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RESEARCH

The Role of Gastric Lipase and Pepsin in Lipid Digestion of a Powder Infant Formula Using a Simulated Neonatal Gastric System

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Abstract

This study has sought to determine the impact of interfacial dynamics on the *in vitro* lipid digestion of a commercial infant formula; in particular, the specific role of interfacial proteolysis on the subsequent rates of reaction of droplet lipolysis. A powder infant formula was used as the as a protein-stabilised emulsion substrate during simulated infant gastric digestion at different *p*H level 3.5, 4.5 and 5.5. The digestate was treated with a fungal lipase and porcine pepsin (used to analogue human gastric lipase and pepsin) respectively and in a combined action. The study found that for fungal lipase treated digestate, the rate and extent of lipolysis were observed to be maxim at *p*H 5.5, in accordance with the optimal pH activity of the lipase. Findings also indicated that the proteinaceous interface did not appear to act as a barrier to lipolysis, since treatment with lipase and pepsin did not result in any significant increase in extent of lipolysis. However, it was observed that surface proteolysis did lead to alteration of the structural fate of the enzyme during digestion when compared to when the emulsion was digested solely by lipase. Findings suggest that lipolysis under these conditions may be independent of the structural dynamics of the emulsion during digestion, as observed within the context of this study design.

Keywords Protein stabilised emulsion · Lipid digestion · pH · Destabilisation of emulsion

Introduction

Gastric lipolysis is typically considered as the first stage of lipid digestion in humans. In adults, gastric lipase hydrolyses triglycerides into component diglycerides and fatty acids, the extent of which typically accounts for 10–30% of total lipid hydrolysis [1] with the remainder taking place during transit through the small intestine. Due to the apparently lesser impact of the gastric stage on overall lipid digestibility, coupled with limited availability of lipase enzymes with appropriate equivalence to the structure and properties of human gastric lipase, the role of gastric lipolysis as an integrated part of *in vitro* studies relating to fat digestion has often been overlooked. More recently however, the increased availability of more appropriate gastric lipase analogues, such as rabbit gastric lipase, has seen gastric lipolysis being increasing considered in terms of its impact on overall lipid digestion.

Observations, based on both *in vivo* and *in vitro* studies have indicated that the fatty acids produced via gastric lipolysis have sufficient surface activity to progressively accumulate at the oil-water interface, as visualised in a number of studies by the formation of surface structures [2, 3]. This accumulation of fatty acids is considered one possible explanation for the limited extent of gastric lipolysis, acting as either a barrier to subsequent lipase adsorption, or alternatively via entrapment of the lipase within the surface layer of emulsion droplets [3]. The modulation of the interfacial layer during the gastric stage of digestion, is also increasingly being considered in terms of its role on subsequent small intestinal lipolysis. Here, there is some indication that the accumulation of surface fatty acids during gastric digestion may serve to ameliorate the initial adsorption of bile salts at the interface, allowing for the initial assembly of mixed micelles and additionally facilitating the adsorption of pancreatic co-lipase/lipase complex [4].

Within this context, it has been increasingly observed that the capacity of gastric lipase to initiate lipolysis is dependent on its ability to adsorb to the interface of emulsion droplets.

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Prior research has indicated that the presence of highly surface active or structured interfacial layers may be inhibitory to gastric lipase adsorption [5], and leading to less fatty acid synthesis within the stomach [6], and with the extant interfacial layer remaining intact on transit into the small intestine [7].

For adults, this is not expected to affect the overall digestibility of consumed fats and oils, since the high surfactancy of the bile salts will typically allow its adsorption to emulsion droplets, regardless of their interfacial condition (noting that interfacial engineering to restrict bile and lipase adsorption is considered a means of limiting lipid digestion). However, for infants, and particularly newborns, the situation is a little more complicated. In neonatal physiology liver function remains immature, which can impact on the availability of digestive secretions in the small intestine. In particular, for lipid digestion there is a greater reliance on the gastric stage of digestion for fatty acid synthesis.

On the basis that the sole source of nutrition during this stage of life maternal milk, it can be argued that the interfacial composition of the milk fat globule membrane is biologically optimised to promote gastric lipolysis. For many years infant formula has provided an alternative source of nutrition for infants where breast feeding is not a viable option. Whilst designed to provide nutritional equivalence to breast milk, the structure of infant formula does show some notable differences to that of maternal milk, most significantly in regards to the properties of the emulsion droplets, in which the interfacial layer of formulated milks predominantly comprises milk proteins as opposed to stabilisation by a phospholipid trilayer in that of maternal milk [7].

Using the argument that the maternal milks are optimally structured for effective digestion, a question arises as to whether the altered interfacial properties of formulated milks can cause variance in lipid digestibility compared to that of breast milk [8]. Prior research using *in vitro* models has indicated that there are clear structural differences of the emulsion droplets during digestion [9], with droplets in formulated milks showing a greater tendency to flocculate in the stomach, followed by extensive coalescence in the small intestine [10], In contrast, the size and stability of maternal milk droplets appears less affected during gastric digestion, in terms of relative digestibility.

This current study focusses on the role of the proteins that are adsorbed at the oil/water interface of infant formula emulsions and specifically whether they inhibit the action of gastric lipases. We also seek to determine the consequence of interfacial gastric proteolysis of protein stabilised droplets as impacting on the relative efficacy of lipolysis. This is based on the hypothesis that whilst intact protein layers may be inhibitory to gastric lipase, partial digestion of the proteinaceous layer may serve to facilitate lipase adsorption. This extends from our previous research [11, 12] indicating that the combined presence of gastric lipase and pepsin results in notably different structural dynamics of a model protein stabilised emulsion when compared to digestion of the same emulsion in the presence of lipase alone. This work builds on our previous findings in attempting to determine whether there is any co-dependency of pepsin and lipase in terms of the rate and magnitude of fatty acids synthesised during gastric digestion.

Thus, in this current work, we simulate digestion of a commercial infant formula *in vitro* using a fungal lipase and followed changes by GC and electron microscopy. We then compared the results with those obtained from a similar *in vitro* digestion of a commercially prepared stable homogenous emulsion that contained no protein and was stabilised by lecithin (Intralipid).

Materials and methods

Materials

A Food-Grade Fungal Lipase derived from m a selected strain of *Rhizopus oryzae* (ATCC 1996) (Connell Brothers Company Australia Pty Ltd., Australia) was chosen. It has been classified as a triacylglycerol acylhydrolase (EC 3.1.1.3) and has been shown to hydrolyse triglycerides at sn-1 and sn-3 positions. It has been reported to be active and stable over a wide *p*H range from 4.5 to 8.5 and with optimal performance at *p*H 7. It is active at temperatures up to 45 ºC with an optimal temperature at 35 to 40 ºC.

Intralipid® was a commercial soya bean oil in water emulsion stabilised by phospholipids from egg lecithin intended for intravenous use (purchased from Fresenius Kabi Australia Pty Limited, NSW Australia). This product contained 20% of soybean oil, 0.12% egg lecithin and 0.22% glycerol. The main phospholipid components in egg lecithin are phosphatidylcholine (PC, 80.5%) and phosphatidylethanolamine (PE, 11.7%). Minor components also include lysophosphatidylcholine (LPC), sphingomyelin (SM), and neutral lipids [13]. The pH has been adjusted to 6.0–9.0 by hydrochloric acid (HCl). The osmolality was 350 mosmol/L. Oil in the diluted Intralipid emulsion was stabilised by egg lecithin containing phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM). The head groups of these phospholipids show no charge at pH 3.5. Thus, the negative surface charge is reflected in the phosphate group.

Myristic Acid was purchased from BDH Ltd, Poole, England.

S-26® Gold Newborn [Wyeth Nutrition (Singapore) Pte. Limited, Singapore, protein 10 g/100 g,, fat 28 g/100 g, carbohydrate 56 g/100 g] was purchased from a local supermarket in August 2018. S-26 Composition as follows: Milk

Solids (Lactose (Milk), Skimmed Milk Powder, Whey Protein (Milk)), Vegetable Oils (Including Soybean), Minerals, Long-Chain Polyunsaturated Fatty Acids Emulsifiers (Soy Lecithin, Monoglycerides), Oligosaccharide (Milk)), Vitamins, Antioxidants.

Palmitic acid and **Pentadecanoic Acid** (~ 99% capillary GC) were purchased from Sigma-Aldrich Pty Ltd., St. Louis, MO, USA.

Porcine Gastric Pepsin P7000 (EC 3.4.23.1) powder with an activity ≥ 250 units/mg solid was purchased from Sigma-Aldrich Pty Ltd., St. Louis, MO, USA.

Deionised Water (Milli-Q water) was purchased from Millipore, Bedford, MA, USA.

All other chemicals were purchased from Sigma-Aldrich Pty Ltd., St. Louis, MO, USA.

Methods

Digestion of a Commercial Powder Infant Formula using a Modified Gastric *In Vitro* Protocol

Simulated gastric fluid (SGF) was prepared by dissolving 200 mg of sodium chloride (NaCl) in Milli-Q water. The *p*H of the solution was adjusted to 2, 3.5, 4.5 or 5.5 with 0.1 M HCl solution, and the total volume made up to 100 mL with Milli-Q water. This *p*H range was aiming at simulating the postprandial variation of *p*H in an infant's stomach, as reported in Lueamsaisuk et al. [11, 12]. The initial digestate was prepared by mixing the diluted infant formula as the substrate, and SGF at a ratio of 20:50 v/v [11]. An amount of 450 mg of dry powdered porcine pepsin (800–2500 units/mg protein) and/or 20 mg of dry powdered *Rhizopus oryzea* lipase (80 U/mg) was first hydrated with a small amount of Milli-Q water (usually 5 to 10 times of the weight of the enzyme) and added to the SGF.

The substrate (i.e., rehydrated formula) was prepared by adding 36 mL Milli-Q water to 5 g infant formula under gentle stirring at 37 °C, to produce a final emulsion containing 3.6% (w/v) fat. Similarly, the substrate in the pre-tests was prepared by (1) diluting 20% (w/v) Intralipid to 3.6% fat with Milli-Q water to 40 mL, to mimic the fat content encountered in the standard infant formula; or (2) adding 1% lactoferrin to the first dilution. The volume of SGF (without the enzymes) was 100 mL in the pre-tests.

The stirring paddle was maintained in a shallow vessel at a rate of 10 rpm to simulate the low shear rates exhibited in the human stomach [14, 15].

The hydrated fungal lipase and porcine pepsin were then added to the digestate which was maintained at 37 °C and incubated for 120 min.

Starting from t = 0 min, and subsequently, at intervals of 15 min, two aliquots (0.5 or 1.0 mL) were taken for gas chromatography, scanning electron or transmitted electron microscopy measurements. This approach was intended to allow continuous and dynamic tracking of changes of emulsion structure and rate of digestion within a single sample, but, as acknowledged in the discussion, sampling can be rendered complex due to the structural changes taking place.

Analysis of Lipolysis Products by GC

Lipid Extraction The extraction process referred to the method in Helbig et al. [16] with small modifications. An aliquot of 0.5 mL sample was taken during the *in vitro* digestion at 0, 15, 30, 45, 60, 90, 105, and 120 min. Each sample was poured into a Kimax tube containing a mix of solvents (1 mL ethanol, 1.5 mL of 1:1 (v/v) diethyl ether and heptane, and 0.1 mL of 2.5 M sulphuric acid]. Each tube was mixed by vortex for about 1 min and centrifuged for another 5 min at 1000 × g at room temperature. The supernatant from each tube was carefully removed and transferred to another Kimax tube. The residual lipidic fraction in the first Kimax tube was extracted with 1 mL of 1:1 (v/v) diethyl ether and heptane again following the previous procedure. The collected supernatant of the two extractions with an addition of 30 μL C15:0 (pentadecanoic acid) internal stock solution (20 g/L) was added was brought up to 3 mL with 1:1 (v/v) diethyl ether and heptane. The internal standard was not added in the pre-tests. Then, 300 mg of anhydrous sodium sulphate was added into each tube to dry any water residue. An amount of 1 mL of the dried supernatant was carefully transferred to a GC sample vial for further analysis. The final concentration of the internal standard was 0.2 g/L in the GC sample vial.

GC Analysis The samples were analysed in a ThermoQuest Trace GC 2000 (Thermo Fisher Scientific, Milan, Italy). An amount of 0.6 μL of the sample was injected into a guard column (Fused silica capillary tubing, deactivated, 0.50 m × 0.53 mm ID, Alltech Associates Inc., Deerfield, IL) by an autosampler Thermo Scientific AS 2000 (Thermo Fisher Scientific, Milan, Italy). The sample passed through a guard column and then into an Agilent capillary non-polar column [DB-5ht, 8 m (length) × 0.32 mm (ID), 0.10 μm (film), -60–400 °C (the temperature limit), Santa Clara, California, United States]. The carrier gas was helium, and it was running at a constant flow rate of 2 mL/min. Temperature ramped from 60 °C (for 1 min) to 380 °C (for 8 min) at a heating rate of 10 °C/min. The total run time for one sample was 41 min. Signals were detected by an FID detector.

The GC trace revealed free fatty acids, monoglycerides, diglycerides and triglycerides. The identification of peaks was performed by comparing the retention time of myristic and palmitic acids used to produce the calibration curve, to those from the analysed samples. Their retention time and

area under the peak were recorded and integrated by using Chromeleon™ 7.2 Chromatography Data System Software (Thermo Fisher Scientific, Milan, Italy).

Transmission Electron Microscopy (TEM)

A 1.5 mL of digestate was sampled at time 0 min and after 120 min. The liquid sample was injected into 3% agarose tubes and sealed with agarose to form an enclosed capsule. The tubes were placed into 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (*p*H 7.2) for at least 24 h. The buffer was then washed in 0.1 M sodium cacodylate (*p*H 7.2) three times for 45 min per wash. The samples were post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for an hour at room temperature and overnight at 4 °C and finally brought up to room temperature for an hour. The buffer was rewashed three times in 0.1 M sodium cacodylate as described above. The samples were then dehydrated through a graded acetone series (25%, 50%, 75%, 95%, 100%, 100%, and 100%) for 45 min for each grade. The samples were then put into resin: acetone (50:50) and placed on a stirrer overnight then replaced by fresh 100% resin and placed on the stirrer for another 8 h. This step was repeated four more times. Then the samples were embedded in moulds with fresh resin and cured at 60 °C oven for 48 h. Light microscope sections were cut at 1 µm using a glass knife on the ultramicrotome (Leica EM UC7, Germany) and heat-fixed onto glass slides. These sections were stained with 0.05% toluidine blue for approximately 12 s and viewed under the light microscope. Those blocks were then trimmed down to the selected areas and cut using a diamond knife (Diatome, Switzerland) at 100 nm and then stretched with chloroform and mounted on a grid using a Quick Coat G pen (Daido Sangyo, Japan). The grids were stained in saturated uranyl acetate in 50% ethanol for 6.5 min and washed with 50% ethanol, and Milli-Q water then stained in lead citrate [17] for another 6.5 min and followed by a wash in Milli-Q water. Then the samples were viewed and imaged with a transmitted electron microscope FEI Tecnai G^2 Spirit BioTWIN (FEI Company, Czech Republic) with a camera Veleta (Olympus SIS, Germany). Samples were analysed in duplicate. This method was a standardised method for analysing milk-like samples for TEM analysis in Manawatu Microscopy & Imaging Centre, Palmerston North, New Zealand.

Statistical Analysis for Infant Formula Tests

With the exception of TEM analysis (duplicate samples), samples were analysed in triplicate.

As confirmed by the use of a standard curve (data not shown), the area under the peak (AUP) with units of mV*min was in proportion to the concentration of free fatty acids (mg/mL) in the samples. Accordingly, AUP was used to represent the rate and degree of gastric lipolysis.

Pentadecanoic acid (15:0) was used as the internal standard in this study. The AUP was then normalised relative to the maximum AUP value of the internal standard in order to compare parameters from different treatments:

$$Corrected(AUP) = \frac{AUP(max)_{internal}}{AUP(i)_{internal}} * AUP(i)$$

Where $AUP(max)_{internal}$ is the maximum area under the peak value of the internal standard, $AUP(i)_{internal}$ is the area under the peak value of the internal standard in each run, and $AUP(i)$ is the area under the peak value of the sample run. The increase of free fatty acids was indicated by corrected AUP instead.

Limited data collection over the first 30 min of digestion meant that it was not possible to effectively quantify the kinetics of lipolysis in the early stages of the reaction. To address this issue, linear regression was instead applied to data points between 30 and 120 min.

Although there were traces of free fatty acids in the digestate when no enzymes were present, the amount was negligible as these were at least one order of magnitude below the value at 30 min. In order to modulate the data from 30 min and onwards, we moved the original point of *x*-axis from 0 to 30 min. Therefore, the interface of the linear regression with the *y*-axis at 30 min was able to provide an indicator as to the initial rate of lipolysis over the first 30 min of digestion.

In order to compare the differences between each *p*H level when infant formula was digested with lipase only, the Student t-test was used for statistical analysis. As there were only mean and standard errors, it was assumed that the variances of the population were equal (homogeneity of variances).

Results

Pre-tests for Intralipid Dilutions

Increase of Free Fatty Acids in Simulated Gastric Digestion

The d_{43} of diluted Intralipid at pH 7 was 0.32 μm (before digestion). The value for lactoferrin coated Intralipid was 0.4 μm. The ζ-potential of the Intralipid emulsion (3.6%, v/v) was −44.06 mV at pH 7 whereas for the lactoferrin coated Intralipid emulsion the value was + 16.6 mV at neutral pH. The droplet size and surface charge data were carried out in a previous study [11]. The extent of lipolysis for Intralipid model emulsions digested in different *p*H levels and enzyme treatments was ~20% (w/w) of total triglycerides (Fig. 1).

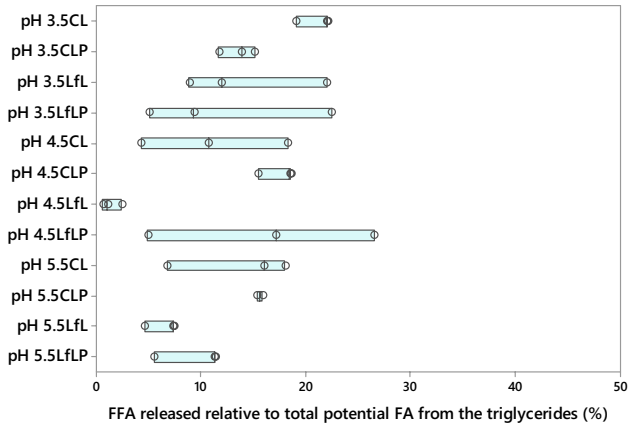

Fig. 1 GC analysis on FFA released relative to total potential FA from the triglycerides from Intralipid model emulsion in a simulated gastric system. CL, Intralipid digested with lipase alone; CLP, Intralipid digested with lipase and pepsin; LfL, lactoferrin coated Intralipid digested with lipase alone; LfLP, lactoferrin coated Intralipid digested with lipase and pepsin. The *p*H values were all initial *p*H levels

This figure was within the range of degree of lipolysis (10–30%) observed in the previous *in vivo* study [18].

Figure 2 showed the original AUP for each treatment. From observation, the digestate of Intralipid with lipase, or lipase and pepsin, did not have visible phase separation during 120 min of simulated digestion. However, when Intralipid was coated with lactoferrin, the digestate showed aggregations from 30 min and onwards. This made sampling problematic as the digestate became increasingly inhomogeneous. It also confirmed that in this study, without an internal standard in each experiment, the high degree of variation was most likely due to issues with sampling, as the sum of the AUP of the GC trace showed the same variation.

Morphological Changes of Intralipid Droplets During Simulated Gastric Digestion Using TEM

Intralipid emulsion droplets did not show any notable difference in the size at the commencement of digestion regardless of enzyme composition or *p*H (Figs. 3A1, B1 and C1, 4A1, B1 and C1). The oil droplets had a diameter of 0.3–0.5 μm (in agreement with previous SEM data) and showed stability against flocculation at all *p*H levels studied. However, at the end of the simulated digestion process, the morphology of most of the oil droplets had changed with the final structure varying according to relative pH. At *p*H 3.5, they formed clusters with individual droplets displaying a high degree of anisotropy. Oil droplets appeared smaller and darker with the bright area inside the droplets, showing electron-dense and less dense regions generated during two hours of digestion (Fig. 3A2-A5). At *p*H 4.5, the appearance of the droplets was more uniform, noting that some electron-dense regions had accumulated at the inner surface of the droplets (Fig. 3B2-B4). The appearance of the large droplets (diameter larger than 5 μm) was indicative of coalescence, with less electron-dense compounds forming in round shapes inside the large droplets (Fig. 3B4 and B5). At *p*H 5.5, some degree of anisotropy was observed, along with regions of highly disordered structures (Fig. 3C2-C5). Although these regions showed appreciable staining, it is difficult to determine whether these are lipids based due to the irregularity of the appearance.

For Intralipid emulsion digested with lipase and pepsin, the oil droplets at the initial state were shown in Fig. 4A1, B1 and C1. After two hours of digestion, oil droplets at *p*H 3.5 and 4.5 became larger. Some were merged into giant droplets with a diameter larger than 5 μm. At *p*H 3.5 the surface of the large droplets became diffuse and cracked (Fig. 4A2-A5, B4 and B5). Vesicles formed inside the large droplets with electron-dense regions observed inside the vesicles (Fig. 4A3, B2 and B3) and then released to the ambient aqueous phase (Fig. 4B4 and B5). Some of the striated structures were generated during simulated gastric digestion, attached on the droplet surface and then released into the ambient phase (Fig. 4B4 and B5). At *p*H 5.5, some others tended to break up and release the electron-dense compounds accumulated inside the droplets (Fig. 4C2-C5). The compounds formed inside the droplets accumulated in round beads and turned out to be very different in shapes from the droplets digested at *p*H 3.5 and 4.5 after two hours. Changes in the morphology of the droplets were more drastic when pepsin was included in the digestion process compared to when there was lipase present alone. Additionally, striated structure compounds only formed in the combined presence of pepsin and lipase.

When Intralipid emulsion droplets were coated with lactoferrin on the interface, droplets became aggregated at the commencement of digestion, with protein layer seen to be located on the interface (Fig. 5A1, B1 and C1). Because of the contrast effect from the protein layer, droplets could be observed much more clearly under TEM. When lipase was present in the digestate alone, the size of the droplets did not change drastically after two hours of digestion at *p*H 3.5 (Fig. 5A2-A5). Electron-dense compounds appeared at the inner interface of the oil droplets (Fig. 5A2), and the droplets were increasingly distorted in shape (Fig. 5A3-A5). At *p*H 4.5, droplets had increased in size and appeared amorphous after two hours of digestion (Fig. 5B2-B5). Droplets at *p*H 5.5 showed diverse structural characteristics. Spherical droplets were still observed, but the staining patterns within these droplets had become distinctively disparate. When both enzymes (lipase and pepsin) were present in the digestate, droplets underwent many notable changes compared to conditions with lipase alone (Fig. 6). Droplets were observed to have merged into large ones, released transparent vesicles

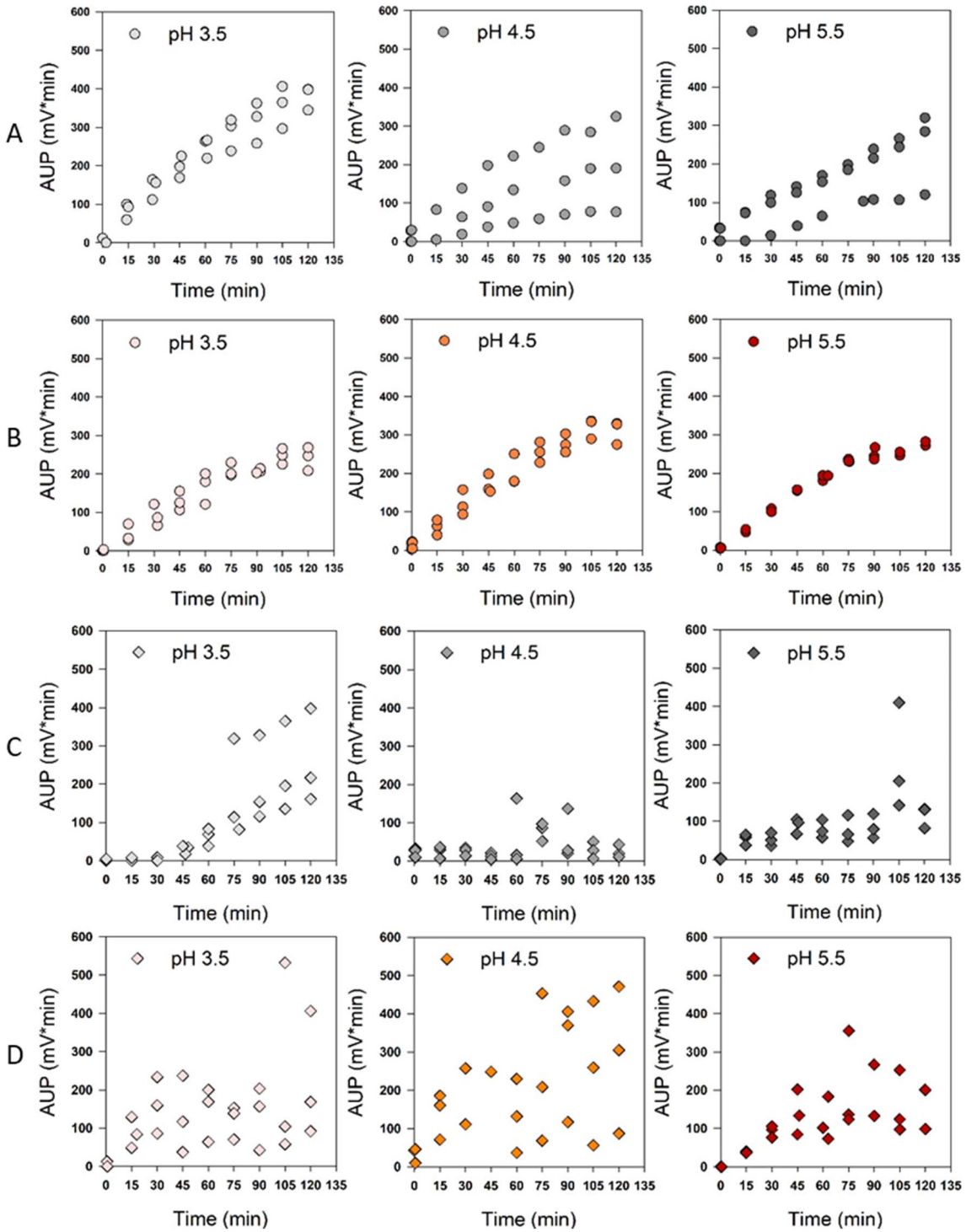

Fig. 2 FFA increase of original AUP values of each *in vitro* digestion treatment. **A** Intralipid digested with lipase; **B** Intralipid digested with lipase and pepsin; **C** lactoferrin coated Intralipid digested with lipase; **D** lactoferrin coated Intralipid digested with lipase and pepsin

and striated like electron-dense compounds and even appearing to have burst. Armand et al. [19] also showed distorted droplets and electron-dense lipolytic products on infant formula gastric digestion in an *in vivo* study in the premature infant.

Infant Formula Tests

Increase of Free Fatty Acids in Simulated Gastric Digestion

The extent of lipolysis was calculated based on the internal

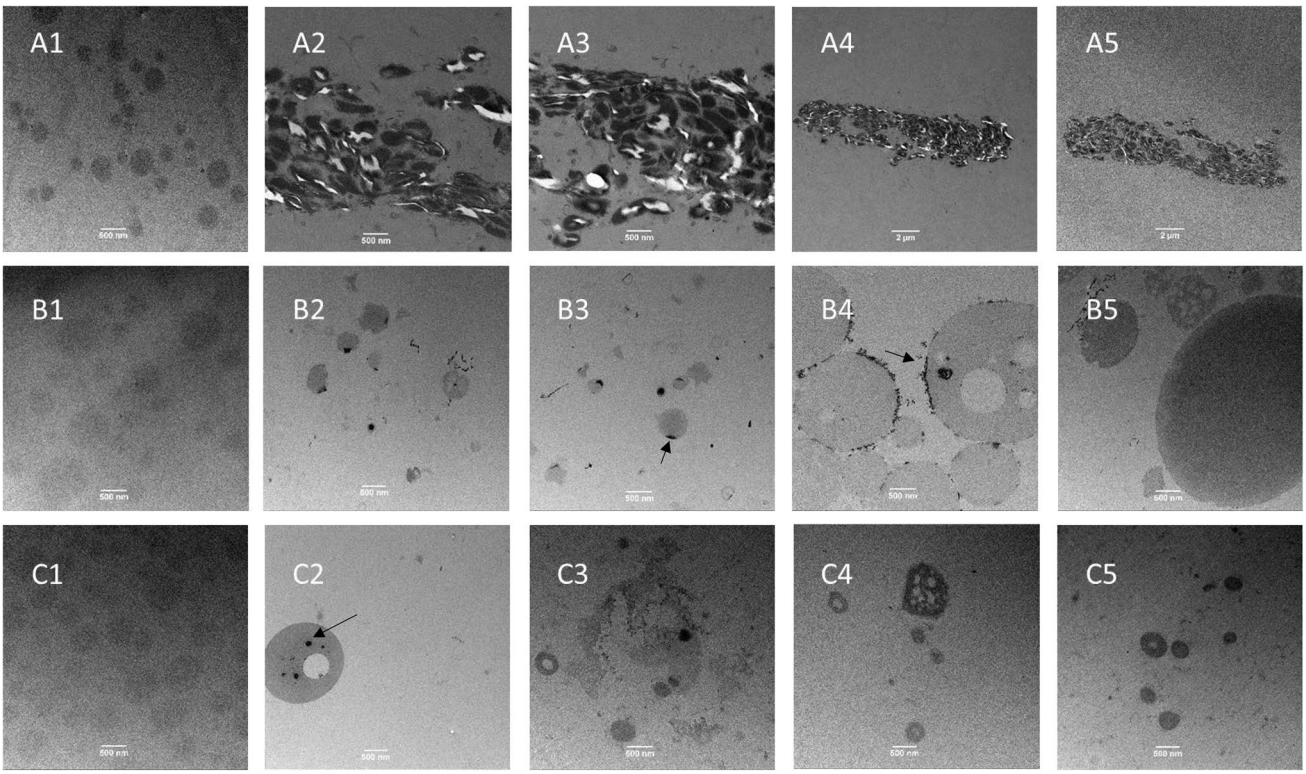

Fig. 3 TEM images of Intralipid emulsion digested with lipase alone. A1, *p*H 3.5 at 0 min; A2-A5, *p*H 3.5 at 120 min; B1, *p*H 4.5 at 0 min; B2-B5, *p*H 4.5 at 120 min; C1, *p*H 5.5 at 0 min; C2-C5, *p*H 5.5 at 120 min. Scalebar = 500 nm. Arrows indicated electron-dense lipolytic products

standard of each run (Fig. 7). The gastric lipolysis for infant formula was under 40% during 120 min of simulated digestion. The results were similar to studies in the past, which showed 10–30% of gastric lipolysis in neonates [18]. Except for *p*H 5.5 digested with lipase and pepsin, the extent of lipolysis increased along with elevated *p*H level. Moreover, the extent of infant formula digested with lipase only was higher than when digested with lipase and pepsin.

Evolution of free fatty acids was treated as the marker of the degree of lipid digestion. Validation of results showed consistent responses with good reproducibility from our method and the GC unit. The results were shown in Fig. 8. The statistical analysis showed that the data from digestion followed a linear relationship with time, except for formula digested at *p*H 4.5 with lipase and pepsin (Table 1). This indicated that, except for treatment at *p*H 4.5 with lipase and pepsin, all other treatments had a significant increase in free fatty acids with time.

The pair-wise comparison of the probability between treatments was shown in Table 2. It could be seen that the FFA increased significantly with time (Fig. 9). From the probability table (Table 2), no significant difference in the slopes was observed between *p*H 3.5 and *p*H 4.5 or *p*H 5.5, or additionally between *p*H 4.5 and *p*H 5.5 in slopes. This indicated that the digestate across the three *p*H conditions showed no significant difference in the rate of lipolysis from 30 min onwards to the end of simulated digestion. However, for the intercept, all three *p*H levels were significantly different from each other. This indicated that in the first 30 min, the rate of lipolysis would have been different among the three *p*H levels where the rate of *p*H 3.5 ($a = 32.287 \pm 30.471$ mV*min) < *p*H 4.5 ($a = 248.715 \pm 32.824$ mV*min) < *p*H 5.5($a = 534.458 \pm 40.924$ mV*min)) (Tables 3 and 4).

When infant formula was digested with lipase and pepsin, the degree of lipolysis was lower relative to digestion with lipase alone. At *p*H 4.5, there was no significant increase with time, and accordingly, we were only able to compare coefficients at *p*H 3.5 and 5.5 (Fig. 10). The slope values at *p*H 3.5 and 5.5 again showed no significant difference. The intercept value of *p*H 5.5 ($a = 67.550 \pm 17.764$ mV*min) was significantly higher than that of *p*H 3.5 ($a = 20.690 \pm 12.692$ mV*min). This indicated at the first 30 min, the rate of lipolysis for the digestate at *p*H 5.5 was slightly higher than the rate at *p*H 3.5. However, after 30 min, the rate of lipolysis was the same.

Furthermore, we were able to compare the intercept and slope values from treatment with lipase alone to the values from treatment with lipase and pepsin using the

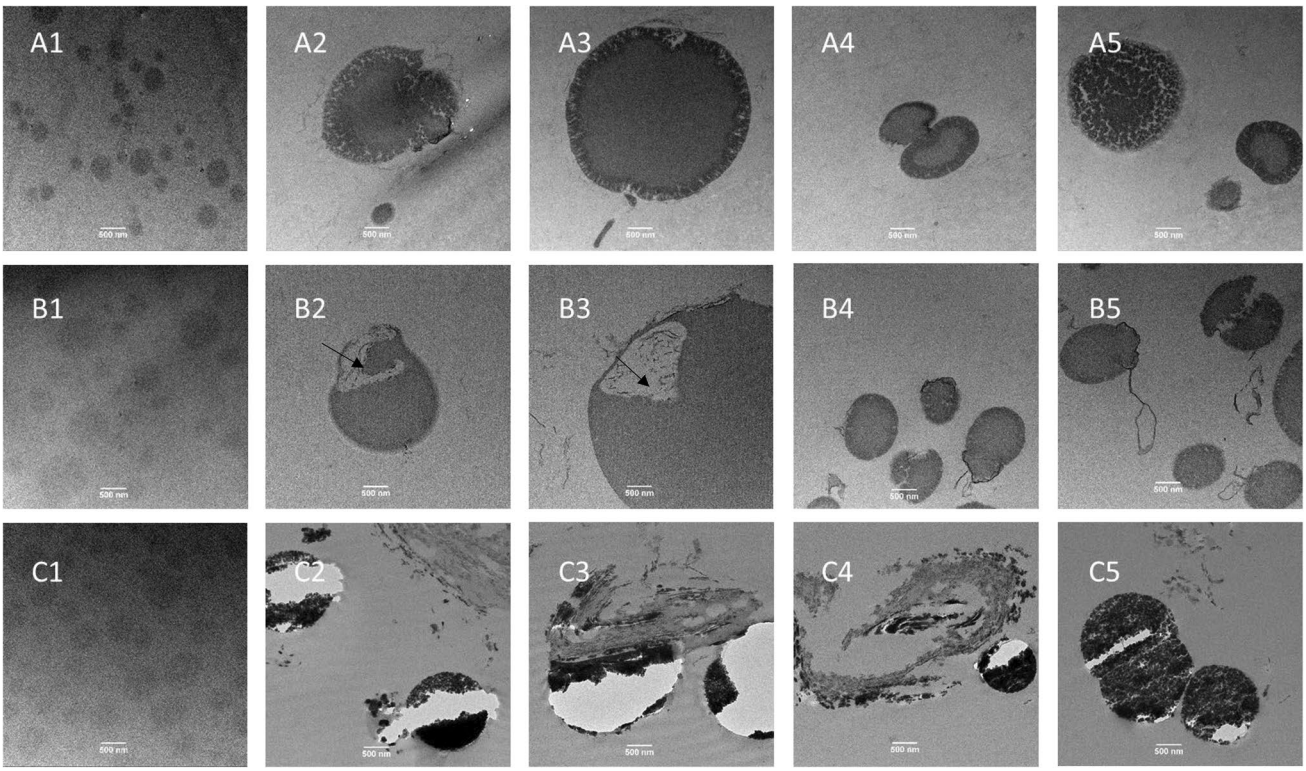

Fig. 4 TEM images of Intralipid emulsion digested with lipase and pepsin. A1, pH 3.5 at 0 min; A2-A5, pH 3.5 at 120 min; B1, pH 4.5 at 0 min; B2-B5, pH 4.5 at 120 min; C1, pH 5.5 at 0 min; C2-C5, pH 5.5 at 120 min. Scale bar = 500 nm. Arrows indicated electron-dense lipolytic products

Student t-test (Fig. 11). The slope values of digestion at *p*H 3.5 with lipase alone (b = -1.679 ± 0.558 mV) or with lipase and pepsin (b = 0.816 ± 0.235 mV) had no significant difference from digestion at *p*H 5.5 with lipase and pepsin (b = 0.975 ± 0.322 mV), but the slope value of digestion at *p*H 5.5 with lipase (b = 2.607 ± 0.716 mV) was significantly higher than those three treatments. The intercept values of digestion at *p*H 3.5 (with lipase alone) at t = 30 min had no significant difference from digestion at *p*H 3.5 or *p*H 5.5 (with lipase and pepsin). However, a significant difference was observed between the intercept values of treatment at *p*H 5.5 (with lipase alone) and those of both *p*H 3.5 and *p*H 5.5 (with lipase and pepsin). This indicated that there was no significant difference between the rate of lipolysis for the first 30 min for digestion at *p*H 3.5 with lipase alone (a = 32.287 ± 30.471 mV*min), and digestion at *p*H 3.5 (a = 20.690 ± 12.692 mV*min) or 5.5 with lipase and pepsin (a = 67.550 ± 17.764 mV*min). This indicated from 30 min onwards, rate of lipolysis for digestion at *p*H 3.5 (with lipase alone or with lipase and pepsin) was the same and was very similar to that of digestion at *p*H 5.5 with lipase and pepsin, whereas the rate for digestion at *p*H 5.5 with lipase was faster than the previous three treatments.

Morphological Changes of Infant Formula During Simulated Gastric Digestion Using TEM

The morphological changes of the infant formula emulsion during simulated gastric digestion were shown in Figs. 12 and 13. Figure 12 showed the changes for lipase only and Fig. 13 for lipase and pepsin. The initial structure (t = 0 min) of the infant formula showed an average droplet size of 0.3–0.4 μm with flocculated droplets evident at *p*H 3.5 or 4.5. At *p*H 5.5, the oil droplets displayed less propensity towards flocculation.

When infant formula was digested with lipase alone, the droplet size was observed to have reduced in size, and the shape of the droplets had turned amorphous after 120 min at *p*H 3.5. The oil droplets did not appear to have any shape when *p*H turned to 4.5 after 120 min of simulated digestion. Also, there appeared to be evidence of some filamentous structures after 120 min of simulated digestion at this *p*H level. A *p*H 5.5, the droplets showed a propensity towards aggregations, displaying increasing anisotropy, but not being significantly reduced in particle size.

When the infant formula was digested with lipase and pepsin, a considerable change in structure was observed. The assembled structure appeared indicative of extensive

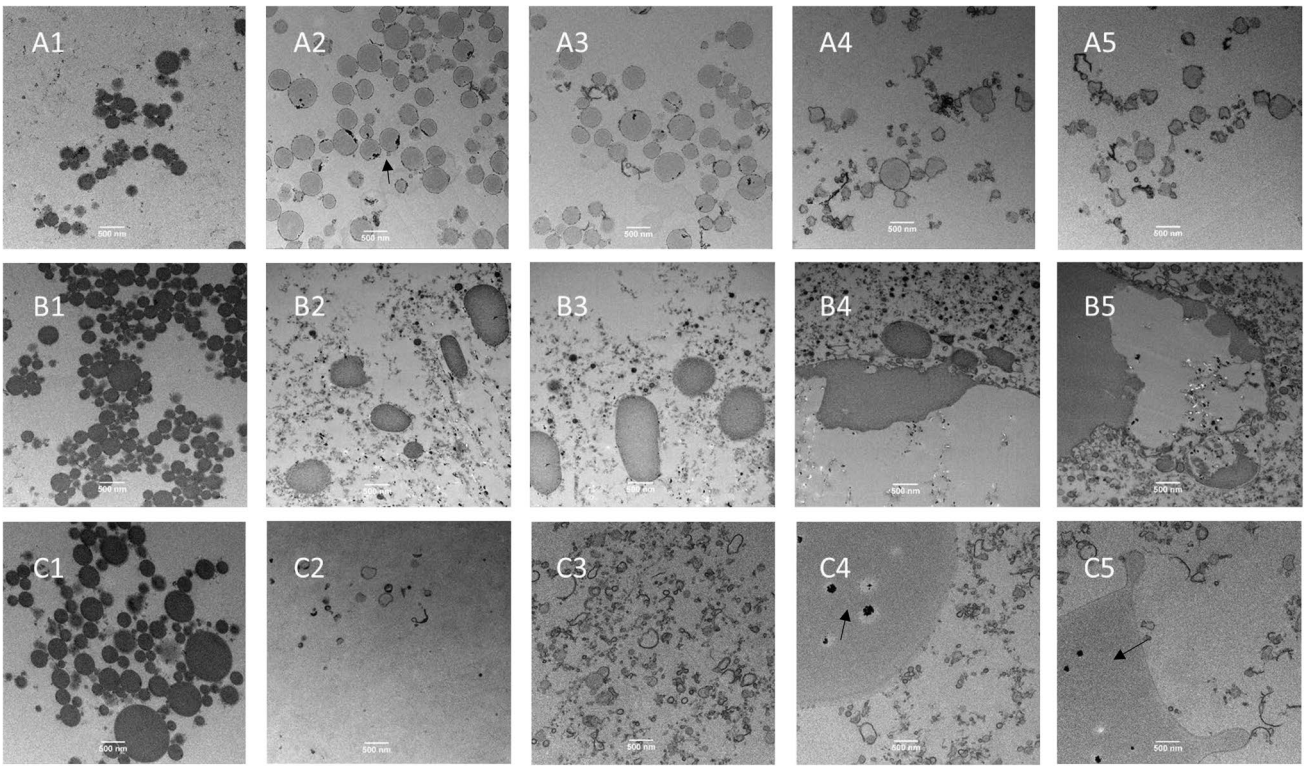

Fig. 5 TEM images of lactoferrin Intralipid emulsion digested with lipase alone. A1, pH 3.5 at 0 min; A2-A5, pH 3.5 at 120 min; B1, pH 4.5 at 0 min; B2-B5, pH 4.5 at 120 min; C1, pH 5.5 at 0 min; C2-C5, pH 5.5 at 120 min. Scale bar = 500 nm. Arrows indicated electron-dense lipolytic products

coalescence of which, surrounded by an interconnected filamentous network, which was attributed to the self-association of hydrolysates of proteins and intact proteins on the interface of oil droplets as well as in the aqueous phase. These structures were seen to be relatively consistent across all *p*H levels studied after 120 min of simulated gastric digestion.

Discussion

Pretests for Intralipid Measurements

In the pretests, we determined that lipolysis was primarily responsible for the changes to the morphology of emulsion droplets during this simulated gastric digestion process. However, correlating the observed changes in droplet structure to the rate and extent of lipolysis was ultimately not possible, due to challenges in obtaining reliable measurements for FFA content during digestion. For the GC measurements, data showed large variations in replicates across most of the treatments (notably those samples observed to undergo phase separation during the process of digestion which hindered reliable sampling). Accordingly, it was difficult to draw reasonable conclusions regarding the extent of lipolysis from the FFA data across variable *p*H conditions, presence of lactoferrin and enzyme combination. Therefore, the GC measurement of FFA increase may not accurately reflect the actual reaction kinetics from the digestion process. The statistical analysis of linear regression for the first 30 min showed that the rate (the slope values) of lipolysis had no significant difference among all treatments. However, the data set showed that linear regression might not be a suitable way to describe the trend of lipolysis in all treatments across the overall reaction time. Since the errors in the experiment were remarkably large, it was also challenging to apply non-linear regression in order to describe the trend of FFA increase.

Findings can be considered in relations to two prior studies by Lueamsaisuk et al. [12, 20] that had shown that the particle size distributions of emulsions comprising Intralipid and lactoferrin (as used in this current study) were affected by both *p*H conditions and combination of pepsin and lipase during simulated digestion over 120 min. The release of FFA was not measured in these previous studies, so it was not possible to unequivocally state that structure dynamics were a consequence of lipolysis. However, it was hypothesised that emulsions not displaying any changes in emulsion structure during digestion were potentially not undergoing lipolysis. In this current study, we confirmed the liberation

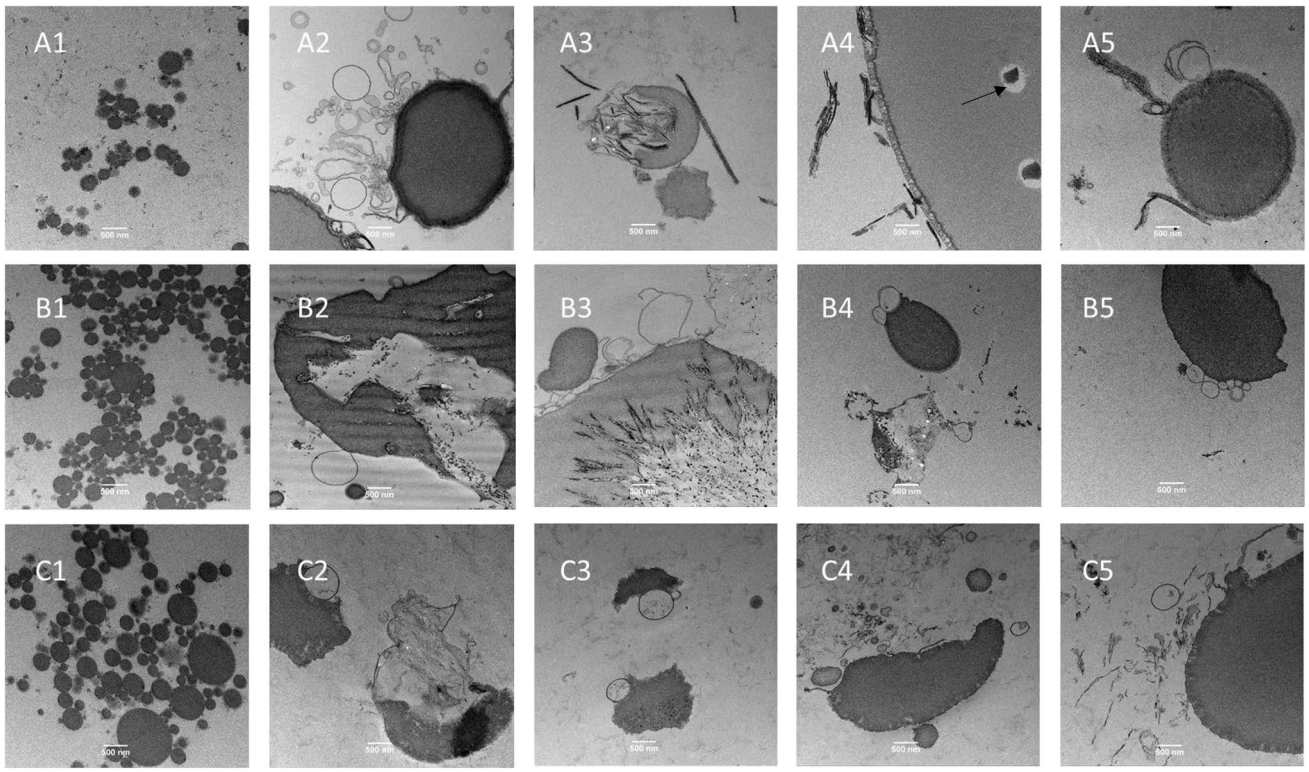

Fig. 6 TEM images of lactoferrin coated Intralipid emulsion digested with lipase and pepsin. A1, pH 3.5 at 0 min; A2-A5, pH 3.5 at 120 min; B1, pH 4.5 at 0 min; B2-B5, pH 4.5 at 120 min; C1, pH 5.5 at 0 min; C2-C5, pH 5.5 at 120 min. Scale bar = 500 nm. Arrows indicated electron-dense lipolytic products

of FFA for all samples regardless of whether the interfacial layer comprised phospholipid or phospholipid bound to lactoferrin. This would appear to confirm that the presence of interfacial protein for these emulsions was not entirely inhibitory to lipolysis when pepsin was excluded from the digestate (i.e., negating the hypothesis that proteolysis of the interface is a requisite for lipolysis to occur for protein stabilised emulsions).

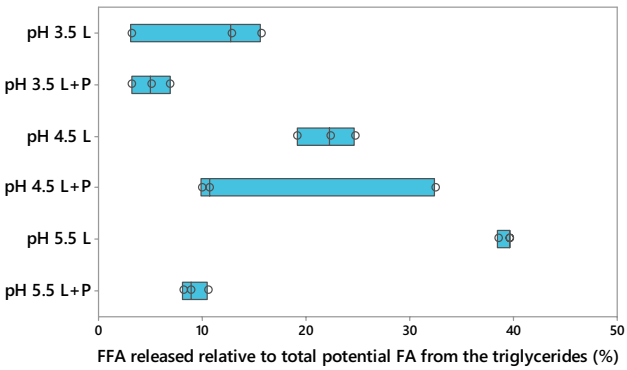

Fig. 7 FFA released relative to the total potential FA from the triglycerides from an infant formula emulsion digested in a simulated gastric system. L, infant formula digested with lipase alone; L + P, infant formula digested with lipase and pepsin. The *p*H values were all initial *p*H levels

In considering the relationship between structural changes observed during digestion and lipolysis, it was interesting to note that the electron-dense structures found near the surface of droplets after digestion (Fig. 5.6–5.9) appeared independent of the inclusion of pepsin in the digestate, *p*H changes, or the curve shape of the FFA increase. Similar structures have been observed in previous studies [19, 21], with the suggestion that these are polar crystal lipolytic products generated from triglyceride hydrolysis. Variance in the extent of structural changes observed in both SEM and TEM under different *p*H conditions may be due to variations in the activities of both pepsin and lipase across this *p*H range. Additionally, differences in droplet structure and morphology may also possibly be due to the impact of *p*H on the assembly of the lamellar polar lipid structures, noting that lower *p*H conditions will increasingly approach the *p*Ka of the fatty acids being liberated and thus increasingly lead to protonation of these molecules.

However, results (both microscopic and GC) would ultimately suggest that the lactoferrin layer bound to the Intralipid oil droplets did not appear to act as a barrier for lipase binding. A study on interfacial protein structure

Table 1 Statistical analysis of linear regression for FFA increase under different treatments

	[a]T1	[a]T2	[a]T3	[a]T4	[a]T5	[a]T6
[b]*a*	38.287	248.715	534.458	20.690	-	67.550
[c]*SEM (a)*	30.471	32.824	40.924	12.692	-	17.764
[d]*b*	1.679	2.650	2.607	0.816	-	0.975
[e]*SEM (b)*	0.558	0.607	0.716	0.235	-	0.322
[f]*df*	1;18	1;19	1;16	1;19	1;19	1;19
[g]*F*	9.058	19.068	13.247	12.080	3.583	9.163
[*]*Sig.*	0.008	< 0.005	0.002	0.003	0.074	0.007
[h]R^2	0.335	0.501	0.453	0.389	-	0.325

[a]T1, *p*H 3.5 digested with lipase; T2, *p*H 4.5 digested with lipase; T3, *p*H 5.5 digested with lipase; T4, *p*H 3.5 digested with lipase and pepsin; T5, *p*H 4.5 digested with lipase and pepsin; T6, *p*H 5.5 digested with lipase and pepsin

[b]*a*, intercept value, unit mV*min

[c]*SEM (a)*, standard error of the mean of *a*

[d]*b*, slope value, unit mV

[e]*SEM (b)*, standard error of the mean of *b*

[f]*df*, degrees of freedom and residual degrees of freedom

[g]*F*, F value

[*]*Sig.*, *p*-value, $\alpha = 0.05$

[h]R^2, coefficient of determination

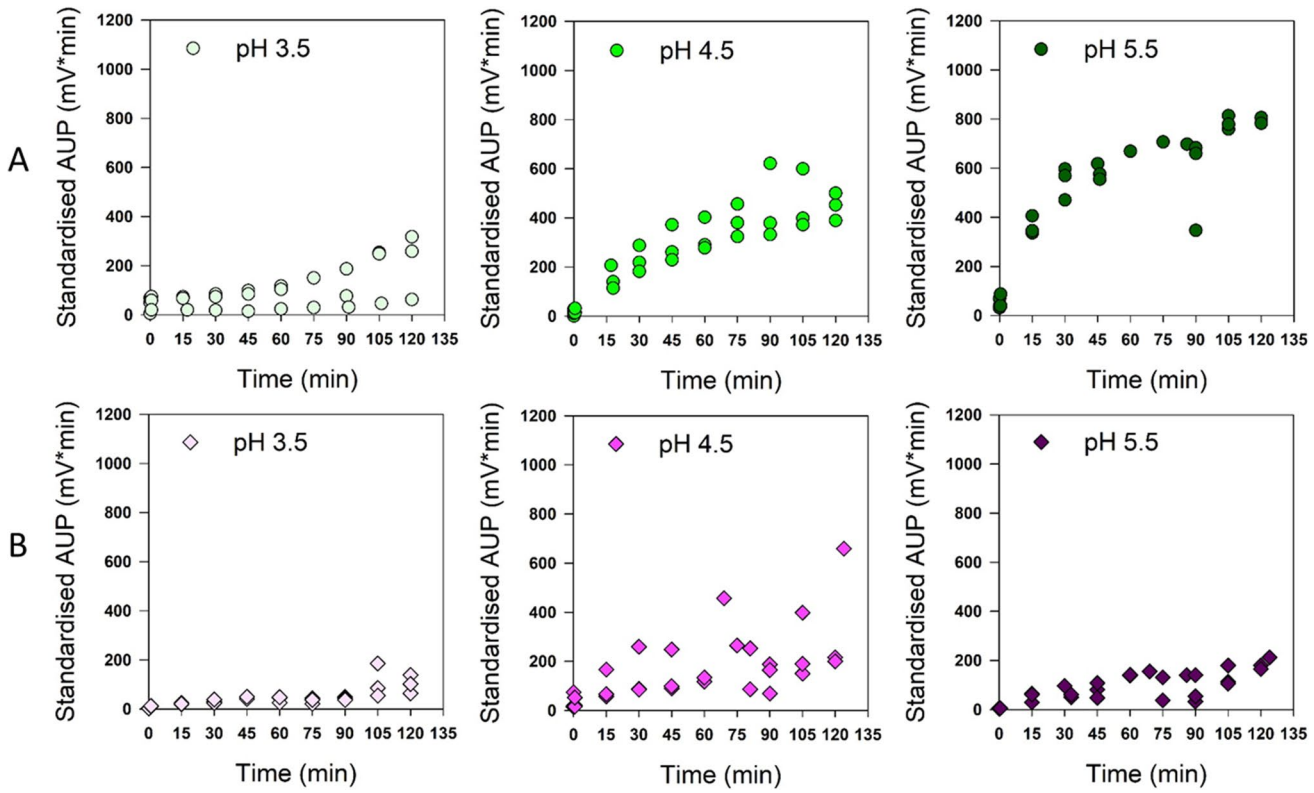

Fig. 8 Free fatty acid increase showed by standardised AUP (mV*min) for a powder infant formula digested in a simulated gastric system. **A** Infant formula digested with lipase alone. B, Infant formula digested with lipase and pepsin. ○: digestion at pH 3.5; ● : digestion at pH 4.5; ●: digestion at pH 5.5; ◇: digestion at pH 3.5; ◆: digestion at pH 4.5; ◆: digestion at pH 5.5.

Table 2 Pair-wise comparison of the probability of coefficients of the linear regression of FFA increase in infant formula digested with lipase alone ($\alpha = 0.05$)

Intercept (a)	*p*H 3.5	*p*H 4.5	*p*H 5.5	Slope (b)	*p*H 3.5	*p*H 4.5	*p*H 5.5
*p*H 3.5	-	< 0.001	< 0.001	*p*H 3.5	-	0.247	0.309
*p*H 4.5	-	-	< 0.001	*p*H 4.5	-	-	0.963
*p*H 5.5	-	-	-	*p*H 5.5	-	-	-

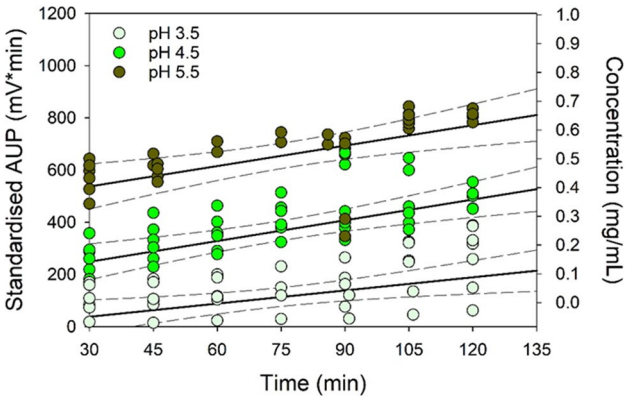

Fig. 9 Linear regression of FFA increase of infant formula digested with lipase alone in a simulated gastric system——— : regression;- - - - : 95% confidence interval;○: digestion at *p*H 3.5;●: digestion at *p*H 4.5;●: digestion at *p*H 5.5

Table 3 Pairwise comparison of the probability of coefficients of the linear regression of FFA increase in infant formula digested with lipase and pepsin ($\alpha = 0.05$)

Intercept (a)	*p*H 3.5	*p*H 5.5	Slope (b)	*p*H 3.5	*p*H 5.5
*p*H 3.5	-	0.038	*p*H 3.5	-	0.692
*p*H 5.5	-	-	*p*H 5.5	-	-

Table 4 Pairwise comparison of the probability of coefficients of the linear regression of FFA increase in infant formula digested with lipase alone or with lipase and pepsin ($\alpha = 0.05$)

Intercept (a)	[a]3.5 L	[b]5.5 L	[c]3.5LP	[d]5.5LP	Slope (b)	[a]3.5 L	[b]5.5 L	[c]3.5LP	[d]5.5LP
[a]3.5 L	-	-	-	-	[a]3.5 L	-			
[b]5.5 L	< 0.001	-	-	-	[b]5.5 L	0.309	-		
[c]3.5LP	0.591	< 0.001	-	-	[c]3.5LP	0.155	0.016	-	
[d]5.5LP	0.406	< 0.001	0.038	-	[d]5.5LP	0.275	0.036	0.692	-

[a]3.5 L, *p*H 3.5 digested with lipase

[b]5.5 L, *p*H 5.5 digested with lipase

[c]3.5LP, *p*H 3.5 digested with lipase and pepsin

[d]5.5LP, *p*H 5.5 digested with lipase and pepsin

provided similar results [22]. This suggested that there were gaps in the oil/water interface where the hydrophobic domain of lipase could access to the phospholipid layer, and thus bind to the interface. Subsequent liberation and adsorption of free fatty acids from lipolysis might then be expected to displace bound protein from the interface, thereby enabling further adsorption of lipase.

In our initial hypothesis, gastric lipolysis kinetics of protein stabilised emulsions are optimised by a co-dependency between pepsin and lipase. Pepsin would facilitate lipolysis by hydrolysing the adsorbed protein layer, partially removing the barrier for lipase to attach on the phospholipid-oil interface, more effectively enabling lipase binding and thus increasing the rate and extent of lipolysis. However, the addition of pepsin did not appear to change this scenario. This may occur due to several reasons. Firstly, as the isoelectric point of lactoferrin is 8.7 [23], lactoferrin carries positive charges from *p*H 3.5 to 5.5. The *p*H level could have a substantial impact on the charge density of proteins as it affects the degree of ionisation of proteins. Therefore, when *p*H increases from 3.5 to 5.5, the charge density of lactoferrin decreases. This may loosen the attachment of lactoferrin to the interface covered by negatively charged phospholipids where lipase could more easily overcome the protein barrier and adsorb on the oil/water interface without the help of pepsin.

Secondly, as *p*H level dropped from 5.5 to 3.5, pepsin activity would be seen to increase towards its optimum at *p*H = 2.0, increasing proteolysis and liberating more peptides. Hydrolysed hydrophobic peptides may serve to reduce the surface tension on the oil/water interface and induce

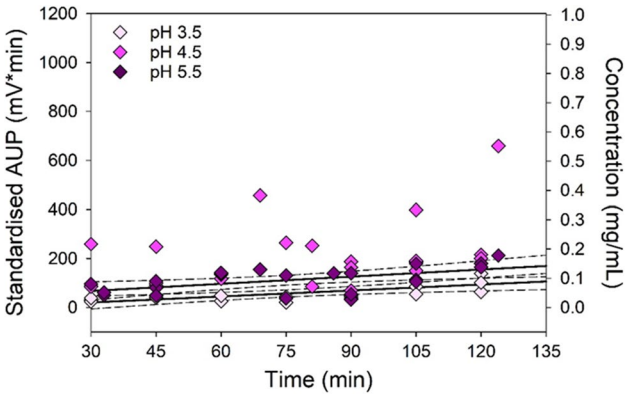

Fig. 10 Comparison of data of FFA increase of infant formula digested with lipase and pepsin in a simulated gastric system——: regression;----: 95% confidence interval (regression of *p*H 4.5 did not show as the regression was not statistically significant);◇: digestion at *p*H 3.5;◆: digestion at *p*H 4.5;◆: digestion at *p*H 5.5

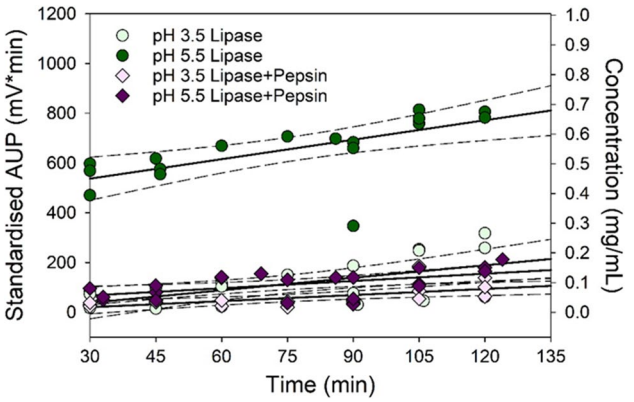

Fig. 11 Comparison of data of FFA increase of infant formula digested with lipase alone or with lipase and pepsin in a simulated gastric system——: regression;----: 95% confidence interval;○: digestion with lipase alone at *p*H 3.5;●: digestion with lipase alone at *p*H 5.5;◇: digestion with lipase and pepsin at *p*H 3.5;◆: digestion with lipase and pepsin at *p*H 5.5

emulsion instability through either flocculation and/or coalescence. For example, a study [24] showed emulsion droplets stabilised by whey protein had stronger interfaces with higher elasticity than hydrolysed proteins. Sarkar et al. [25] also found emulsion droplets stabilised by *β*-lactoglobulin had extensive flocculation and coalescence in the *in vitro* gastric digestion. Such destabilisation result may usually lead to an increase of droplet size and reduction in surface area for lipase adsorption. Separating the particular roles of pepsin and lipase on lipid hydrolysis kinetics has been found to be particularly challenging, both due to the profound changes in emulsion structure during digestion and as a consequence of the disparate optimum activities displayed by both enzymes. Arguably, the results would be better interpreted if the sampling problem could be resolved with an internal standard induced for GC analysis.

That said, it is worth noting that changes to emulsion droplet size relative to FFA release have been observed in several earlier studies, although the separation of lipases and pepsin was not considered in these studies [3, 9, 26–29]. Some of this previous research also found the release of FFA reached a plateau status after 20 to 60 min after the initiation of gastric digestion (*in vitro* and *in vivo*) [3, 9, 26, 27, 29]. This indicated that the reaction might reach equilibrium or that the lipolytic products had inhibited the lipase activity at the first hour of gastric digestion [3]. The study by Pafumi et al. [3] showed that lipases were trapped in the protrusions formed by lipolytic products on the oil/water interface and therefore inhibited lipolysis.

These findings were somewhat different from what was observed in this study. This was arguably a surprising outcome, given the significant structural changes observed for the same systems when applying variable pH and enzyme conditions [30]. One of the reasons, as stated above, was the difficulty in sampling (and thus likely impacted by the structural changes evidenced in our prior study). The large standard errors were also seen in those studies. It should be noted that lipolysis takes place at the oil/water interface which is very different from the conventional enzymatic reactions in the aqueous environment following Michaelis-Menten kinetics. Therefore, it could be very possible that the concentration of FFA from the lipolysis in this study has not yet reached the saturation point for product inhibition, also noting that this study utilised a fungal lipase as an analogue for human gastric lipase, and therefore may not provide full equivalence regarding the type and concentrations of FFA released.

Infant Formula Tests

The gastric digestion of formula emulsions was studied under *in vitro* conditions, with the rationale that separate applications of lipase and combined lipase-pepsin would provide an indication of their respective roles in the lipolysis of emulsion droplets. Digestion behaviours, both in terms of generation of fatty acid and emulsion structure were seen to vary according to both *p*H and enzyme combination.

Lipolysis from 0 to 30 min – Lipase Alone

For emulsion digestion in the absence of pepsin, findings from GC indicated that the rate of lipolysis showed significant difference among three different *p*H conditions for the samples containing lipase in the absence of pepsin, as determined by the intercept values at t = 30 min (Fig. 4). Nothing that the extent of lipolysis increased as *p*H was increased from 3.5 to 5.5, this would suggest that this effect

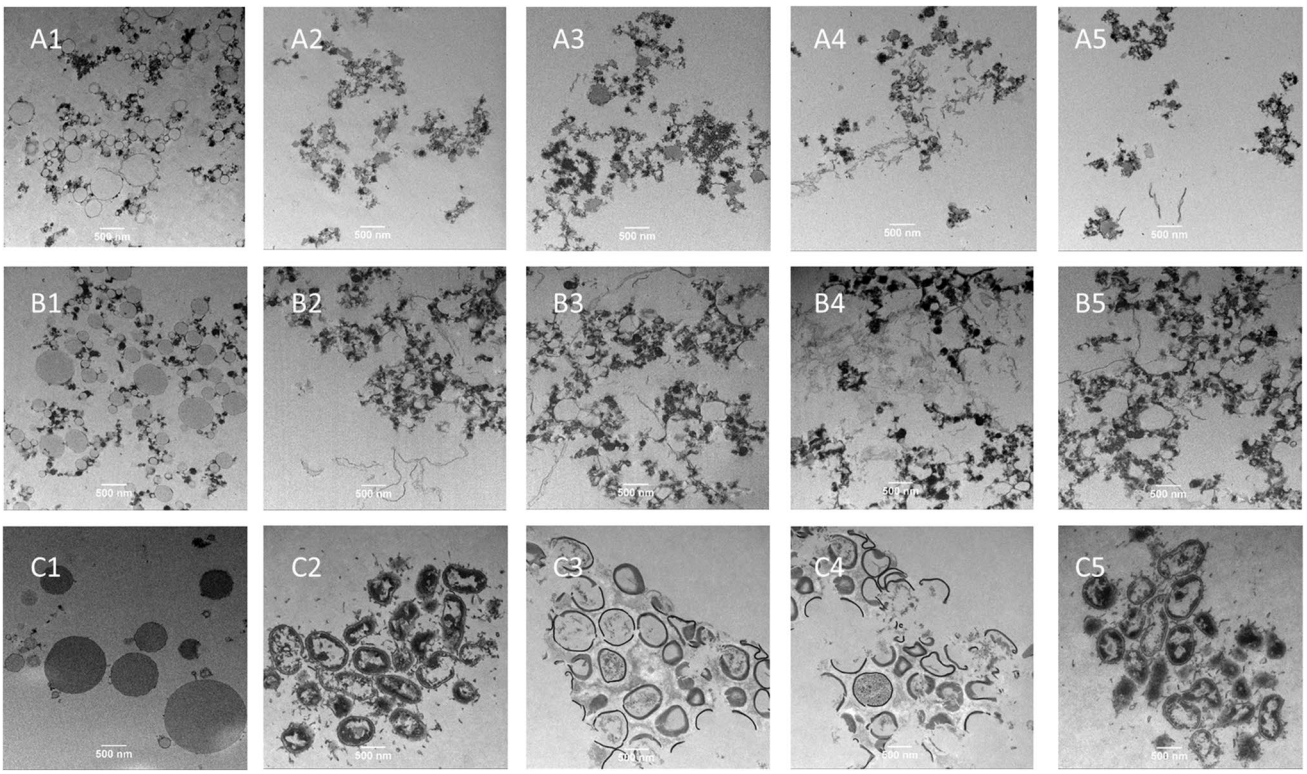

Fig. 12 Images of TEM of Infant formula digested with lipase alone in a simulated gastric system. A1, *p*H 3.5 at 0 min; A2-A5, *p*H 3.5 at 120 min; B1, *p*H 4.5 at 0 min; B2-B5, *p*H 4.5 at 120 min; C1, *p*H 5.5 at 0 min; C2-C5, *p*H 5.5 at 120 min; Scale bar = 500 nm

was primarily governed by the relative *p*H activity of the enzyme. As HGL lipase is an acid-stable lipase and possesses an optimal *p*H of 3.0 to 6.0 [30–33], this allows it to digest triglycerides across a broad spectrum of digestive *p*H, though it does become less active towards fasting *p*H conditions [30]. In contrast, the fungal gastric lipase used in this study is optimally active at *p*H 7.0 (pK_a of histidine is 6.5 in its Ser-His-Asp catalytic triad) [34, 35]. Thus, it is not necessarily surprising that the relative rate of digestion would decline under increasingly acidic conditions, as indicated in Fig. 4.

The extent of lipolysis observed at *p*H 5.5 also indicated that the proteinaceous interface of the droplets was not inhibitory to lipase adsorption. Decreasing *p*H towards the isoelectric point might, however, be expected to influence both the interfacial structure of the protein and continuous phase structure which could in turn influence the lipolysis behaviours observed. Figure 6 has shown that, at the commencement of digestion, at *p*H 5.5 the emulsion droplets were stable and non-interacting. In contrast, at *p*H 4.5 and 3.5, flocculation of the emulsion was clearly observed. As *p*H approached the isoelectric point of the proteins, interactions between the adsorbed protein layer of the emulsion droplets and the surrounding serum protein phase could lead to the formation of coagulated protein-droplet structures. It could be speculated that the access of the lipase enzyme is optimised when droplets are non-aggregated, and for which the entirety of the surface is available for lipase adsorption. In contrast, aggregated structures may reduce the availability of binding sites for lipase adsorption. While it is argued that the relative *p*H activity of the enzyme, the predominant effect of extent of lipase during the first 30 min of digestion, and the variations in emulsion structure, as observed in Fig. 6, may separately impact on the degree of lipolysis during the early stages of digestion.

Lipolysis from 30 to 120 min – Lipase Alone

While significant differences in rates of lipolysis were observed over the first 30 min of digestion, it is interesting to note that the relative rate of change of lipolysis after 30 min through to the end of digestion was shown to be equivalent across all three *p*H conditions.

One further curious observation was that no plateau levels of FFA were observed for any of the samples regardless of relative *p*H over 120 min of digestion. Approximately 10–30% of lipolysis occurs during gastric digestion, with the remainder occurring in the small intestine. The lower the levels of lipolysis in the stomach are usually attributed to the accumulation of FFA at the interface of fat droplets [3]. In

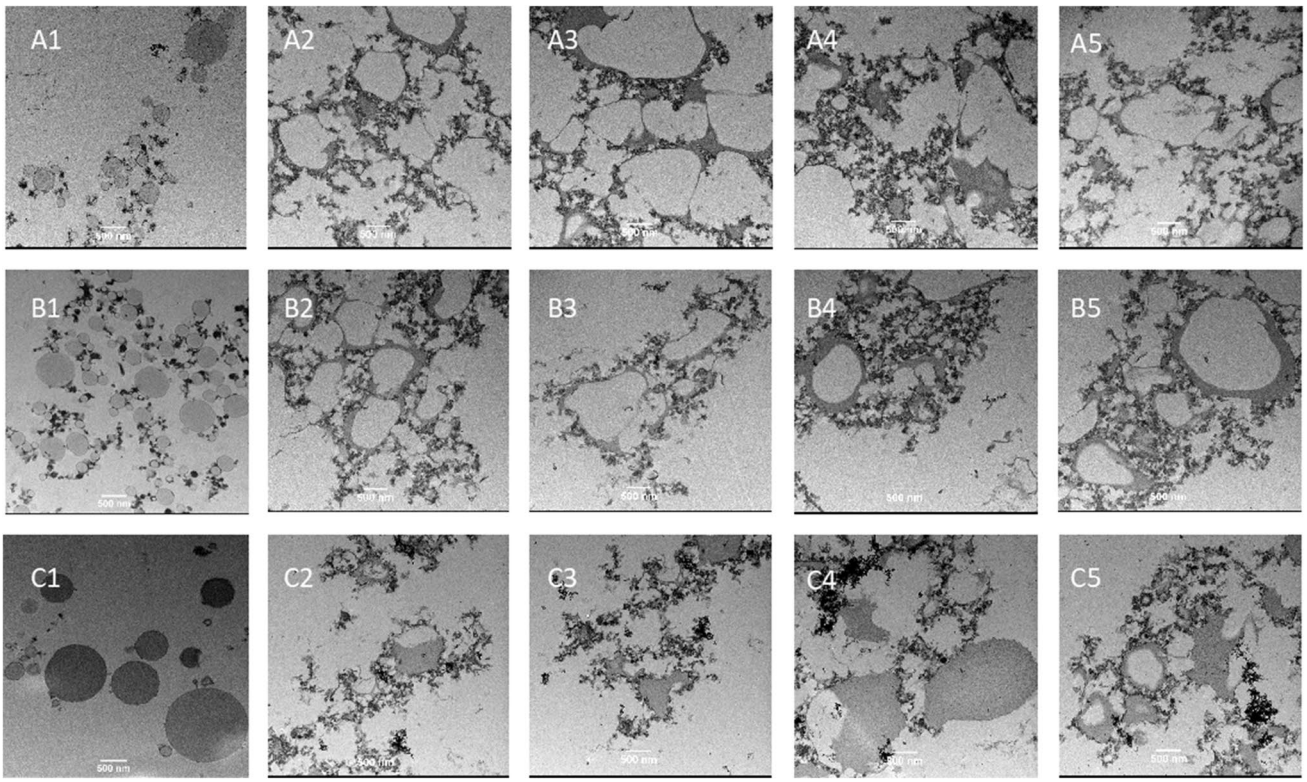

Fig. 13 Images of TEM of Infant formula digested with lipase and pepsin in a simulated gastric system. A1, *p*H 3.5 at 0 min; A2-A5, *p*H 3.5 at 120 min; B1, *p*H 4.5 at 0 min; B2-B5, *p*H 4.5 at 120 min; C1, *p*H 5.5 at 0 min; C2-C5, *p*H 5.5 at 120 min; Scale bar = 500 nm

the absence of bile salts, which assist in the solubilisation and removal of FFA from the interfacial layer, the accumulation of surface fatty acids ultimately becomes inhibitory to the adsorption of gastric lipase and limits its access to the triglyceride substrate. That no plateau level was observed was perhaps surprising and may be due to fungal, rather than human gastric lipase, being used in the study. Additionally, it may be argued that the reaction has not proceeded to the point of droplet surface saturation by free fatty acids had occurred.

While GC data shows that the rate of increase of FFA to be comparable across all *p*H conditions, noticeable structural differences were observed to the emulsion droplets during this period. At *p*H 3.5 and 4.5, after 120 min of digestion, flocculated structures could still be observed. As has already been discussed, the formation of coagulated protein-fat networks under conditions approached protein pI were observed at commencement of digestion. In the absence of pepsin, it seems reasonable that these structures would persist for the duration of incubation, and lipolysis appears to have little influence on the emulsion structure under these conditions.

After 120 min, droplets at *p*H 5.5 was also observed to be agglomerated (noting that aggregation was not observed at the onset of digestion). Flocculation under theses *p*H conditions is considered a direct consequence of lipolysis. Based on the observations in Fig. 5.6, it is suggested that fatty acid displacement of the adsorbed protein layer of emulsion droplets during lipolysis reduces both charge stabilisation and droplet repulsion. These adsorbed polar lipids can form a variety of crystalline and soft condensed layers at the surface of oil droplets depending on fatty acid types [3]. This surface crystallisation may be a mechanism for the droplet anisotropy observed in Fig. 5.6 and is similar to the observations made by Gallier et al. [21] in their study where a lipid-structured phase was observed at the interface of milk fat globules when digested milk in a gastric digestion study conducted in rats. Moreover, such interfaces can be rendered sticky, leading to droplet aggregation as observed by Pafumi et al. and others [3, 21].

Lipolysis from 0 to 120 min – Lipase and Pepsin

The inclusion of pepsin to the gastric fluid was seen to alter the digestion behaviours of the emulsion depending on *p*H conditions. As indicated in the findings, the impact on lipolysis was most noticeable at *p*H 5.5, displaying a considerable decrease in rate and extent of lipolysis during the first 30 min of digestion when compared to the sample digested by lipase alone. At *p*H 3.5, no significant changes were observed relative to the sample with lipase alone, nothing that the overall

extent of lipolysis was lowest under theses *p*H conditions. At *p*H 4.5, the raw data suggests that inclusion of pepsin results in a reduction in the extent of lipolysis relative to the sample containing lipase alone, however, the high degree of variability across samples at this *p*H makes it difficult to verify this finding.

The observation that the inclusion of pepsin to the simulated gastric fluid appears to reduce the rate and extent of lipolysis (at least at *p*H 5.5) appears contradictory to the initial hypothesis of the research that interfacial pepsinolysis would facilitate lipase adsorption for protein-stabilised emulsions by rendering the adsorbed layer more hydrophobic. There are several possible mechanisms to explain these observed behaviours. The first of these relates to the design of the study, and the use of the fungal lipase as an alternative to human gastric lipase. From a biological viewpoint, human gastric lipase has resistance to pepsinolysis, which is essential for it to retain activity during digestion. In contrast, the fungal lipase used in this study may be less resistant to pepinsolysis, leading to a loss of activity. This would arguably account for the low extent of lipolysis observed under all *p*H conditions. However, it should be noted that fungal lipases (including the *Rhizopus oryzae* derivative used in this study) have been used widely in *in-vitro* studies in combination and speculated to not be compromised in terms of activity due to hydrolysis of the enzyme by pepsin. Furthermore, while a significant reduction in the extent of lipolysis was observed at *p*H 5.5 when pepsin was present, pepsin activity at this *p*H would be greatly reduced, and thus least likely to affect the functionality of the lipase.

Another possible explanation for the differences in measured results is related to sampling issues during analysis, which in turn can be related to significant changes to emulsion structure during digestion when pepsin was included in the simulated gastric fluid. The sampling method of emulsions for GC analysis was made challenging due to increasing separation of the emulsion during gastric digestion under conditions where both enzymes were present.

This separation is believed to be primarily a consequence of proteolysis of the interfacial layer, which is responsible for the change in structures observed in TEM in Fig. 7. After 120 min digestion completed, absence of the initial spherical droplet distribution was observed across all three *p*H conditions. Instead, a network of aggregated filaments was observed surrounding highly anisotropic structures. These filaments were attributed as assemblies of protein formed through a combination of *p*H and protein hydrolysis. The darker anisotropic regions within the protein network, most notably at *p*H 5.5, are assumed to be coalescent fat droplets. However, at *p*H 3.5 and 4.5, the voids within the protein network appeared unstained, suggesting an absence of fat. These are interpreted as being ghost structures caused by the increasing destabilisation of the emulsion leading to extensive coalescence and phase separation of oil from with the structures. The observations are supported by previous work using particle size analysis which showed increasing particle size of formula milk during digestion with lipase and pepsin [12].

Proteolysis of the interfacial layer could cause detachment of polar polypeptides and retention of hydrophobic domains at the oil/water interface promoting flocculation of droplets, even for *p*H conditions away from the *p*I of the proteins. Secondly, loss of mechanical stabilisation of the interface arising from proteolysis promotes coalescence of droplets which is exacerbated by increased droplet-droplet contact as a result of flocculation.

As stated earlier, the loss of emulsion structure during digestion was problematic in trying to achieve homogeneous sampling throughout incubation. However, it is also worth noting that the extensive coalescence observed in the TEM images (Fig. 7) would have the effect of decreasing the relative surface area of the emulsion during digestion, thereby reducing the availability of binding sites for lipase adsorption. This would itself contributed to a decrease in relative extent of lipolysis, providing a third possible explanation as to the apparent reduction in overall lipolysis relative to emulsion digested with lipase alone.

Conclusions

In summary, this study provided further insight into the effect of protein interface in the process of gastric lipolysis by studying a commercial powder infant formula in a simulated gastric system. The results showed that proteins on the interface of the oil droplets do not appear to block lipases from accessing their substrate at the oil core. The proteins on the interface of emulsions seems to slow down the reaction of lipolysis. The rate of lipolysis was dominated by the ambient pH level when there was only the presence of lipase in the simulated digestive system. The higher the pH, the closer it was to the optimum activity, and the faster the rate could be achieved. When pepsin and lipase were present in the model, pepsin did not appear act as a facilitator to lipolysis at the oil/water interface. On the contrary, pepsin could be argued as being inhibitory to lipase on the interface, as fungal lipases might not be as highly glycosylated as human gastric lipase. Additionally, proteolysis of the interfacial layer could cause the loss of mechanical strength of the protein interface, leading to coalescence of droplets and a resulting reduction in available surface area for lipase adsorption. The above reasons could all contribute to the complexity of the kinetic and structural properties of infant milk gastric lipolysis. However, the limitation of the lipase used in this study and the limitation of the *in vitro* study itself require further proof from studies conducted using better lipase analogues to human gastric lipase and *in vitro* researches.

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Data Availability The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Declarations

Competing Interests The authors declare no competing interests.

Conflict of Interest Author Alastair MacGibbon is employed by Fonterra Co-operative Group Ltd; Author Matt Golding has received research support from Fonterra Co-operative Group Ltd. All other authors have no relevant financial affiliations.

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