bode et al., 2004). Immediately before the start of the experiment the intestinal compartment was filled with 45 mL pancreatin solution, 45 mL bile solution, and 74 mL of SIES. During the experiment pancreatin and bile solutions were both secreted at 2 mL/min. Intestinal pH was maintained at 7.0 by the addition of 1 M sodium bicarbonate. Digestion was simulated for 300 min to mimic the orocecal transit time of infants (Bode et al., 2004). Luminal digesta (200 μ L) were taken every 30 min and immediately mixed (1:1 v/v) with enzyme inhibitor and stored at -20°C . Enzyme inhibitors for gastric digesta consisted of orlistat 0.06 g/L and pepstatin 0.51 mg/L in 0.2 % EtOH. For intestinal digesta only orlistat was used as inhibitor at 0.06 g/L. Bioaccessible lipids were collected continuously over 30 min intervals and 15 mL aliquots were stored at -20°C . From 150 min onwards, the pyloric valve remained open to simulate the postprandial re-contraction of the stomach (i.e. the housekeeping wave) that empties the residual gastric content into the small intestine.

2.8. Lipid analysis

Fatty acids (FA) in digesta and bioaccessible fractions were quantified by gas-chromatography with flame ionization detection (GC-FID). First, internal standard 1,2-dinonadecanoyl-sn-glycero-3-

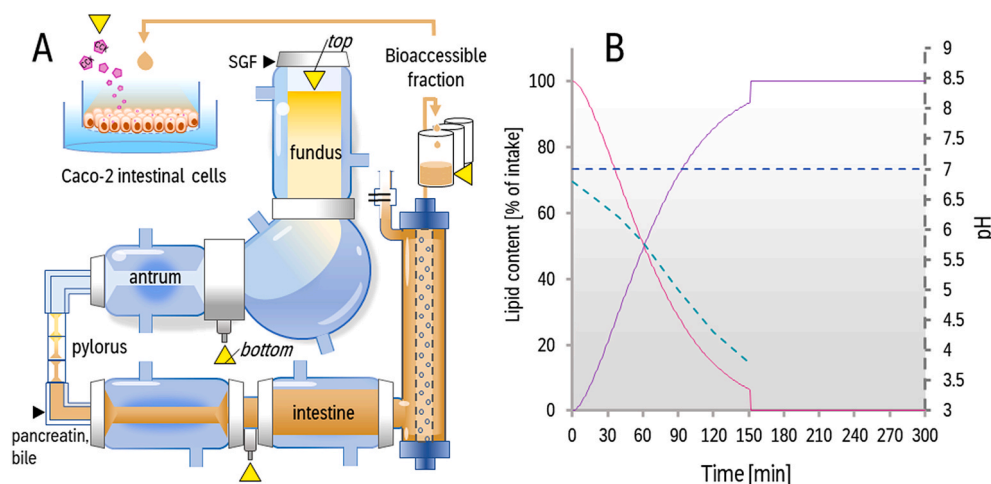


Fig. 1. Schematic of the tiny-TIM and intestinal cell model. Depicting a phase-separated meal within the stomach, where a concentrated lipid layer forms at the top in the fundus, leaving a watery residue in the antrum. Sampling points are indicated by yellow triangles. The filtered intestinal content, representing the bio-accessible lipid fraction, is incubated with intestinal cells after which cholecystokinin is measured (A). Transit of a homogeneous emulsion through the different parts of the model: lipids in the gastric compartment are emptied according to the equation described by Elashoff, Reedy, & Meyer (1982, red). Lipid are emptied and digested in the small intestine (purple). Dashed line shows the pH during gastric (green) and intestinal (blue) digestion (B).

phosphocholine (Avanti Polar Lipids Inc., Alabaster, USA) was added to 1 mL sample in a glass tube to a final concentration of 0.085 mg/mL. 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine, as opposed to free non-adeylic acid was selected as internal standard for its stability during long-term storage. Lipids were then extracted from the tube using a modified Bligh and Dyer protocol (Bligh & Dyer, 1959), with 2 mL dichloromethane, 2 mL methanol, and 1 mL 1% (w/v) EDTA solution. The extraction mixture was centrifuged at 3000 rpm for 10 min, and the resulting dichloromethane layer was removed and dried using a SpeedVac®. Subsequently, the dried lipids and internal phosphocholine standard were hydrolyzed and converted to free FA methyl esters (FAME) by addition of 2 mL methanol and 40 µL concentrated sulphuric acid, followed by heating at 100 °C for 60 min (W.W. and C., 1993). After hydrolysis, 2 mL of hexane and 0.5 mL of 2.5 M sodium hydroxide were added. FAME in the upper hexane layer were quantified using GC-FID (Shimadzu Corporation, Kyoto, Japan), using a CP-SIL88 column (60 m × 0.25 mm id, 0.20 µm film thickness: Agilent Technologies Inc., Santa Clara, USA). FAME were identified based on their retention times using external reference standards GLC-569B and GLC-461 (Nu-Chek Prep Inc., Elysian, USA). Due to discrimination of FAME's smaller than C14 in the injector of GC-FID. The area of these FAME's is corrected by using area ratio of these FAME's against C16:0 in the standard GLC-461. The relative concentration of the identified FAME's in the samples is calculated via the peak area and via the internal standard the concentration of fatty acids in the sample was calculated.

2.9. Determination of emptied and bioaccessible lipids

Lipolysis started in the gastric compartment, and gastric pre-digested lipids were gradually emptied into the intestinal compartment. There, emptied lipids where further lipolyzed and solubilized by pancreatic enzymes and bile. The resulting free fatty acids and monoacylglycerides were separated from intact lipids by continued filtration, which yielded the bioaccessible lipid fraction. The sum of lipids in the intestinal compartment and the filtrate represented the gastric emptied lipids. The proportion of total dietary FA that were present in the small intestinal compartment at (*t*) were calculated according to equation (2):

$$L_{(t)}[\%] = \frac{([L]_{(t)} - [Lblank]_{(t)}) \times 164}{[Lmeal]_0 \times Mmeal_0} \times 100 \quad (\text{eq.2})$$

Where $[L]_t$ is the concentration of FA in the intestinal sample at timepoint *t*, $[Lmeal]_0$ and $Mmeal_0$ are the total FA concentration and mass of the meal at the start of the experiment respectively. 164 (mL) is the static volume of the intestinal compartment. The cumulative proportion of dietary bioaccessible FA, at timepoint (*t*) was calculated according to equation (3):

$$\sum L_{(t)}[\%] = \frac{([L]_{(t)} \times M_{(t)} - [Lblank]_{(t)} \times Mblank_{(t)})}{[Lmeal]_0 \times Mmeal_0} \times 100 \quad (\text{eq.3})$$

Where $[L]_t$ is the concentration of FA in the filtrate sample and $m(t)$ is the mass of collected filtrate at timepoint (*t*). Emptied dietary lipids at timepoint (*t*) were calculated by summing the proportions of total fatty acids in the intestinal compartment (eq. (2)) and the cumulative bioaccessible FA (eq. (3)).

2.10. Determination of lipid halftimes

Lipid gastric emptying and bioaccessibility halftimes were determined by fitting the respective data using a four-parameter logistic regression model according to equation (4).

$$L(t)[\%] = Lmin + \frac{Lmax - Lmin}{1 + \left(\frac{t}{L\frac{1}{2}}\right)^{Slope\frac{1}{2}}} \quad (\text{eq.4})$$

Wherein: $Lmin$ and $Lmax$ give the plateaus at the lower and upper ends of the curve respectively. $L\frac{1}{2}$ gives the time point [min] at which the proportion of total FA reaches 50% of the upper plateau (i.e. the half-time). $Slope\frac{1}{2}$ represents the slope of the curve at $L\frac{1}{2}$. Quest Graph™ Four Parameter Logistic (4 PL) Curve Calculator (AAT and Bioquest, 2023) was used to calculate the values for $Lmin$, $Lmax$, $L\frac{1}{2}$ and the $Slope\frac{1}{2}$. The relative percentual difference (RPD) in emptied and bio-accessible lipid halftimes ($L\frac{1}{2}$) of sIFM, cIF and HM were calculated according to equation (5).

$$RPD L\frac{1}{2} \text{ vs. sIF} = \frac{L\frac{1}{2} x}{L\frac{1}{2} sIF} \times 100 \quad (\text{eq.5})$$

Where $L\frac{1}{2}$ sIF is the lipid halftime of sIF and $L\frac{1}{2} x$ is the lipid halftimes for milk *x*, with *x* being sIFM, cIF or HM.

2.11. Microstructure observation

Microscopic images of digesta were made using an Olympus CKX41 inverse light microscope with a U-LS30-3 camera (3.1 Mp) at 40 × magnification.

2.12. Intestinal cell model

Human colorectal adenocarcinoma Caco-2 cells have been shown to have enteroendocrine activity (Shobatake et al., 2019). In our study Caco-2 cells were maintained in DMEM at 37 °C in a humidified atmosphere of air:CO₂ (95:5, v/v). At passage 64–67, 2×10^5 cells were seeded per insert of 12-well transwell plates and cultured for 21 days. Prior to incubation with digesta, medium was replaced by FCShi- and NEAA-free medium for 1 h, after which cells were washed with PBS. TinyTIM filtrate aliquots at time intervals: 0–30, 30–60, 60–90, 90–120 min of digestion (containing the bioaccessible lipids) were thawed, and per time interval, a weighted pool consisting of 5% w/w of total filtrate from each repetition was prepared. Bioaccessible lipids in filtrates collected from 0 to 120 min are considered most relevant in their potential to affect the CCK mediated gastric emptying rate of the residual gastric content, because from 120 min onwards, the residual gastric content represents less than 14 % of the ingested milk and is therefore unlikely to substantially affect overall gastric emptying. The filtrate' enzymes, bile and lipolytic products can induce cytotoxicity, which was mitigated by heat inactivation at 100 °C for 4 min and dilution 1:8 (v/v) with FCShi- NEAA-free medium (Vors et al., 2012). A 30 min incubation of 8-fold diluted heat inactivated pooled filtrate did not affect cellular metabolic activity as measured by WST assay and as such, was not considered cytotoxic. 1 mL of diluted pooled filtrate was applied apically to the cells. After 30 min of incubation, apical incubate was collected and secreted CCK was quantified using RayBio® Human CCK enzyme immunoassay kit (Raybiotech, Norcross, United-States) according to the manufacturer's instructions.

2.13. Statistics

Dynamic *in vitro* lipid digestion experiments were performed as independent triplicates. Pooled filtrates were applied to intestinal cells in independent quadruplicates. Gastric FA concentration and blank corrected CCK release are displayed as mean ± standard error of the mean (SEM), statistical significance of differences between means we determined by one-way ANOVA with Tukey HSD *post-hoc* test. All statistical analysis were performed with IBM SPSS Statistics 19.

3. RESULTS

3.1. Creaming rates

The creaming rate of lipid droplets in the studied milks (eq. (1))

represents their potential for lipid top layer formation. Creaming rates for sIF, sIFM, cIF and HM were: 0.01 mm/h, −0.01 mm/h, −1.31 mm/h and −2.12 mm/h respectively. Mode diameters are given in Table 1 and other equation parameters were: medium viscosity at 4×10^{-3} Pa s, and serum and particle densities at 1.03×10^3 kg/m³ and 0.88×10^3 kg/m³ respectively (Meng et al., 2022).

3.2. Lipid top layer formation

The FA concentrations in top- and bottom-sampled gastric digests were determined as indicators of the lipid emulsion state, being either: homogeneous or forming a lipid top layer. At 30 min of gastric digestion, HM had formed a lipid top layer, with a higher FA concentration in the top vs. bottom and a HM bottom FA concentration that was lower than those of both standard IFs (Fig. 2). At 60 min, cIF showed lipid top layering resembling that of HM, with a higher top FA concentration vs. the bottom and a lower bottom FA concentration for both cIF and HM compared to sIF and sIFM (Fig. 2). sIF (until 90 min) and sIFM top and bottom FA concentrations were not different, indicating homogeneous lipid emulsions. At 120 min, when 86% of lipids had been emptied from the stomach, sIF showed top lipid layering, as the FA concentration in the top was higher vs. the bottom (Fig. 2). Lipid top layering could be ranked as HM > cIF > sIF > sIFM.

3.3. Microstructure formation

Lipid droplet size and aggregate formation of the milks during gastrointestinal transit were assessed using light microscopy. At 30–120 min of gastric digestion, larger lipid droplets were present in the top vs. the bottom for HM (Fig. 3). Between 60 and 90 min (pH 5.8–5.0) large aggregates started to appear in the IFs. For sIFM the aggregation started at 60 min and for sIF and cIF at 90 min. Aggregates appeared larger for sIF compared to sIFM at 90 min and larger for sIFM compared to cIF. Aggregate formation could be thus ranked as: sIF > sIFM > cIF > HM. Under intestinal conditions, aggregates were resolved.

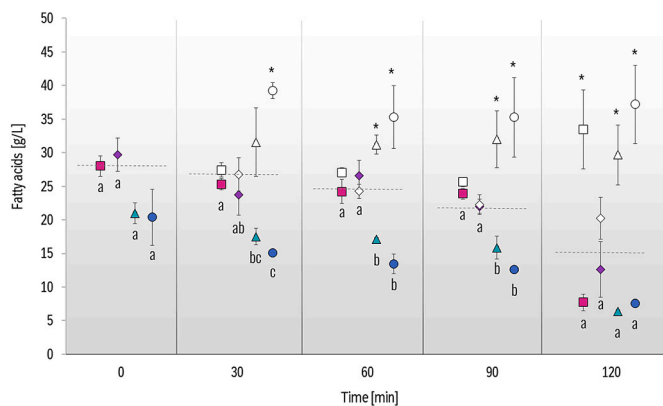


Fig. 2. Time course of fatty acid concentration in the top and bottom of the gastric compartment. Standard infant formula (sIF, squares), standard IF with added MFGM (sIFM, diamonds), concept IF (cIF, triangles) and pooled human milk (HM, circles): mean $\pm$ SEM ($n = 3$). Gastric content was sampled from the top (fundus, white markers) and bottom compartments (antrum, colored markers). Different letters represent significant differences in bottom gastric fatty acid concentration between the milks, determined by one-way ANOVA with Tukey *post hoc* test. (*) indicates a significant difference in the fatty acid concentration at the top and bottom parts of the gastric compartment for the given milk, determined by Students' *t*-test. Horizontal dashed lines indicate the theoretical fatty acid concentration across the gastric compartment during digestion.

3.4. Lipid emptying rates

The FA content of the intestinal compartment and bioaccessible fractions during digestion was quantified to determine the gastric lipid emptying rate. Lower proportions of FA were emptied into the intestine for HM compared to all IFs at 60, 90 and 150 min. At 120 min, both cIF and HM showed a lower proportion of emptied FA compared to sIF and sIFM (Fig. 4A). The data were fitted (eq. (4)) to calculate gastric lipid emptying halftimes ($L_{1/2}^{\text{emptied}}$). The $L_{1/2}^{\text{emptied}}$ was not different between sIF and sIFM. $L_{1/2}^{\text{emptied}}$ tended to be greater for cIF vs. both standard IFs (cIF vs. sIF $+15.4 \pm 2.8$ %, $p = 0.084$, cIF vs. sIFM $+17.7 \pm 2.8$ %, $p = 0.071$) and was greater for HM vs both standard IFs (HM vs. sIF $+31.1 \pm 5.8$ %, Fig. 4C). The lipid gastric emptying rate of cIF and HM was thus lower than that of the standard IFs.

3.5. Lipid bioaccessibility rates

The FA content of the bioaccessible fractions during digestion was quantified to determine the lipid bioaccessibility rate. The proportions of bioaccessible FA were lower for cIF and HM compared to sIF and sIFM from 90 to 240 min, and lower for HM compared to sIF at 60 min (Fig. 4B). The data were fitted (eq. (4)) to calculate lipid bioaccessibility halftimes ($L_{1/2}^{\text{bioaccessible}}$). The $L_{1/2}^{\text{bioaccessible}}$ was not different between sIF and sIFM, nor between cIF and HM. Both cIF and HM $L_{1/2}^{\text{bioaccessible}}$ were greater compared to sIF and sIFM (e.g. cIF vs. sIF $+26.7 \pm 3.6$ %, HM vs. sIF: $+46.0 \pm 5.2$ %, Fig. 4C). The lipid bioaccessibility rates of cIF and HM were thus lower than those of the standard IFs. The magnitude of the differences in $L_{1/2}^{\text{bioaccessible}}$ between sIF and sIFM (small lipid droplets) compared to HM and cIF (large lipid droplets) were notably greater compared to the differences in their $L_{1/2}^{\text{emptied}}$. $L_{\text{max}}^{\text{bioaccessible}}$, (eq. (4)) was calculated to be 90.0 ± 4.2 %, 87.0 ± 1.9 %, 89.2 ± 2.7 % and 99.4 ± 2.8 % for sIF, sIFM, cIF and HM respectively and these did not differ between the milks, neither was there a difference in lipid bioaccessibility measured at 300 min (Fig. 4B).

3.6. Lipolysis rates

To assess whether differences in lipolysis rates between the milks contributed to the outcomes in $L_{1/2}^{\text{bioaccessible}}$, the relative percent difference versus sIF in $L_{1/2}^{\text{emptied}}$ (eq. (5)) was subtracted from the relative percent differences versus sIF in $L_{1/2}^{\text{bioaccessible}}$ ($RPD_{L_{1/2}^{\text{emptied}}} \text{ vs. sIF} \text{ minus } RPD_{L_{1/2}^{\text{bioaccessible}}} \text{ vs. sIF}$). This showed that the relative contribution of lipolysis rate to the $L_{1/2}^{\text{bioaccessible}}$ was greater for cIF and HM compared to sIF and sIFM (cIF vs. sIF $+11.3 \pm 0.8$ %, HM vs. sIF: $+14.9 \pm 0.9$ %, Fig. 4D). The lipolysis rates of cIF and HM were thus lower than those of sIF and sIFM.

3.7. Cholecystokinin response

Bioaccessible fractions from digested milks were incubated on intestinal epithelial cells to assess CCK response. Bioaccessible fractions of sIF and sIFM, collected from 30 to 60 min, elicited a CCK response that was ~ 4.2 times, and ~ 2.7 times higher compared to HM and cIF respectively. The bioaccessible FA concentrations of sIF and sIFM were higher from 30 to 60 min (2.1 ± 0.5 and 2.0 ± 0.1 g FA/L respectively) compared to cIF and HM (1.3 ± 0.1 g and 1.0 ± 0.2 g FA/L respectively). Bioaccessible fractions from sIFM, collected from 60 to 90 min resulted in a higher CCK response compared to HM. There were no differences in CCK response to bioaccessible fractions from sIF and sIFM per time point, as well as between cIF and HM. (Fig. 5A). The CCK response showed a significant linear correlation with free FA content (Fig. 5B).

4. Discussion

We hypothesized that the variations in size and coating of the infant formulas and human milk lipid droplets will lead to differences in their

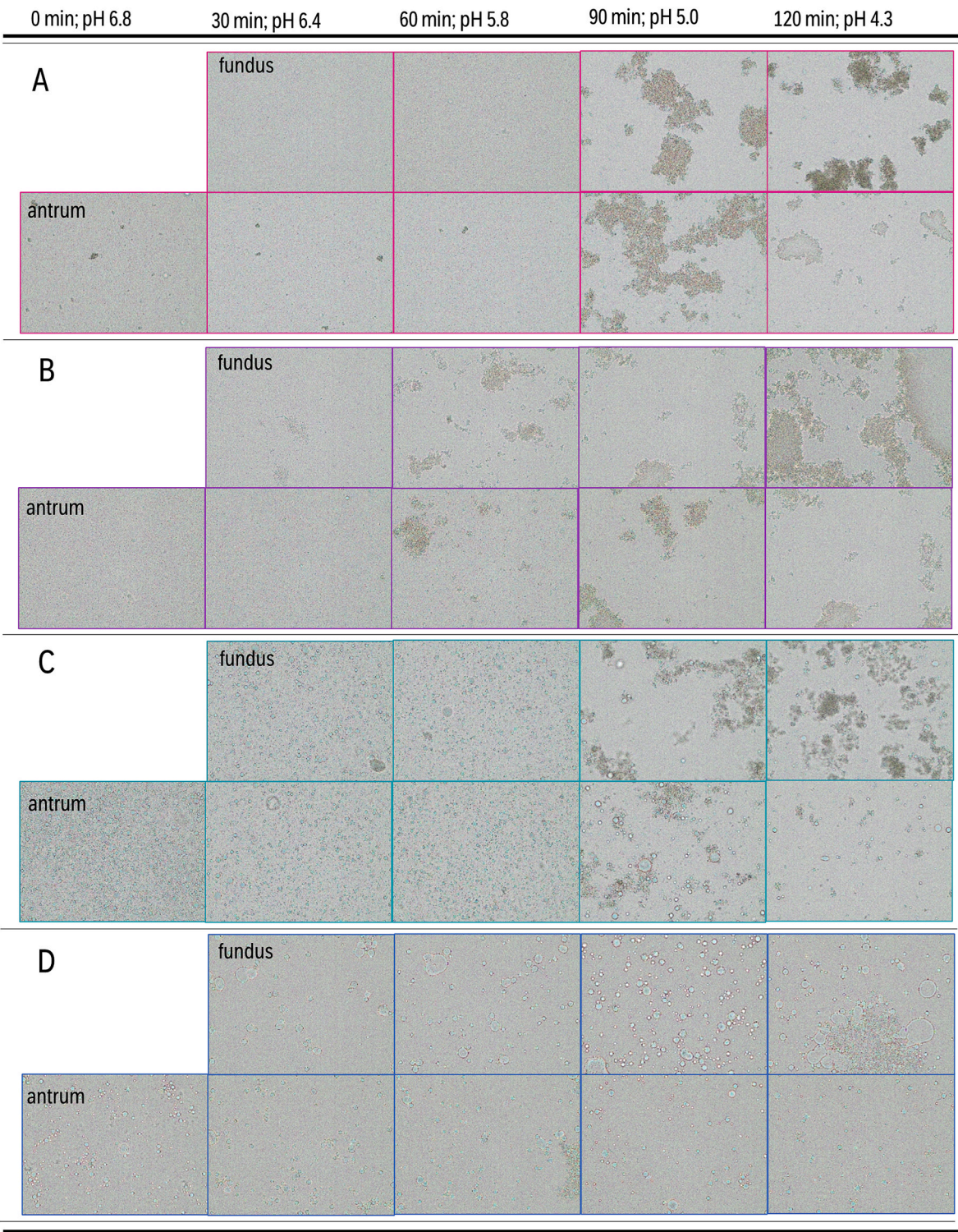


Fig. 3. Light microscopic images of gastric digests. Digests were sampled from the top (fundus) and from the bottom (antrum) part of the gastric compartment. **A:** standard infant formula (IF), **B:** standard IF with added MFGM, **C:** concept IF, **D:** pooled human milk. Sample from left to right taken at: 0, 30, 60, 90, 120 min of gastric digestion.

gastrointestinal lipid handling and bioaccessibility rates. Our present *in vitro* study indeed demonstrated a different lipid handling and bioaccessibility rate for sIF compared to cIF and HM. Furthermore, we showed that these effects were the result of the differences in lipid droplet characteristics and not solely the presence of MFGM, as gastrointestinal handling and bioaccessibility rate were not different between sIF and sIF with non-coating MFGM.

We investigated whether gastric lipid top layering is affected by milk lipid droplet characteristics. In cIF and HM, rapid upward migration (*i.e.* creaming) of large lipid droplets resulted in a lipid top layer. In contrast, the sIF and sIFM lipid emulsions were homogeneous until, at 120 min, the appearance of large gastric aggregates had resulted in lipid top layer formation for sIF, but not for sIFM. The rapid creaming of cIF and HM was likely the result of their initial lipid droplet sizes, as their creaming

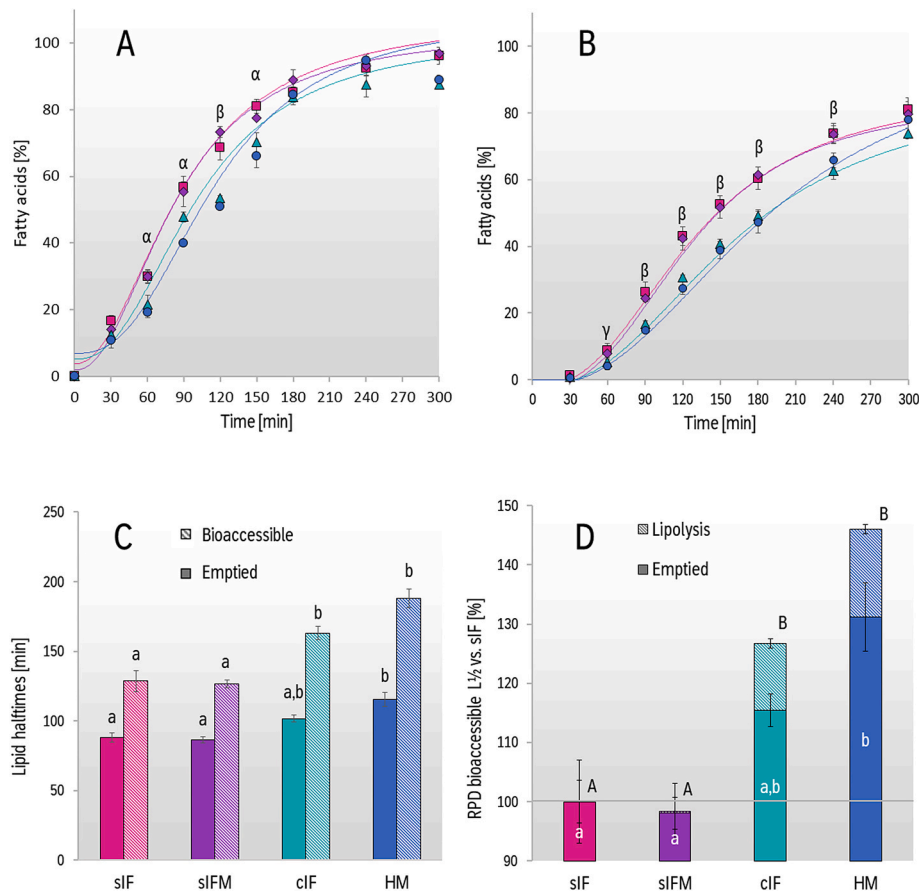


Fig. 4. Time course of emptied and bioaccessible lipid transit during tiny-TIM during digestion. Proportions of total dietary fatty acids: gastric emptied (A) and bioaccessible (B). Lipid half-times ($L_{1/2}$ eq. (4)) for emptied (filled bars) and bioaccessible lipids (dashed bars) (C). Relative percent difference (RPD) in bio-accessible $L_{1/2}$ compared to standard infant formula (sIF), filled bars represent the RPD in emptied lipid half-times, dashed bars represent the contribution of lipolysis to the RPD in bioaccessible half-times (D). Standard infant formula (sIF, red), standard IF with added MFGM (sIFM, purple), concept IF (cIF, green) and pooled human milk (HM, blue). All data as mean $\pm$ SEM ($n = 3$). Curves (A + B) represent fitted data using four parameter logistic regression. Significant differences determined by one-way ANOVA with Tukey *post hoc* test. α indicates significant differences for HM vs sIF, sIFM and cIF. β indicates significant differences for cIF and HM vs. sIF and sIFM. γ indicates a significant difference between HM vs. sIF. Lowercase and uppercase letters represent significant differences in emptied and bioaccessible half-times (C) or emptied and lipolysis RPD (D).

rates were calculated to be 124 and 200 times greater compared to sIF respectively.

The lipid top layering of sIF at 120 min could be explained by the increase in particle size after aggregation of lipid droplets, that likely increased the creaming rate of the resulting aggregates. In contrast, sIFM remained homogeneous, possibly due to a smaller size of gastric aggregates compared to sIF. Overall, gastric aggregates were largest for sIF followed by sIFM and were smallest for cIF followed by HM, which suggests that the MFGM coating limited aggregation and is consistent with previous studies (Liang et al., 2018; Ma et al., 2023; Zhu et al., 2021). The MFGM derived phospholipid coating is potentially limiting aggregation due to the low isoelectric point at pH $\sim$ 4.2 and inertness to pepsinolysis of the phospholipids, thereby maintaining electrostatic repulsion of the lipid droplets under gastric conditions (Lopez et al., 2017). Rapid gastric top layering (10 min postprandial) after HM consumption was also observed in adults (Camps et al., 2021). Contrary to our study however, at that same timepoint, consumption of standard IF resulted in a greater gastric top layer compared to HM. The early onset of standard IF top layering in this adult study vs. the current *in vitro* study, was likely a consequence of increased acid and pepsin secretions in the adult stomach compared to infants (Gan et al., 2018), which accelerates aggregation and lipid layer formation.

In our current study, all milks followed the same gastric volume emptying time by means of the programmed settings in the tiny-TIM, as

where protein dominant IFs and HM do not differ in their volume gastric emptying rate (Billeaud et al., 1990). However, we found differences in the lipid emptying rates of the milks, which were lower for HM compared to sIF, and with cIF showing a tendency in the same direction as HM. sIF and sIFM lipid emptying rates were not different, as, at the moment of lipid top layer formation for sIF at 120 min, 86% of gastric lipid content had been emptied, with the remaining 14% not affecting overall lipid emptying. To our knowledge, no studies have been performed on the lipid emptying of IF or HM in infants. Our study shows a biphasic gastric lipid emptying pattern for cIF and HM, consisting of an initial lipid depleted phase, followed by a lipid rich phase, while standard IFs lipid emptying was more linear.

The effects of lipid top layering on gastric lipid emptying rate that we observed are reflective of an upright body position due to the physical orientation of the tiny-TIM model. Infants however, are commonly lying in the supine position. Multiple studies consistently found that gastric emptying is slower in the supine versus upright position (Halemani et al., 2023). The effects observed by us on lipid gastric emptying may therefore be enhanced *in vivo* as a slower gastric emptying time in the supine position would result in a greater fraction of intragastric lipid to remain in the stomach and be affected by lipid layering.

Following gastric emptying, we found that the lipolysis rates of cIF and HM were lower than those of sIF and sIFM. As lipolysis is an interfacial process, lipid droplet size and coating are known to influence

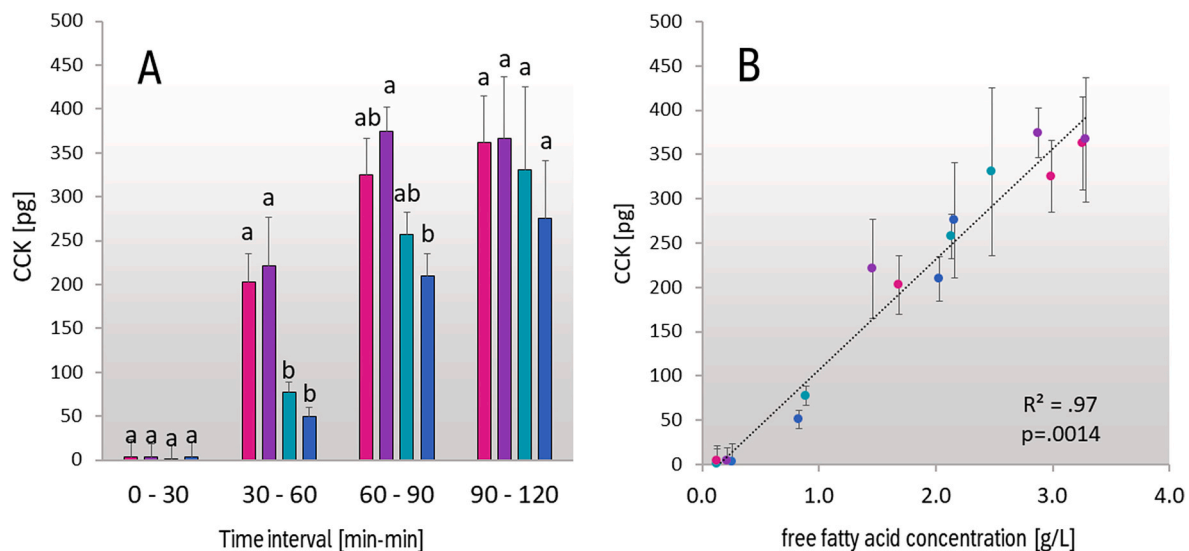


Fig. 5. Intestinal cholecystokinin (CCK) response from bioaccessible lipid fractions. CCK response following incubation of Caco-2 cells with 1:8 (v/v) diluted and heat inactivated bioaccessible lipid fractions collected throughout early gastrointestinal digestion (0–120 min) (A): mean $\pm$ SEM (n = 3). Standard infant formula (sIF, red), standard IF with added MFGM (sIFM, purple), concept IF (cIF, green) and pooled human milk (HM, blue). Different letters represent significant differences determined by one-way ANOVA with Tukey *post hoc* test. Pearson correlation coefficient (R^2) and p-value between incubated bioaccessible free fatty acids and subsequent CCK response (B).

the rate of both gastric and intestinal lipolysis by affecting the available surface area for lipase adsorption and hydrolysis (Abrahamse et al., 2021; Fondaco et al., 2015). Together, the lower gastric lipid emptying and lipolysis rates resulted in lower lipid bioaccessibility rates for both cIF and HM compared to sIF and sIFM.

Total lipid bioaccessibility at the end of the simulated digestion did not differ between the milks, which is consistent with previous studies that compared IFs containing milk fat with HM (Hageman et al., 2019).

Lipolysis and lipid layering are dependent on the colloidal properties of lipid droplets. Freezing human milk at -80°C minimally alters its lipid droplet properties, preserving its colloidal characteristics similar to fresh milk (Lina Zhang et al., 2022).

The bioaccessible fractions of sIF and sIFM elicited a greater intestinal CCK response compared to those of cIF and HM. Lipid in the bio-accessible fractions consist mainly of free FA and monoacylglycerides with long chain free FA being CCK secretagogues (Feltrin et al., 2004). The CCK response by gut epithelial cells to bioaccessible free FA, showed a linear relationship. The bioaccessible FA concentrations of sIF and sIFM were higher from 30 to 60 min compared to cIF and HM. Amino acids also act as CCK secretagogues when incubated on Caco-2, but only in molar concentrations that are ~ 20 times higher compared to long chain free FA (Song et al., 2015). As such, the higher bioaccessible FA concentrations for sIF and sIFM likely resulted in the greater CCK response. *In vivo*, CCK is secreted by I-cells located in the upper duodenum in response to the presence of free fatty acids released from the stomach, and during early intestinal lipolysis (Scheuble et al., 2018). A faster gastric lipid emptying and gastric and intestinal lipolysis is therefore likely to result in a greater CCK response from sIF compared to HM which is in line with findings in infants (Salmenpera et al., 1988).

In adults, it was demonstrated that a higher CCK response after consumption of lipid emulsions containing small droplets, was associated with delayed gastric emptying (Hussein et al., 2015; Maljaars et al., 2012), and a lower rise in postprandial circulating triglyceride (*i.e.* lipemia) compared to large lipid droplets (Baumgartner et al., 2017; Hussein et al., 2015). These findings may not directly translate to infants however, as their enteric gastric emptying regulation is still immature. Indeed, nutritional factors that impact gastric emptying in adults, did not impact gastric emptying in infants (Bourlieu et al., 2014; Ramirez et al., 2006). Animal studies demonstrated functional immaturity of

enteroendocrine regulation until the weaning period (Weller, 2006). Ramirez et al. speculated that there would be limited evolutionary need for enteroendocrine mediated adaptability to changes in nutritional content, because HM is the sole source of early life nutrition (Ramirez et al., 2006). Although more research on infant enteric regulation is needed, it appears that the infants' gastrointestinal tract is less responsive to enteroendocrine inhibitory feedback than the adults'. Therefore, gastric lipid top layering, lipid emptying and lipolysis, are dominant in determining the bioaccessibility rate of milk lipids in infancy. Based on this, we conclude that in infants, cIF and HM bioaccessibility rates are lower compared to sIF and sIFM. A lower lipid bioaccessibility rate helps prevent excessive levels of potentially harmful lipolytic products which may be particularly beneficial for infants since their capacity to absorb lipids is still immature (Burge et al., 2021; Lindquist & Hernell, 2010; Rings et al., 2002). A lower bioaccessibility rate is also expected to result in a lower, more sustained postprandial lipemia. This is in line with findings in infants, where a tendency for lower postprandial lipemia for breastfed compared to standard IF fed infants was found (Teller et al., 2017). Further research is needed to explore the influence of lipid droplet characteristics on intestinal absorption and transport, as these processes may additionally affect postprandial lipemia.

Differences in the pacing of postprandial lipemia are associated with differences in lipid partitioning, *i.e.* the utilization of lipids for energy production (β -oxidation) or lipid storage in adipose tissue (Michalski et al., 2013; Vors et al., 2013). Higher levels of serum β -oxidation markers have been observed in breastfed infants compared to formulafed infants (He et al., 2019; Slupsky et al., 2017). Additionally, mice fed cIF had higher levels of hepatic protein involved in β -oxidation compared to standard IF fed mice (Ronda et al., 2020). It can therefore be hypothesized that lower bioaccessibility rates and postprandial lipemia with cIF and HM, predisposes the infant to utilizing lipids for β -oxidation, which could contribute to a higher energetic efficiency and the observed beneficial growth effect in infants that consumed cIF or HM compared to those who consumed sIF during early life (Abrahamse-Berkeveld et al., 2023). Furthermore, β -oxidation yields ketone bodies, providing a supplemental energy source for the rapidly growing infant brain which consumes up to 66% of total body energy, as opposed to only $\sim 20\%$ in adults (Cunnane & Crawford, 2014). More research on a potential effect of lipid bioaccessibility on lipid partitioning, infant

growth and brain development is warranted.

5. Conclusion

This study highlights the impact of lipid droplets characteristics on the kinetics of lipid bioaccessibility and their potential physiological effects. Large phospholipid coated lipid droplets of the concept IF and HM formed a lipid top layer and were consequently retained in the stomach longer compared to standard IF. Under intestinal conditions, concept IF and HM lipid droplets showed a lower lipolysis rate and CCK response compared to standard IF. These processes resulted in a lipid bioaccessibility rate for concept IF that was lower than standard IF and closer to that of HM without affecting total lipid bioaccessibility. A more gradual lipid availability, can result in a more beneficial lipid utilization which could impact lipid metabolism, growth and brain development.

CRediT authorship contribution statement

G.G.M. Thomassen: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **E. Abrahamse:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization. **M. Mischke:** Writing – review & editing. **M. Becker:** Methodology, Formal analysis. **N. Bartke:** Writing – review & editing, Project administration. **J. Knol:** Writing – review & editing, Supervision. **I.B. Renes:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The research was funded by Danone Nutricia Research. All authors are employees of Danone Nutricia Research.

Data availability

Data will be made available on request.

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