Milk mimicry – Triglyceride mixtures that mimic lipid structuring during the digestion of bovine and human milk

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A B S T R A C T

It is well recognised that the digestion of milk lipids releases fat-soluble bioactives, primarily in the intestines where 70–90% of lipid digestion occurs. The digestion of milk lipids under intestinal conditions yields fatty acids and monoglycerides that self-assemble into liquid crystalline phases that are species-dependent, suggesting that they play a role in directed nutrition. Yet to date, no studies have proposed mimicking milk liquid crystalline structuring by precisely tailoring the lipids in the digesting emulsions. In this work, the preparation of lipid emulsions that mimic lipid self-assembly in bovine and human milk was examined. Mixtures of between four and eight off-the-shelf homotriglycerides were prepared based on the total fatty acid content of bovine and human milks and these were emulsified using casein. If kept at or above body temperature after emulsification the emulsions were stable and 84 ± 6% (bovine) and 86 ± 3% (human) of the lipids could be digested under intestinal conditions over 2 h. The evolution of liquid crystalline phases was determined as a function of extent of lipid digestion enabled by the well-defined nature of the lipid emulsion and the emulsifying buffer solution. It was found that eight triglycerides with a fine balance of medium- and long-chain triglycerides were required to mimic the bicontinuous cubic phases observed in digesting commercial bovine milk. In contrast, the micellar cubic phases observed in the digestion of donor human milk could be mimicked with simpler lipid mixtures comprising four–seven triglycerides comprising mostly long chain and unsaturated triglycerides. These simplified mixtures are therefore representative colloidal structures that mimic the lipid self-assembly behaviour in digesting dairy emulsions, which can be used as oral delivery vehicles of fat-soluble bioactives targeted towards particular populations including infants in the first year of life.

1. Introduction

Milk is a vital nutrient delivery system for mammals but despite its ubiquity the physico-chemical behaviour of milk under physiological digestion is still poorly understood, limiting our ability to fully harness its potential as an active biomaterial. Milk is a complex mixture of emulsified triglycerides, caseins, whey proteins, vitamins and minerals specifically tailored to the biology of mammalian species to meet the nutrient requirements of their infants. In the case of humans, it is well established that nutritional outcomes for children who are unable to be fed mothers’ milk tend to be poorer than their breast-fed counterparts (Lawrence, 1994; Martin, Ling, & Blackburn, 2016). In nature, milk delivers poorly-soluble lipophilic nutrients [fats in the form of triglycerides, polyunsaturated fatty acids (PUFAs), sterols, vitamins A, D, E and K] that otherwise display solubility-limited absorption in the intestines where the majority of lipid digestion and nutrient absorption occurs (Ballard & Morrow, 2013; Bernbäck, Blackberg, & Hermell, 1989; Carey, Small, & Bliss, 1983; Carriere, Barrowman, Verger, & René, 1993; Hinsberger & Sandhu, 2004). These lipophilic nutrients are initially bound in emulsified milk fat globules, comprising one of the most complex naturally-occurring fat mixtures (Christie & Clapperton, 1982; Fox & McSweeney, 2006; Innis, 2011; Jenness, 1979; Jensen et al., 1995; Jensen & Newburg, 1995; Morera Pons, Castellote Bargalló, Campoy Folgoso, & López Sabater, 2000; Morera Pons, Castellote Bargalló, & López Sabater, 1998a, 1998b; Zou et al., 2013). Lipid digestion hydrolyses the milk fats into 2-monoglycerides and fatty acids, which releases lipophilic nutrients for absorption in the intestine. In order to understand the role of milk as a nutrient delivery vehicle, it is therefore crucial to understand how these complex digesting lipids interact with each other and with lipophilic bioactives to drive nutrient absorption. However, most contemporary studies into the influence of lipid digestion on intestinal colloids focus on a single lipid acyl chain length or a single digestion product, often incorporating oleyl (C18:1) glycerides and fatty acids (Hjelm, Schteingart, Hofmann, & Sivia, 1995; Hjelm, Schteingart, Hofmann, & Thiyagarajan, 2000; Hofmann, 1968; Kossena, Boyd, Porter, & Charman, 2003; Mele et al., 2018; Nakano...
et al., 2002; Phan, Salentinig, Gilbert, et al., 2015; Phan, Salentinig, Hawley, & Boyd, 2015a; Rezhdo et al., 2017; Salentinig et al., 2014; Shao, Bor, Al-Hosayni, Salentinig, & Yaghmur, 2018; Yaghmur, Al-Hosayni, Amenitsch, & Salentinig, 2017; Yaghmur et al., 2006), providing an inadequate picture of the intestinal emulsions crucial to lipophilic nutrient absorption. This work represents a step towards generating representative colloidal mixtures that mimic mammalian milks from a lipid self-assembly perspective through the creation of simplified but representative lipid mixtures.

During digestion of the lipids in donor human milk and commercial bovine milk, the digestion products self-assemble into liquid crystalline structures that occur in a well-defined sequence (Fig. 1) (Chulow, Salim, Hawley, & Boyd, 2018; Salentinig, Phan, Hawley, & Boyd, 2015; Salentinig, Phan, Khan, Hawley, & Boyd, 2013). Lamellar (L) phases are observed throughout the digestion of the milks of both species and these have been shown to be calcium soaps formed by the binding of native calcium ions in the milk by ionised fatty acids released during digestion (Chulow et al., 2018). In the case of bovine milk prominent hexagonal (H$_2$) and bicontinuous cubic (V$_2$, Im$3m$ space group) phases form after the formation of calcium soaps with the presence of a weak transient micellar cubic (I$_2$, Fd$3m$ space group) phase sometimes observed prior to the formation of the hexagonal phase. In the case of donor human milk only a persistent inverse micellar cubic (I$_2$, Fd$3m$ space group) phase is observed after the formation of calcium soaps. In the current study, casein-stabilised lipid emulsions with increasing numbers of homotriglycerides were digested to determine which lipid acyl chain types were crucial to these self-assembly processes. The mixtures were designed to mimic the total fatty acid content of bovine and human milk with greater numbers of triglycerides leading to increased lipid diversity/complexity in the digestion products. The thermal properties of the lipid mixtures and their digestibility under in vitro intestinal conditions were quantified. These data were then compared with small angle X-ray scattering (SAXS) profiles recorded during digestion to determine if the emulsions followed the same progression of lipid self-assembly as human and bovine milks.

2. Experimental section

**Materials** – Tricaprin (C10:0, 98%), trilaurin (C12:0, >98%), trimyristin (C14:0, >95%), tristearin (C18:0, >80%) and triolein [C18:1, 80% with the major impurity being trilinolein (C18:2)] were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Two batches of tricaprylin (C8:0) were used for these experiments, both with stipulated purities on the order of >99% and these were purchased from Sigma Aldrich (St Louis, MO, USA) and Nu-Chek Prep (Elysian, MN, USA). Tributyrin (C4:0, 97%), tripalmitin (C16:0, >85%), casein from bovine milk (technical grade) and trizma® maleate were purchased from Sigma Aldrich (St Louis, MO, USA). Calcium chloride dihydrate (Univar AR grade) was purchased from Ajax Fine Chemicals (Seven Hills, NSW, Australia). Sodium chloride (99.7%) and propylene glycol (99.5%) were purchased from Chem Supply (Gillman, SA, Australia). Sodium hydrosxide pellets and sodium azide (>99%) were purchased from Merck (Darmstadt, Germany). Hydrochloric acid (36% aqueous solution) was purchased from Labserv (Longford, Ireland). Lactose monohydrate

![Fig. 1. Structuring of milk lipids during digestion to be mimicked in this study a) Commercial bovine milk and b) human donor milk (data replotted with permission from (Salentinig et al., 2015)). The plots on the left indicate the appearance of X-ray diffraction peaks corresponding to the liquid crystalline phases as a function of digestion time. Peak labels indicate the liquid crystalline structure of the self-assembling milk lipids during digestion as follows: I = V$_2$ inverse bicontinuous cubic phase (Im$3m$ space group), F = I$_2$ micellar cubic phase (Fd$3m$ space group), H = H$_2$ inverse hexagonal phase, L = lamellar phase associated with calcium soaps of fatty acids released during digestion.]
Food Hydrocolloids 110 (2021) 106126

Preparation of mixed homotriglyceride emulsions – Homotriglyceride mixtures were dispersed in a continuous phase comprising casein and lactose in tris maleate digestion buffer. The lipid content in the emulsions was 700 mg of lipid in 20 g of dispersion (3.5 wt %) and the relative amount of each lipid was based on the reported mole% of acyl chains in bovine and human milk from a recent reverse-phase high performance liquid chromatography study (Zou et al., 2013). The casein emulsifier content was matched to reported values for bovine and human milk to provide a similar emulsification capacity for the appropriate emulsions (Jenness, 1979; Jost, 2007). Triglycerides were transferred into 20 mL scintillation vials and the target masses of triglyceride in each mixture are outlined in Table 1. Propylene glycol (35 ± 1 mg) was added to aid dispersion and inhibit crystallisation of the triglycerides in the emulsions. Tributyrin (C4:0) and tricaprylin (C8:0) were measured out with volumetric pipettes using their manufacturer reported densities of 1.03 and 0.956 g cm⁻³, respectively. Triolein (C18:1) and propylene glycol were weighed into the vials using a Pasteur pipette for transfer to within ±1.0 mg of the target mass. All other triglycerides were weighed onto weighing paper before being transferred into the vials, with the difference between the lipid-laden and final mass of the weighing paper recorded to measure the amount of lipid transferred from the weighing paper. All solid lipids were weighed to within ±1.0 mg of the target mass. Once all lipids were transferred into the vials, the mixtures were melted in an oven at ~75 °C for 20–40 min. The molten lipids were swirled to thoroughly mix them before they were allowed to cool to ambient temperature (19–22 °C) and all mixtures were observed to resolidify into a waxy solid upon cooling to ambient temperature. The lipid mixtures were then stored in a freezer at ~20 °C until used. Aqueous solutions of casein and lactose in digestion buffer (50 mM tris maleate, 150 mM sodium chloride, 5 mM calcium chloride, 6 mM sodium azide, pH 6.5) were used to emulsify the lipids. For bovine milk-mimicking mixtures the buffer comprised 2.50 g of casein, 2.50 g of lactose monohydrate and 95.00 g of digestion buffer. For human milk-mimicking mixtures the buffer comprised 0.20 g of casein, 7.00 g of lactose monohydrate and 92.80 g of digestion buffer. Casein was initially dispersed in the digestion buffer by tip ultrasonication with a Misonix S-4000 Ultrasonic Liquid Processor. The mixtures were ultrasonicated with an amplitude of 30 for a total sonication time of 2 min 30 s (pulse cycle = 5 s on/5 s off) three–four times, agitating the mixtures between ultrasonication cycles until all of the casein granules were observed to be dispersed. Lactose monohydrate was then added and any water lost to evaporation during ultrasonication was replaced with ultrapure water. The casein/lactose/tris buffer thus prepared (19.3 g) was added to the lipid mixtures (0.7 g) in sealed 20 mL scintillation vials and the lipids were melted in an oven at 75 °C. The lipid mixtures were then emulsified using tip ultrasonication whilst still hot. The emulsions were ultrasonicated with an amplitude of 30 for a total sonication time of 30 s (pulse cycle = 2 s on/2 s off) three times, agitating the mixtures between ultrasonication cycles. The mixtures were then immediately loaded into the digestion vessel pre-equilibrated to 37 °C.

Preparation of pancreatic lipase – Porcine pancreatin was freeze-dried for storage before use using the following procedure (Phan, Salentinig, Hawley, & Boyd, 2015b). Batches of pancreatin obtained from MP biologicals (20 g each) were poured into 50 mL Falcon tubes and mixed with water (25 mL). The contents of the Falcon tubes were thoroughly mixed using a combination of agitation and vortex mixing to ensure complete dispersion of the pancreatin powder. The mixtures were then centrifuged in an Eppendorf Centrifuge 5804 R at 2205 × g for 15 min before the supernatant was collected and subjected to a second centrifugation. The supernatant from the second centrifugation was partitioned into 5 mL aliquots in 20 mL scintillation vials, which were capped with paraffin that was pierced with a needle 10–15 times. The contents of the vials were frozen by placing them in dry ice before they were loaded into a VirTis Advantage freeze-drier and freeze-dried for a minimum of 60 h. After freeze-drying the pancreatin was stored in the freezer until ready for use. For use in lipolysis experiments, the freeze-dried pancreatin was reconstituted by adding digestion buffer (50 mM tris maleate, 150 mM sodium chloride, 5 mM calcium chloride, 6 mM sodium azide, pH 6.5) to pre-weighted freeze-dried pancreatin in the following ratio 1 mL buffer : 217 mg freeze-dried pancreatin. The lipolytic activity of the resulting reconstituted pancreatin suspensions was tested by digesting tributyrin (6 g, 5.8 g/l tris maleate digestion buffer with a pH of 7.5 (18 mL) at 37 °C using reconstituted pancreatin suspension (2 mL). The activity of the batches of freeze-dried pancreatin prepared were found to vary with supplier batch between 500 and 800 TBUs where TBU is a tributyrin unit 1 μmol titratable butyric acid produced per minute of tributyrin lipolysis.

In vitro lipolysis measurements - A pH-stat apparatus (Metrohm Titirando 902) was used to perform in vitro lipolysis. Emulsions (18 mL) to be digested were loaded into a jacketed glass digestion vessel held at 37 °C by a water heater/circulator bath. After equilibration to the measurement temperature, the pH of the emulsion was adjusted to pH 6.500 ± 0.005 with the minimal amount of aqueous sodium hydroxide or hydrochloric acid solutions (0.2–5.0 M). Reconstituted pancreatin suspension was injected into the emulsion to initiate digestion and the rate of fatty acid evolution was monitored as a function of time by measuring the amount of sodium hydride titrant (0.2 M) required to maintain the pH of the digesting emulsions at 6.5 by neutralising titratable fatty acids released. To determine the total amounts of fatty acids released a back titration was performed at the end of the lipolysis measurements to raise the pH of the digested emulsions to 9.0 and ionise all fatty acids evolved in the mixture. Analogous lipolysis measurements were performed on the bovine and human casein/lactose/tris buffers used to prepare the emulsions for substitution of triglycerides in the digestion buffer (no added lipids from the corresponding emulsions). This accounts for the production of other digestion products such as amino acids/peptides from hydrolysed casein in the buffer solution, hydrolysed proteins in the porcine pancreatin mixture and for the intrinsic buffer capacity of the buffer. The forward and back titration measurements on the buffers (n = 3) were used to determine the total fatty acids released during the titration of the digesting homotriglyceride mixtures (n = 2 for each lipid composition giving n = 10 for bovine and n = 8 for human) as previously reported (Stillhart, Dürr, & Boyd, 1985).

Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Mimic</th>
<th>Amount of Triglyceride in 20 g emulsion (μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C4:0</td>
<td>C8:0</td>
</tr>
<tr>
<td>Bovine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 TG</td>
<td>92</td>
<td>30</td>
</tr>
<tr>
<td>7 TG</td>
<td>94</td>
<td>51</td>
</tr>
<tr>
<td>6 TG</td>
<td>98</td>
<td>53</td>
</tr>
<tr>
<td>5 TG</td>
<td>102</td>
<td>–</td>
</tr>
<tr>
<td>4 TG</td>
<td>115</td>
<td>–</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 TG</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>6 TG</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5 TG</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4 TG</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* As tributyrin (C4:0) and tricaprylin (C8:0) were measured out with volumetric pipettes the uncertainty quoted is likely an overestimate.
Kuentz, 2014). Briefly, the average amount of titrant added as a function of time in the forward and back titration of the buffers were subtracted from the corresponding forward and back titration profiles for each sample. The total amount of fatty acid added in both forward and back titrations (minus buffer) was then used to scale the forward titration profiles such that the final data point was equal to the total amount of titrant added. The extent of digestion was then expressed as the percentage of lipids digested determined from the known molar quantity of lipids weighed out for each individual lipid mixture assuming that 1 mol of triglyceride would be digested to give 2 mol of fatty acid if fully digested. In previous studies on mammalian milks, it was either assumed that the extent of lipid digestion reached 100% when the titration curve plateaued (Salenting et al., 2015; Salenting et al., 2013) or digestion buffer was used as an approximation for the dispersant used in the background titrations without lipid required to determine the extent of digestion, which also led to extents of digestion around 100% (Clulow et al., 2018). The use of the same buffer in the digestion measurements both with and without lipid in this work provides a more accurate measurement of the extent of digestion and as such direct comparisons with previously reported extents of lipid digestion for bovine and human milk, which are approximations, have not been drawn.

Small angle X-ray scattering (SAXS) experiments with in vitro lipolysis – Small angle X-ray scattering measurements were performed on the SAXS/WAXS beamline at the Australian Synchrotron. 2D X-ray scattering patterns were recorded on either a Pilatus-1M or a Pilatus-2M detector with incident X-rays with energies between 11 and 13 keV (wavelengths λ between 0.954 and 1.127 Å). Sample-detector distances between 1500 and 1700 mm gave minimum Q values around 0.02–0.03 Å⁻¹ and a sufficiently wide Q-range (Q = 4π/λsin(θ) where the scattering angle is 2θ) to observe at least three diffraction peaks from the liquid crystalline phases formed during lipid digestion (Qmax = 0.60 Å⁻¹ with the Pilatus 1M detector and 1.20 Å⁻¹ with the Pilatus 2M detector) (Clulow et al., 2018; Salenting et al., 2015; Salenting et al., 2013). To couple the in vitro lipolysis measurement to the SAXS measurements, a quartz capillary (external diameter 1.5 mm) was mounted in the path of the synchrotron X-ray beam. A peristaltic pump was used to flow a fraction of the digesting mimic mixtures through the capillary at a rate of ~10 mL min⁻¹ and back into the digestion vessel, whilst X-ray scattering patterns were measured once every 20 s (5 s acquisition time). The reconstituted pancreatic suspensions were injected remotely using a syringe driver and the progress of digestion was monitored using cameras inside the experimental hutch. Bovine mimic emulsions and commercial bovine milk were digested for just over 60 min and human mimic emulsions were allowed to digest for 90 min given their apparent slower rate of initial digestion. It should be noted that the SAXS measurements on the 8 TG bovine mimic emulsion were terminated after 50 min because an intermittent fault in the detector was observed, which meant that not all data was recorded. Missing frames from this measurement are indicated in Figure S4 f) (online supplementary material) showing that no frames were missing during the first 45 min of digestion.

Scatterbrain software (version 2.82, developed by the SAXS/WAXS beamline team) was used to export profiles of scattered X-ray intensity (I(Q)) versus the scattering vector Q. Intensity maps were generated as a function of time using IgorPro graphing software (Version 7.0.8.1). The relative position of diffraction peaks observed were used to identify the lipid liquid crystalline phases that formed during milk digestion. Phases were identified when at least three characteristic diffraction peaks from a given phase were observed (with the exception of sponge phases that only displayed one broad diffraction peak) and the lattice parameters of the phases were determined by fitting the location of these characteristic peaks in Q using the multipeak fitting algorithm in IgorPro (version 7.0.8.1). The following equations were used to determine the lattice parameters (a) from the characteristic peak multiplier (x) and the corresponding peak Q value (Qpeak) (Hyde, 2001; Seddon & Templer, 1995):

\[ a = \frac{4\sqrt{3}}{Q_{\text{peak}}} \text{ where } x = 1, 2, \text{ or } 3 \text{ (first three peaks)} \]

Micellar cubic (I2, Fd3m) phase: \[ a = \frac{4\sqrt{3}}{Q_{\text{peak}}} \text{ where } x = 3, 8 \text{ or } 11 \text{ (first three peaks)} \]

Bicontinuous cubic (V2, Im3m) phase: \[ a = \frac{4\sqrt{3}}{Q_{\text{peak}}} \text{ where } x = 2, 4, \text{ or } 6 \text{ (first three peaks)} \]

Hexagonal (H2) phase: \[ a = \frac{4\sqrt{3}}{Q_{\text{peak}}} \text{ where } x = 1, 3 \text{ or } 4 \text{ (first three peaks)} \]

**Thermal analysis –** Differential scanning calorimetry was performed using a PerkinElmer DSC8500 instrument maintained by the Helen Macpherson Smith Trust laboratory within the Monash Institute of Pharmaceutical Sciences. Pre-mixed lipid mixtures (3–5 mg) were weighed into aluminium sample pans, which were then hermetically sealed with a hand press. Samples were loaded at 30 °C and then subjected to two heating and cooling cycles between 80 °C and –30 °C with a heating/cooling rate of 10 °C min⁻¹. After the second cooling cycle the sample was returned to the loading temperature before the next sample was loaded. All data shown herein come from the second heating and cooling cycles.

**3. Results**

### 3.1. Thermal properties

The melting and freezing behaviours of the triglyceride mixtures between –35 and 80 °C were measured using differential scanning calorimetry and the resulting thermograms are given in Fig. 2. The triglyceride mixtures were prepared in the same manner as those emulsified for digestion studies with 700 mg of lipid being measured into vials with 35 mg of propylene glycol before the mixtures were melted and mixed at 75 °C before they were resolidified.

The bovine milk-mimicking triglyceride mixtures all displayed two melting transitions between ~20 and 0 °C and a prominent melting peak with low temperature shoulders between 30 and 65 °C [Fig. 2 a)]. The more complex bovine mimics containing 6-8TG also displayed a weak melting transition between 10 and 20 °C, which is likely associated with the melting of tricaprin (C10:0), which is present in these mixtures but not the 4-5TG bovine mimics. Upon cooling all of the mimics displayed two crystallisation transitions between 40 and 20 °C, with further broad crystallisation transitions below –10 °C [Fig. 2 b)]. The human milk-mimicking triglyceride mixtures all displayed two melting transitions between ~20 and 5 °C upon heating [Fig. 2 c)]. A weak and broad melting transition was evident in all samples between 20 and 35 °C, which adjoined a prominent melting peak with a low temperature shoulder between 35 and 60 °C. Upon cooling three distinct crystallisation transitions were observed between 40 and 0 °C with a further broad crystallisation observed below –10 °C [Fig. 2 d)].

The point at which the lipids were fully molten upon heating and began to crystallise upon cooling were determined from the points at which the associated endo/exotherms deviated from the baseline. All of the milk-mimicking lipid mixtures became completely molten between 59 and 64 °C [Fig. 2 e)] and began to crystallise upon cooling at close to body temperature between 39 and 35 °C. The lipid mixtures could therefore be supercooled below the fully melted temperature by around 20 °C before they began to recrystallise when the cooling rate was 10 °C min⁻¹.

### 3.2. In vitro digestion

The milk-mimicking emulsions were digested under in vitro intestinal conditions and the extent of digestion [Fig. 3] was measured through the liberation of fatty acids that were titrated using a pH stat apparatus. The extent of digestion was expressed as the percentage of digested triglyceride assuming that each mole of triglyceride in the digesting mixture

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This text is a sample of a scientific article that includes detailed descriptions of experimental methods, data analysis, and results. It is designed to be read naturally, providing insights into the research on food hydrocolloids and their digestion, with a focus on lipid digestion and characterization techniques. The text includes references to previous studies and methodologies used to ensure accuracy and comparability in the research. The analysis of data through various techniques such as titration, SAXS/WAXS, and differential scanning calorimetry (DSC) provides a comprehensive understanding of lipid digestion and its consequences on milk emulsions.
released 2 mol of titratable fatty acid. All of the bovine milk-mimicking emulsions were digested at a similar rate and all of the human milk-mimicking emulsions were digested at a similar rate, irrespective of the number of triglycerides in each mixture as shown in Fig. 3 (all individual digestion profiles are shown in Fig. S1, electronic supplementary material). For the bovine milk-mimicking triglyceride mixtures, 34 ± 4% of the triglycerides were rapidly digested in the first 4 min of digestion. This was followed by gradual slowing of digestion up to 40 min digestion time (Fig. S2, electronic supplementary material) after which 75 ± 6% of the triglycerides in the emulsion had been digested. After 40 min of digestion the rate of digestion was slow and near-linear up to 2 h at which point 84 ± 7% of the triglycerides in the mixtures were digested. When examining the individual extents of digestion of the emulsions, the emulsions containing more triglycerides tended to be digested more completely during the two-hour digestion period (Fig. S3, electronic supplementary material) but each mimic had a replicate close to the average extent of digestion after 2 h. The human milk-mimicking triglyceride mixtures were initially digested at a faster rate than the bovine milk-mimicking emulsions as shown in Fig. 3, with the initial rapid stage of digestion lasting for 2 min resulting in 23 ± 1% digestion. However, the next phase of digestion between three and 30 min was slower than for the bovine milk-mimicking emulsions. Despite this, the extent of digestion after 40 min was 69 ± 4%, which is within the experimental uncertainty of the bovine milk-mimicking mixtures at the same digestion time. Similarly, the final extent of digestion of the human milk-mimicking emulsions after 2 h of digestion of 86 ± 3% was also the
same as that of the bovine milk-mimicking mixtures within experimental uncertainty. The digestion profiles for the human milk-mimicking emulsions were more consistent than for the bovine milk-mimicking emulsions, which is consistent with their triglyceride compositions being more similar.

3.3. Liquid crystalline phase analysis by SAXS — bovine milk mimics

The bovine and human milk-mimicking emulsions were digested in vitro and the X-ray scattering patterns of the digesta were measured simultaneously. The scattering patterns of the digesting bovine milk-mimicking mixtures are shown in Fig. 4 and by combining these scattering patterns and digestion profiles as a function of time (Fig. 3), the self-assembly behaviour of the lipids was directly related to the extent of digestion (Fig. 5). The relative positions of the diffraction peaks observed were used to identify the phases forming during digestion.

The scattering patterns of all of the bovine milk-mimicking emulsions contained a number of common features. Firstly, persistent lamellar phases with diffraction peaks around $Q = 0.13-0.15, 0.26-0.30$ and $0.39-0.45 \text{ Å}^{-1}$ (peak ratio 1:2:3) were observed in all of the bovine milk-mimicking emulsions both before and throughout digestion (extent of digestion $= 0 - (76 \pm 6)\%$). Prior to the onset of digestion lamellar phases with lattice parameters around $42.1-42.2$ and $47.2-47.7$ Å were observed in the bovine milk-mimicking emulsions with 5–8TGs and these remained persistent throughout the digestions (Fig. S4 in the electronic supplementary material). The least complex 4 TG mimic initially contained lamellar phases with shorter lattice parameters of 38.9 and 40.7 Å and only the phase with longer lattice parameter appeared to persist throughout the digestion, with a lamellar phase with lattice parameters of 40.3–41.5 Å observed throughout digestion. After lipase was injected to initiate digestion, more intense diffraction peaks corresponding to additional lamellar phases with lattice parameters between 42.3 and 46.9 Å were observed to evolve throughout the course of digestion of each emulsion. The final lattice parameters of these additional phases were on the order of 44.8–45.7 Å at the end of digestion.

Another common feature in all of the digesting bovine milk-mimicking mixtures was the formation of an inverse hexagonal (H$_2$) phase comprising rod-like water channels arranged in a hexagonally close-packed array within the fat droplets after the formation of the lamellar phases (Seddon, 1990). The peaks corresponding to these phases around $Q = 0.11-0.12, 0.19-0.20$ and $0.22-0.24 \text{ Å}^{-1}$ (peak ratio $= 1: \sqrt{3}:2$) were observed after $(31 \pm 4)-(35 \pm 4)\%$ of the lipids present were digested and persisted until the end of each digestion. Initially, multiple overlapping diffraction peaks were observed, corresponding to multiple hexagonal phases with different lattice parameters between 58.8 and 66.1 Å. These diffraction peaks converged towards a single low $Q$ peak throughout digestion in most cases, indicating a reduction in disparity in lattice parameter and a shift towards larger lattice parameters of 61.4–67.4 Å.

The phase behaviour of the different bovine milk-mimicking emulsions diverged in the low $Q$ region of the scattering profiles at $Q \leq 0.10 \text{ Å}^{-1}$ after the formation of the hexagonal phases. The bovine milk-mimicking mixture containing four triglycerides (4 TG) was seen to form a transient micellar cubic (I$_{2d}$, Fd$ar{3}$m space group) phase with diffraction peaks around $Q = 0.059, 0.102$ and $0.118 \text{ Å}^{-1}$ (peak ratio $= \sqrt{3} : \sqrt{8} : \sqrt{11}$). The micellar cubic phase formed after the hexagonal phases when 66 ± 5% of the lipids in the emulsion were digested and the
diffraction peaks were observed until 72 ± 6% of the lipids had been digested. A broad diffraction peak centred around $Q = 0.060-0.067 \text{ Å}^{-1}$ began to form as the micellar cubic phase disappeared (extent of digestion = 72 ± 6%) and this persisted until the end of digestion. Such broad correlation peaks at low $Q$ in the diffraction patterns of glyceryl monooleate/diglycerol monooleate and glyceryl monooleate/polyunsaturated fatty acid dispersions have been associated with sponge ($L_3$) phases, which are disordered networks of water channels penetrating throughout the lipid droplets like the cavities of a sponge, separated by lipid bilayers (Angelova, Angelov, Garamus, & Drechsler, 2019; Vallydeperas et al., 2016). In this regard they have the same structural components as bicontinuous cubic phases but are lacking in liquid crystalline order, which leads to broad correlation peaks representing the average spatial separation between lipid bilayers. The position of the broad correlation peak in the digesting bovine milk mimics between $Q = 0.056$ and 0.067 Å$^{-1}$ were in the same $Q$ range as the corresponding diffraction peaks observed for the bicontinuous cubic ($V_2, \text{Im}3\text{m}$ space group) phase observed in bovine milk, suggesting similar inter-bilayer spacings in the $L_3$ structure but without the long-range order.

Correlation distances between lipid bilayers in the sponge phase between 93.4 and 111.0 Å were observed across all emulsions with the lattice parameter becoming greater throughout the course of digestion in all emulsions except for the most complex 8 TG mimic. In the case of the 5 TG bovine mimicking emulsion, the persistent sponge ($L_3$) phase formed first when 45 ± 3% of the lipids in the emulsion were digested before very weak diffraction peaks were observed for a transient micellar cubic ($I_2, \text{Fd}3\text{m}$ space group) phase from 64 ± 5% lipid digestion until the end of digestion (76 ± 6% lipid digestion). The 6 TG and 7 TG bovine mimics only formed the persistent sponge ($L_3$) phase after 50 ± 3 and 68 ± 5% lipid digestion, respectively, with no micellar cubic phase observed. Finally, the 8 TG bovine milk-mimicking emulsion formed a bicontinuous cubic ($V_2, \text{Im}3\text{m}$ space group) phase after 62 ± 4% of the lipid had been digested and this persisted until the end of digestion. A weak broad diffraction peak associated with a sponge phase was also observed beneath the diffraction peaks of the $V_2$ phase towards the end of digestion from 74 ± 6% lipid digestion onwards.

**Fig. 4.** Increasing the complexity of triglyceride mixtures designed to mimic the total fatty acid content of bovine milk leads to bovine milk mimicry. Peaks corresponding to each liquid crystalline phase are marked at the top of each subfigure as $S$ = sponge ($L_3$) phase, $L$ = lamellar phases associated with crystalline triglycerides and calcium soaps of liberated fatty acids, $H$ = hexagonal ($H_2$) phase, $F$ = micellar cubic ($I_2, \text{Fd}3\text{m}$) phase and $I$ = bicontinuous cubic ($V_2, \text{Im}3\text{m}$) phase. The colour scale is logarithmic with darker colours indicating higher X-ray intensities. The bottom right panel shows the corresponding scattering patterns for commercial bovine milk for comparison. The number before “TG” indicates the number of triglycerides in each mimic, readers are referred to Table 1 for the lipid compositions for each individual mimic mixture. Readers are referred to Fig. S4 in the electronic supplementary material for examples of individual scattering profiles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
3.4. Liquid crystalline phase analysis by SAXS – human milk mimics

The human milk-mimicking emulsions all displayed similar phase behaviour, which is commensurate with their similar lipid compositions (Figs. 6 and 7). As with the bovine milk-mimicking emulsions all of the human milk-mimicking emulsions contained lamellar (Lₐ) phases with peaks around $Q = 0.13-0.15, 0.26-0.29$ and $0.41-0.44$ Å⁻¹ before and throughout digestion [0 - (82 ± 3)% lipid digestion]. Weak diffraction peaks corresponding to lamellar phases were present before lipid digestion (Fig. S5 in the electronic supplementary material) with lattice parameters of $40.5-42.7$ Å, with the least complex 4 TG emulsion having the lamellar phase with the lowest lattice parameter. These phases were
persistent throughout digestion with a slight elongation of lattice parameter in most cases to 40.4–45.3 Å throughout digestion. Immediately after the onset of digestion additional diffraction peaks corresponding to lamellar phases evolved with lattice parameters on the order of 44.5–48.7 Å, with these phases having final lattice parameters of 45.0–45.9 Å at the end of digestion.

The only other phase observed in the digesting human milk-mimicking emulsions was the micellar cubic (I$_2$, Fd3m space group) phase and in each case multiple sets of diffraction peaks corresponding to such phases were observed throughout digestion. Initially, diffraction

Fig. 6. Triglyceride mixtures with 4-7 homotriglycerides designed to mimic the total fatty acid content of human milk lead to human milk mimicry. Peaks corresponding to each liquid crystalline phase are marked at the top of each subfigure as L = lamellar phases associated with crystalline triglycerides and calcium soaps of liberated fatty acids and F = micellar cubic (I$_2$, Fd3m) phase. The colour scale is logarithmic in each case with darker colours indicating higher X-ray intensities. The number before “TG” indicates the number of triglycerides in each mimic, readers are referred to Table 1 for the lipid compositions for each individual mimic mixture. Readers are referred to Fig. S5 in the electronic supplementary material for examples of individual scattering profiles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
peaks around \( Q = 0.107–0.111, \ 0.125–0.130, \ 0.213–0.222 \) and \( 0.250–0.260 \ \text{Å}^{-1} \) (peak ratios = \( \sqrt{8} : \sqrt{11} : \sqrt{33} : \sqrt{44} \)) were observed. In the 4 TG human milk-mimicking emulsion the micellar cubic phase was observed at the earliest extent of digestion of 16 ± 1%. The 5-7TG human milk-mimicking emulsions began to display the micellar cubic phase when (24 ± 1) – (25 ± 1)% of the lipids were digested. In all emulsions the I\(_2\) phase persisted until the end of digestion (extent of digestion = 82 ± 3%). In all of the human milk-mimicking emulsions there were two sets of diffraction peaks corresponding to the Fd3m space group at various points in the digestions. When initially formed the I\(_2\) phases had lattice parameters on the order of 160.7–162.0 Å, which quickly expanded to higher lattice parameters of 163.7–168.9 Å. This high lattice parameter phase remained dominant in the earlier stages of digestion in most cases but the 4 TG human milk-mimicking emulsion displayed two sets of diffraction peaks corresponding to the Fd3m space group at various points in the digestions. After 74 ± 3% of the lipids in the 5 TG, 6TG and 7 TG human milk-mimicking emulsions were digested a clearly distinct second set of diffraction peaks at slightly higher \( Q \) values corresponding to shorter lattice parameters of 156.5–164.1 Å became more prominent and at the end of all digestions there were two clearly distinct I\(_2\) phases present. As the lipid composition of the emulsions became more complex the peaks also began to broaden with the diffraction patterns becoming more diffuse but still distinguishable as seen in Fig. 6.

### 4. Discussion

#### 4.1. Milk mimic preparation and behaviour prior to digestion

The aim of this work was to determine the composition of simplified homotriglyceride emulsions that could mimic the lipid self-assembly behaviour of bovine and human milk during digestion. This involved preparing emulsions of triglycerides with higher melting points than standard milk fats and monitoring their phase behaviour as a function of the extent of lipid digestion. To this end, all lipids were molten at 75 °C when dispersed by tip sonication and the resulting hot emulsions were subsequently loaded immediately into a thermostatted digestion vessel at 37 °C to prevent significant crystallisation of the triglycerides. However, lamellar phases were observed in all of the milk-mimicking emulsions prepared prior to the addition of lipase (Figs. S3 and S4 in the electronic supplementary material) and this indicated a degree of crystallisation of the emulsified triglycerides. This correlates with the thermal analysis presented in Fig. 2 e), which indicated an onset of crystallisation at 37 °C. This onset of crystallisation was observed in the semi-regiorandom nature of the acyl chain distributions in native milk fats which frustrates crystallisation and makes their maximum melting points around 40 °C (Fox et al., 2006; Jensen et al., 1995; Jensen & Newburg, 1995; Jost, 2007; MacGibbon, 1988).
milky triglycerides into lamellar structures within both human and bovine milk fat globules has been reported upon storage below body temperature (Lopez, Bourgaux, Lesieur, & Ollivon, 2007; Lopez, Briard-Bion, Bourgaux, & Pérez, 2013). The lipid mixtures of this work comprising mixed homotriglycerides with higher melting points up to 73°C (Table S2) were therefore much more prone to crystallisation upon cooling (Lutton, 1950). The levels of crystallisation were however insufficient to trigger significant partial coalescence and breaking of the emulsions at body temperature (Boode & Walstra, 1993). The lamellar phases are designated L throughout this work because the characteristic diffraction peaks around $Q = 1.3–1.7\,\text{Å}^{-1}$ required to assign the crystal polymorph as α, β or β' were not within the measured Q-range of the SAXS experiments performed. The lattice parameters of the lamellar phases formed prior to digestion are consistent with the long spacings of crystallised triglycerides used to make the emulsions, particularly tri-myristin (C14:0 – 35.8–41.2 Å), tripalmitin (C16:0 – 40.6–45.6 Å) and tristearin (C18:0 – 45.2–50.6 Å) (Lutton, 1950). In the 5-8TG bovine milk-mimicking emulsions the lattice parameters observed were consistent with the crystallisation of polymorphs of tripalmitin and tristearin. For the 4 TG bovine milk-mimicking emulsion the shorter lattice parameters measured for the lamellar phases were more consistent with the crystallisation of tripalmitin and trimyristin, which is consistent with there being no tristearin in the 4 TG lipid mixture. In the case of the human milk-mimicking emulsions, the initial lamellar phases observed before digestion are consistent with the crystallisation of tri-myristin and tripalmitin, which are present in all of the mimic mixtures. The triglyceride crystals formed are likely to be mixed triglyceride crystals with the average lattice parameters representative of those triglycerides incorporated into the lipid mixtures. The persistence of these lamellar phases throughout digestion provides a reason for the lipid digestion being incomplete within the two-hour digestion, with solid triglycerides being digested at a slower rate than that of liquid triglycerides (Bonnaire et al., 2008; Keogh et al., 2011).

4.2. Formation of calcium soaps during early lipolysis

The lamellar phases that evolved after the addition of lipase have been shown previously to be the result of calcium soap formation as ionised fatty acid digestion products combine with calcium ions in the digesting emulsions and are removed from the surfaces of the digesting fat globules (Clulow et al., 2018; Sassene et al., 2014). The associated lattice parameters of these phases between 42.3 and 46.9 Å for the bovine milk-mimicking emulsions lie close to the lattice parameters of calcium myristate (C14:0, 40.2 Å) and palmitate (C16:0, 45.4 Å), whilst those of 44.5–48.7 Å for human milk-mimicking emulsions are more consistent with calcium palmitate and stearate (C18:0, 50.6 Å) (Clulow et al., 2018). In reality, the calcium soaps that form in the mixture will depend upon the total lipid composition, with the lattice parameter dominated by the lipids in higher quantities in the emulsions and so it is not surprising that calcium palmitate would play a dominant role.

It is noteworthy that there was limited formation of non-lamellar liquid crystalline structures at extents of digestion commensurate with gastric digestion in adults (10–25%) (Carriere et al., 1993; Lengsfeld et al., 2004) and premature infants (∼6% for medium chain tri-glycerides) (Roman et al., 2007). Calcium soaps were the only phases observed up to 25% lipid digestion in the bovine milk-mimicking emulsions, with most of the human milk-mimicking emulsions showing the onset of formation of inverse micellar cubic phases at around 25% lipid digestion and only the 4 TG human-mimic showing formation of the micellar cubic phase earlier at around 15% lipid digestion. It is therefore highly unlikely that significant lipid self-assembly would be observed under gastric conditions as the calcium soaps require a neutral/basic environment to form (Salentinig et al., 2015; Salentinig et al., 2013) and these are the dominant, if not only, phases formed at the low extents of digestion reported for the gastric phase.

4.3. Evolution of liquid crystalline phases in later lipolysis is controlled by lipid packing

Turning to the bovine milk-mimicking emulsions, the formation of hexagonal phases that shift to larger lattice parameters after the initial formation of calcium soaps is consistent with the previously reported behaviour of bovine milk (Fig. 1) (Clulow et al., 2018; Salentinig et al., 2013). The lattice parameters of the hexagonal phases formed in the bovine milk-mimicking emulsions are also consistent with the prior reports, which indicate lattice parameters of 56 Å (Salentinig et al., 2013) and 59–65 Å (Clulow et al., 2018) depending on the triglyceride/lipase ratio. Whilst transient micellar cubic phases have been reported to form during the digestion of bovine milk, this usually occurs as a prelude to the formation of hexagonal phases and the associated diffraction peaks are typically very weak (Clulow et al., 2018; Salentinig et al., 2013). Furthermore, the lattice parameters of the I2 (Fd3m) phases observed in this study are greater than those previously reported in commercial bovine milk of 150 Å (Salentinig et al., 2013) and 162 Å (Clulow et al., 2018). The formation of the I2 phases in the 4 TG and 5 TG bovine milk-mimicking emulsions of this work were therefore not consistent with the behaviour of bovine milk.

The formation of bicontinuous cubic phases are hallmarks of the end stages of digestion of commercial bovine milk, typically the Im3m structure (Fig. 1) but Pn3m structures have also been observed (Clulow et al., 2018; Salentinig et al., 2013). The only bovine milk-mimicking emulsion to form a bicontinuous cubic phase was the most complex 8 TG emulsion, with the 4-7TG bovine milk-mimicking lipid mixtures forming disordered sponge phases only. The liquid crystalline structures observed during the digestion of the triglyceride emulsions is dictated by the molecular packing of the digestion products, the surfactants in this system that self-assemble into the phases observed (Fig. 8). The average shape of the surfactants in each phase can be defined geometrically by the critical packing parameter $\text{CPP} = V/(\mathcal{A}_3\lambda_3)$, where $V$ is the volume of the apolar alkyl tail of the lipid, $\mathcal{A}_3$ is the optimum interfacial area per hydrophilic headgroup at the oil-water interface and $\lambda_3$ is the average length of the lipid tail (Israelachvili, Mitchell, & Ninham, 1976; Mitchell & Ninham, 1981). As the volume of the apolar lipid part of the surfactants within the phases increases, the local curvature of the oil-water interface becomes increasingly curved towards the aqueous phase. This leads to a phase progression from flat bilayers (lamellar, $\text{CPP} \approx 0$) to curved bicontinuous bilayers ($I_2/\mathcal{V}_3$), to cylinders ($H_2$) and finally to spheres ($I_2$) with the greatest interfacial curvature as the CPP increases above 1 (Barauskas & Nylander, 2008; Hyde, 2001; Seddon, 1990; Seddon & Templer, 1995). Broadly speaking, the longer the chain length of the lipid tail the greater the tail group volume at a given temperature and the larger the CPP. Lipid unsaturation also plays a prominent role in tail volume, with the presence of cis-double bonds such as in oleic acid introducing kinks in the alkyl chains, introducing packing frustration and increasing tail group volumes. Fatty acids also have smaller apparent headgroup areas than monoglycerides, with increasing oleic acid/monoolein ratios observed to drive structural progression from bicontinuous bilayer cubic ($V_2$) phases to inverse micellar cubic ($I_2$) phases in dispersed lipid particles (Nakano et al., 2002).

A breakdown of the triglycerides in terms of their chain length [short (<C6), medium (C8–C12) and long (>C14)] and saturation is compared with the non-lamellar liquid crystalline phases observed during digestion in Table 2. For the bovine milk-mimicking emulsions it is clear that the presence of medium-chain triglycerides plays a strong role in determining the structures that form. The 4 TG and 5 TG bovine milk-mimicking mixture, which did not contain any medium-chain triglycerides formed inverse micellar ($I_2$) cubic phases, whilst the 6–8TG bovine milk-mimicking emulsions did not form this phase. This supports the assertion that the medium chain triglycerides lower the average CPP of the digesting lipids, preventing the formation of the inverse micellar phase with high interfacial curvature. As the medium-chain triglyceride...
content was progressively increased in the 6–8TG bovine milk-mimicking emulsions the average CPP of the lipids decreases to the point where the inverse bicontinuous cubic (V$_2$) phase was formed during digestion. Coexisting hexagonally-packed rods (H$_2$) and sponge (L$_3$) phases were observed in all of the bovine milk-mimicking emulsions during digestion, suggesting that these phases both possess interfacial curvature between the two cubic structural extremes (V$_2$ and L$_3$). The ratio of the long- and medium-chain lipids in these emulsions therefore seems to play a dominant role in defining the structural progression, given that the digestion products of short-chain lipids are more water soluble and would be expected to partition more extensively into the aqueous phase. It is also noteworthy that the balance in CPP required to form the ordered V$_2$ (Im$	ext{3m}$) bicontinuous cubic phase is so fine that adding 3% tricaprylin (C8:0) to the 7 TG bovine milk-mimicking mixture to form the 8 TG mixture is sufficient to tip the balance.

In contrast, all of the human milk-mimicking emulsions followed the phase behaviour expected from the prior report on structuring in digesting human milk (Salentinig et al., 2015). Each of the emulsions formed persistent micellar cubic I$_2$ (Fd$	ext{3m}$) phases as the digestions proceeded after the initial appearance of calcium soaps. The lattice parameters of the micellar cubic phases in donor human milk changed from 157 to 153 Å during digestion (Salentinig et al., 2015), which is consistent with the decrease in lattice parameter observed in the digesting human milk-mimicking emulsions towards the end of digestion. In terms of the lipid packing arguments described (Fig. 8), the human milk-mimicking emulsions all contain similar molar ratios of medium-/long-chain and saturated/unsaturated triglycerides and their structural progressions are commensurately similar. The high molar quantities of both long-chain and unsaturated lipids ensure a high average CPP of the digesting lipids, which drives the formation of the inverse micellar (I$_2$) phases of greater interfacial curvature.

4.4. Comparing mimics with milks and future outlook

These findings provide important insights for the mimicry of milk lipid self-assembly during digestion, particularly in the context improving infant nutrition. Infant formulae often comprise blends of vegetable oils mixed with exogenous surfactants (lecithins) and bovine-derived proteins designed to recreate the total lipid composition of human milk in a readily digestible form. It was recently reported that infant formulae do not all form the same micellar cubic phases that human milk has been shown to form upon lipid digestion (Pham et al., 2020). This study used a combination of high-performance liquid chromatography (HPLC) and principle component analysis (PCA) to

Table 2
Correlation of the lipid content of the triglyceride emulsions prepared with the non-lamellar liquid crystalline phases observed during digestion. For comparisons with lipid self-assembly during digestion of bovine and human milk, readers are referred to the recent study of Pham and co-workers, which correlates the formation of liquid crystalline phases with the compositions of fatty acid and monoglyceride digestion products in a variety of milks and milk substitutes (Pham et al., 2020).

<table>
<thead>
<tr>
<th>Species</th>
<th>Bovine</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mimic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 TG</td>
<td>12(4)</td>
<td>7 TG</td>
</tr>
<tr>
<td>6 TG</td>
<td>14</td>
<td>5 TG</td>
</tr>
<tr>
<td>4 TG</td>
<td>17</td>
<td>4 TG</td>
</tr>
<tr>
<td>7 TG</td>
<td>0</td>
<td>6 TG</td>
</tr>
<tr>
<td>5 TG</td>
<td>18</td>
<td>5 TG</td>
</tr>
<tr>
<td>4 TG</td>
<td>15</td>
<td>4 TG</td>
</tr>
<tr>
<td>Short-chain TGs (mole %)</td>
<td>12(4)</td>
<td>7(2)</td>
</tr>
<tr>
<td>Medium-chain TGs (mole %)</td>
<td>16(4)</td>
<td>7(2)</td>
</tr>
<tr>
<td>Long-chain TGs (mole %)</td>
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<td>7(2)</td>
</tr>
<tr>
<td>Saturated TGs (mole %)</td>
<td>79</td>
<td>79</td>
</tr>
<tr>
<td>Unsaturated TGs (mole %)</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>V$_2$ (Im$	ext{3m}$) observed</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>L$_3$ (Fd$	ext{3m}$) observed</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>
demonstrate that the liquid crystalline structures formed during digestion of different milk and milk substitute emulsions were more strongly correlated with the fatty acid digestion products than the monoglycerides. In particular, milks releasing more long-chain unsaturated (oleic (C18:1) and linoleic (C18:2)) fatty acids upon digestion were found to form inverse micellar cubic (I2) phases. In contrast, milks releasing more long-chain saturated [myristyl (C14:0), palmityl (C16:0) and stearyl (C18:0)] fatty acids were found to form hexagonal (H2) and inverse bicontinuous cubic (V2) phases of lower interfacial curvature. The results herein have shown that matching the total fatty acid content of human milk in an emulsion comprising four–seven homotriglycerides will mimic the self-assembly of human milk when casein (at human milk concentrations) is used as the emulsifier. These findings are supported by the human milk-mimicking emulsions prepared for this work, which all mimic the structural behaviour of human milk and all contained ~50 mol % long-chain unsaturated triolein (C18:1). Furthermore, the bovine milk-mimicking emulsions containing greater contents of saturated long- and medium-chain triglycerides all formed structures of lower interfacial curvature. Whilst the monoglycerides released during digestion are double key structural components in the self-assembled structures (Hyde & Andersson, 1984; Nakano et al., 2002; Shao et al., 2018; Yaghmur et al., 2017; Yaghmur et al., 2006), there is a growing body of evidence that the self-assembly of lipids during digestion is directed by the more abundant fatty acid digestion products (Pham et al., 2020). This may need to be factored into the design of lipid emulsions where the formation of particular liquid crystalline phases during digestion is desired. Given the apparently prominent role of fatty acids in the lipid self-assembly, the precise intestinal pH will play a role in self-assembly by tuning the ratio of protonated/deprotonated fatty acids (Salentinig et al., 2015; Salentinig et al., 2013). The contribution of both exogenous and endogenous emulsifiers to the self-assembly processes occurring will also be an ongoing focus of this research.

Lipid structuring in digesting emulsions is a key material property for the delivery of lipophilic bioactive (nutrients or drugs) in foods (Mezzenga, Schurtenberger, Burbidge, & Michel, 2005). Milk is nature’s delivery vehicle for lipophilic nutrients and therefore provides us with a blueprint to provide effective lipophile delivery (Mezzenga et al., 2019). The distinct structural progressions observed during the digestion of the milks of different species suggest that different colloidal structures are favoured for lipophile uptake by different species. The composition-structure relationships determined in this work shed light on the balance of lipid compositions required to mimic changes in liquid crystalline structure using simplified but structurally-representative homotriglyceride mixtures. This allows the targeted formation of food-based delivery vehicles from off-the-shelf triglycerides, which can then be optimised for the delivery of particular lipopolysaccharides or to particular recipients. An important application of the mimicity concept is in the nutrition of human infants. It is known that the nutritional outcomes for infants fed formula are worse than those of their breast-fed counterparts and it is also known that not all infant formulae mimic the lipids present during human milk during digestion. Designing infant formulae or milk fortifiers that effectively mimic the nanoscale structures observed in human milk is therefore a logical step towards improving milk substitutes for infants and improving their associated health outcomes.

5. Conclusions

Lipid emulsions comprising homotriglycerides have been created that mimic the lipid self-assembly behaviour in digesting bovine and human milk. After an initial rapid burst of digestion, the human milk-mimicking emulsions were digested more slowly than the corresponding bovine emulsions. However, all of the emulsions reached a similar final extent of digestion with 84 ± 6% of bovine milk-mimicking lipids and 86 ± 3% of human milk-mimicking lipids digested within 2 h. A lipid mixture comprising eight triglycerides was required to produce the bicontinuous cubic phases observed towards the end of commercial bovine milk digestion, which was more complex than the mixtures of four–seven triglycerides required to produce the micellar cubic phases characteristic of donor human milk under digestion. These results are the first step towards developing readily-obtainable, simplified, yet representative mimics for the self-assembly of complex milk lipid mixtures during digestion – a critical step in analysing the ability of complex lipid mixtures to effectively deliver fat-soluble bioactives to the gut.

Declaration of competing interest

The authors have no conflicts of interest to declare.

CRediT authorship contribution statement

Andrew J. Clulow: Conceptualization, Funding acquisition, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration. Malinda Salim: Investigation, Writing - review & editing. Adrian Hawley: Investigation, Funding acquisition, Resources, Writing - review & editing. Ben J. Boyd: Conceptualization, Funding acquisition, Resources, Project administration, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2020.106126.


Hyde, S. T., & Andersson, S. (1984). A cubic structure consisting of a lipid bilayer...


