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On the role of bile salts in the digestion of emulsified lipids

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Abstract

The objective of this study was to understand quantitatively the role that bile salts play in the digestion of emulsified lipids. The behaviours of digestion by pancreatin (1.6 mg/mL) of sodium-caseinate-stabilized emulsions (0.5 wt% protein) and bile-extract-stabilized emulsions (0.2–5 mg/mL) as influenced by the addition of aqueous bile extract were studied under simulated intestinal conditions (37 °C; pH 7.5; 39 mM K_2HPO_4, 150 mM NaCl; with continuous agitation at ~ 150 rev/min for 3 h). The droplet characteristics (size and ζ-potential) of the sodium caseinate- and bile extract-stabilized droplets were evaluated by light scattering techniques. The kinetics of the total fatty acids released by hydrolysis of the emulsified lipids was monitored by the pH-stat method with or without the presence of continuous phase bile extract. The results suggested that the presence of unadsorbed bile extract markedly enhanced the rate and the extent of lipid digestion. This could be attributed to considerable removal of lipolysis products (free fatty acids, mono- and/or di-acylglycerols) in mixed micelles, which are known to inhibit lipid digestion, by the unadsorbed bile salts. This study provides new insights for the lipid digestion of food formulations.

Keywords:

Bile extract, Emulsion, Lipolysis kinetics, Continuous phase, Interfacial layer, Sodium caseinate

1. Introduction

The rising levels of obesity and overweight populations are some of the most serious global public health challenges and are creating a huge healthcare cost burden. For this reason, there is a need for the development of effective microstructural strategies to delay the digestion of energy-dense lipids, suppressing appetite and thereby reducing subsequent calorie intake. Consequently, understanding the fundamental aspects of the digestion of emulsified lipids under conditions that simulate the human gastrointestinal tract is of paramount importance to gain insights into the physicochemical and biochemical processes in the physiological milieu that further bioengineer the initial food structure (Golding, et al., 2011; Mackie & Macierzanka, 2010; Sarkar, et al., 2015; Singh & Sarkar, 2011; Singh, Ye, & Horne, 2009). In the last few years, a significant level of understanding on the gastrointestinal structuring of emulsions after consumption, which typically includes different extents and types of droplet flocculation and coalescence, adsorption/desorption of emulsifiers and binding of metabolites, has been gained (Golding, et al., 2011; Hur, Decker, & McClements, 2009; Julia Maldonado-Valderrama, et al., 2008; Sarkar, Goh, & Singh, 2009; Sarkar, Goh, Singh, & Singh, 2009; Sarkar, Horne, & Singh, 2010a, 2010b; Torcello-Gomez, Maldonado-Valderrama, Martin-Rodriguez, & McClements, 2011).

In healthy humans, 70–90% of lipid digestion takes place in the small intestine; it is essentially an interfacial process that involves a complex interplay between lipase/colipase and bile salts. Bile salts are a very peculiar type of biosurfactant that, unlike classical surfactants, do not have a hydrophobic head and a hydrophilic tail group. The facial amphilicity of bile salts originates from the flat steroidal structure, with the polar hydroxyl groups on the concave side and methyl groups on the convex side (Euston, Baird, Campbell, & Kuhns, 2013; Galantini, et al., 2015; J. Maldonado-Valderrama, Muros-Cobos, Holgado-Terriza, & Cabrerizo-Vílchez, 2014). Because of their high surface activity, bile salts play a

crucial role in lipid digestion by pushing initial adsorbed materials from the interface and permitting lipase/colipase complexes to act on the bile-coated oil droplets. Recent research has focused mainly on bile-salt-mediated displacement studies, in which an understanding of the orogenic mechanism of this displacement (Julia Maldonado-Valderrama, et al., 2008) and the important role of the initial charge (Sarkar, Horne, et al., 2010a) and the type of protein layer (Bellesi, Pizones Ruiz-Henestrosa, & Pilosof, 2014) in determining the kinetics of the sequential adsorption or displacement of the adsorbed layer by intestinal bile salts have been revealed. On the other end, the aggregation and self-assembly behaviour of bile salt solutions and their role in absorption/transport have been well established (Holm, Müllertz, & Mu, 2013; Madenci & Egelhaaf, 2010). The aggregation of bile salts in solution is due to hydrophobic interactions and hydrogen bonds between the polar hydroxyl and carboxylate groups (Madenci, et al., 2010). Bile salts are believed to facilitate the solubilisation of lipid digestion products into lamellar phase or mixed micelles. This solubilisation results in the removal of digestion products, such as free fatty acids, mono and diacylglcerols from lipid droplets and accelerates further digestion and absorption of lipidic excipients (Small, Cabral, Cistola, Parks, & Hamilton, 1984). However, there is scant information available on the quantitative role of aqueous (unadsorbed) bile salts in the lipid digestion and subsequent fatty acid release as compared to that of the adsorbed phase.

Therefore, the objective of this study was to compare the kinetics and the degree of fatty acid release from sodium-caseinate-stabilized emulsions and porcine-bile-extract-stabilized emulsions and to unravel the role of “free” bile extract in the aqueous phase, using a simple pH-stat-based autotitration technique and theoretical consideration of apparent lipolysis rates. We have introduced the use of porcine bile salts-stabilized emulsions as a relatively new template for understanding digestion of emulsified lipids, to gain some insights into possible role of the presence of bile salts in adsorbed or continuous phase during

digestion. Previous studies have generated useful insights into the displacement of protein or phosphatidyl choline-stabilized interface by pure bile salts, such as sodium cholate, sodium deoxycholate, sodium taurcholate, and sodium glycodeoxycholate (Euston, et al., 2013; Wickham, Garrood, Leney, Wilson, & Fillery-Travis, 1998). To our knowledge, this is the first study where we used porcine bile extract to initially stabilize oil droplets and understand their in vitro lipolysis in absence or presence of continuous phase bile salts.

As lipid digestion is an interfacial process, the surface area of lipid droplets is expected to have an impact on the binding of lipase and the formation of fatty acids, with smaller droplets resulting in an increased level of fatty acid release and vice versa (Armand, et al., 1992). Hence, we also compared the droplet characteristics of protein-stabilized and bile-extract-stabilized emulsions using light scattering techniques.

2. Materials and methods

2.1. Materials

Sodium caseinate (Nacas) was obtained from Fonterra Co-operative Group Ltd, Auckland, New Zealand. Porcine bile extract B8631 and porcine pancreatin (P1750, 4 × USP) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Porcine BE used in this study had a total bile salt content of 49 wt%, of which the majority of the bile acid species were glycodeoxycholic acid (10–15 wt%) followed by taurodeoxycholic acid (3–9 wt%) and deoxycholic acid (0.5–7 wt%) (Zangenberg, Müllertz, Kristensen, & Hovgaard, 2001). The key phospholipid was phosphatidyl choline (6 wt%) and the content of Ca^{2+} was less than 0.06% (w%). Based on the phospholipid/ bile acid ratio, it can be suggested that the phospholipid was present as mixed micelles in conjunction with bile salt (Wickham, et al., 1998). Commercial soy oil (refined, bleached and deodorized) was obtained from Davis Trading Company, Palmerston North, New Zealand. All other chemicals were of

analytical grade and were obtained from either BDH Chemicals (BDH Ltd, Poole, England) or Sigma-Aldrich Chemical Company unless otherwise specified. Prior to experiments, solutions were freshly prepared using Milli-Q water (water purified by treatment with a Milli-Q apparatus; Millipore Corp., Bedford, MA, USA) as the solvent.

2.2. Preparation of emulsions

Aqueous solutions of Nacas (0.5 wt%) were prepared by dispersing Nacas in Milli-Q water and stirring gently for at least 2 h at 20 °C to ensure complete dissolution. Similarly, aqueous solutions of bile extract (BE) (0.1, 0.5 and 1.0 wt%) were prepared by dispersing quantities of BE in Milli-Q water and stirring for 3 h at 45 °C until all the BE had dissolved. The pH was adjusted to 7.0 using 1 M NaOH or 1 M HCl. Initially, pre-emulsions were prepared by blending 20.0 wt% soy oil with 80.0 wt% aqueous Nacas solution or BE solution using a conventional high speed mixer (Silverson L4RT, OFI Testing Equipment, Inc., Houston, TX, USA) at 6500 rev/min for 3 min. These coarse emulsions were then passed twice through a mini two-stage valve homogenizer (12.5H, Rannie, Copenhagen, Denmark) operating at 250 bar and 50 bar in the first and second stages respectively. The Nacas and BE emulsions were prepared at least in duplicate.

2.3. Droplet size determination

The mean droplet size distribution was monitored by static laser light scattering using a particle analyser (Mastersizer 2000, Malvern Instruments Ltd, Malvern, Worcestershire, UK). The relative refractive index (N) of the emulsion was taken as 1.095, i.e. the ratio of the refractive index of soy oil (1.456) to that of the aqueous phase (1.33). The absorbance value of the emulsion droplets was taken as 0.001. The sizes of emulsion droplets were reported as

the surface-weighted mean diameter $d_{3,2}$ (μm) and were calculated using the equation $d_{3,2} = \Sigma n_i d_i^3 / \Sigma n_i d_i^2$, where n_i is the number of particles and d_i is the diameter of emulsion droplets.

2.4. ζ-Potential measurements

The zeta-potential (ζ-potential) of emulsions was determined by a laser Doppler velocimetry and phase analysis light scattering (M3-PALS) technique using a Malvern Zetasizer Nano ZS (ZEN 3600) instrument (Malvern Instruments Ltd). One millilitre of sample diluted to approximately 0.005 wt% droplet concentration was placed in a folded capillary cell (Model DTS 1070, Malvern Instruments Ltd). An individual ζ-potential measurement was calculated from the mean and the standard deviation of at least five readings from an individual sample.

2.5. Preparation of simulated intestinal fluid (SIF) and mixing of emulsions with SIF

The SIF contained 39 mM K_2HPO_4 and 150 mM NaCl and the pH was maintained at 7.5 (Convention, 1995). For in vitro intestinal digestion with SIF, freshly prepared emulsions were diluted with SIF buffer (without added pancreatin) and water at a ratio of 1:4, which resulted in a final oil concentration of 4 wt%. Post dilution, the final concentrations of BE in the BE-stabilized emulsions (0.1, 0.5 and 1.0 wt%) were 0.2, 1 and 2 mg/mL respectively. The mixture of BE emulsion or Nacas emulsion with SIF was digested by the addition of pancreatin (1.6 mg/mL) in powdered form in the presence of 0.2, 1, 2 or 5 mg/mL of BE during digestion. In some experiments involving the digestion of Nacas emulsions, the aqueous phase bile salts were removed by centrifugation at 48,000 g for 30 min before addition of the pancreatin. During the digestion of the emulsions, small aliquots were withdrawn periodically for analysis.

2.6. Free fatty acid release

The free fatty acids (FFAs) generated from emulsified lipids during the digestion of the emulsions in SIF were measured by auto-titration. The emulsion–SIF mixture (4 wt% oil) was mixed in a flask in a water bath at 37 °C. The system was then adjusted to pH 7.5 using NaOH or HCl solution, followed by the addition of pancreatin powder (48 mg of powder to 30 mL of diluted sample). The intestinal digestion was carried out over 3 h while maintaining the pH at 7.5 by the addition of 0.05 M NaOH, using a pH-stat automatic titration unit (TitraLab 856, Radiometer Analytical SAS, Lyon, France), to neutralize the FFAs generated by lipolysis. The volume of 0.05 M NaOH consumed was recorded and calculated as the amount of FFAs hydrolysed from the emulsions.

A series of standard oleic acid solutions containing 0–800 μmol of oleic acid was prepared and titrated with 0.05 M NaOH to create a standard curve. Briefly, a stock standard oleic acid solution containing 5.9 mM oleic acid (molecular weight = 282.47 g/mol) was prepared by dissolving oleic acid in methanol that was pre-adjusted to pH 7.5 using 0.05 M NaOH. Different volumes of the stock standard oleic acid solution were then mixed with the pre-adjusted methanol (pH 7.5) and titrated with 0.05 M NaOH to pH 7.5. The amounts of NaOH consumed were plotted as a function of oleic acid concentration to create the standard curve, which was expressed as the molarity of oleic acid (μM) versus the amount of 0.05 M NaOH consumed (mL). The volume of NaOH consumed was converted to the amount of FFAs (as μM FFAs/mL emulsion) based on the standard curve and was then plotted as a function of the digestion time. The percentage of FFA released was calculated from the number of moles of 0.05 M NaOH required to neutralize the FFA that could be produced from the triacylglycerols if they were all digested (assuming the generation of 2 FFAs per triacylglycerol molecule by the action of lipase action) using Equation 1 (Li & McClements, 2010):

$$\%FFA = 100 \times \left(\frac{V_{NaOH} \times M_{NaOH} \times M_{W\,Lipid}}{2 \times W_{Lipid}} \right) \quad (1)$$

where, V_{NaOH} is the volume (mL) of sodium hydroxide, M_{NaOH} is the molarity of the sodium hydroxide solution used (0.05 M), MW_{Lipid} is the average molecular weight of soy oil (0.874 kg mol^{-1}, (Ionescu, 2005)) and W_{Lipid} is weight of lipid initially present in the reaction vessel. In many if not most emulsions, the fatty acid released (Φ) gradually increases with time t , potentially attaining the total release (Φmax). The kinetic parameters for the initial FFA release were calculated using Equation (2) (Ye, Cui, Zhu, & Singh, 2013):

$$\ln[(\emptyset_{max} - \emptyset_t)/\emptyset_{max} = -kt \quad (2)$$

where k is the first-order rate constant for FFA release (s^{-1}) and t is the digestion time (s). The other kinetic parameters, i.e. the time at which 50% of the total FFAs were released ($t_{1/2}$, min) and the total FFA level (Φ_{max}, μM/mL), were obtained from the FFA curves (Li, et al., 2010).

2.7. Statistical analyses

The results were statistically analysed by analysis of variance using Minitab 15.1.0 software (Minitab Inc., State College, PA, USA). Differences were considered to be significant at $p \leq 0.05$. Means and standard deviations of at least five measurements carried out on two freshly prepared emulsions are reported.

3. Results and discussion

3.1. Droplet characteristics of Nacas and BE emulsions

We first discuss the droplet behaviour of the emulsions in the presence of added aqueous BE without any added pancreatin. This sets the scene for understanding the impact of BE alone

on emulsions stabilized by Nacas or BE without any interference from proteolytic or lipolytic activity. The average droplet sizes and the ζ-potentials of Nacas emulsions in the presence of various levels of aqueous BE are shown in Fig. 1. Initially, the emulsion droplet size of the Nacas emulsion was 0.33 μm and the droplet size distribution was monomodal (data not shown). As shown in Fig. 1, there was no significant change in the droplet diameter on the addition of 0.2–5.0 mg/mL of aqueous BE ($p > 0.05$). This suggested that BE did not induce any droplet aggregation or coalescence in the Nacas emulsions, which was in line with the behaviour of other milk-protein-stabilized emulsions in the presence of BE (Mun, Decker, & McClements, 2007; Sarkar, Horne, et al., 2010a).

As shown in Fig. 2, the average droplet sizes of emulsions stabilized by BE were smaller than 0.3 μm. Even at a low concentration of < 0.1 wt%, BE was able to form finely dispersed emulsion droplets that showed no visible droplet aggregation and coalescence. In fact, on subsequent centrifugation and removal of the continuous phase, the droplet sizes of the cream phase of BE emulsion droplets redispersed in Milli-Q water still remained unchanged ($p > 0.05$), which further highlights that BE is an efficient emulsifier and is capable of adsorbing rapidly and forming stable emulsions (McClements, 2005).

As expected, the ζ-potential of the Nacas emulsion was negative at neutral pH (Fig. 1); interestingly, the negative charge increased slightly from –26 to –30 mV as a function of an increased concentration of aqueous BE. The overall change in ζ-potential on the addition of BE ($\Delta\zeta = -4$ mV) was in line with the results reported by Mun, et al. (2007). This could be attributed to possible displacement of the original Nacas from the interface by some anionic components within the BE, which is unlikely to bind to the anionic Nacas-coated interface, as reported in the previous studies (Euston, et al., 2013; Sarkar, Horne, et al., 2010a). However, it is worth noting that, even in the presence of 5 mg/mL of aqueous BE, the surface charge of the Nacas emulsion droplets did not reach the magnitude of that of the BE-stabilized

emulsions, with a ζ-potential of > –40 mV (Fig. 2). This suggested that, despite interfacial displacement of the original Nacas by the added BE, there were possibly some remnants of existing adsorbed Nacas at the oil/water interface, thus resulting in a mixed Nacas–BE interface at the droplet surface (Fig. 1). In contrast, a strong electrostatic repulsion between fully BE-coated droplets could be envisaged, with ζ-potential values greater than –45 mV (Fig. 2). The ζ-potential values for BE-coated droplets did not change significantly as a function of BE concentration ($p > 0.05$), which is in line with the droplet size measurements, suggesting that 0.2 mg/mL BE was able to saturate the droplet surface. The ζ-potential of the cream phase of BE-stabilized emulsion droplets that were redispersed in Milli-Q water remained highly negative, which indicated that the unadsorbed BE in the continuous phase did not affect the surface charge significantly (Fig. 2).

It is well known that the surface area of lipid droplets affects the rate and the extent of lipid digestion significantly, with smaller emulsion droplets being digested more rapidly owing to the increased surface area for the initial binding of the pancreatic lipase to the emulsified lipid substrate (Armand, et al., 1999; Li, et al., 2010). Our results suggested that the difference (if any) between the kinetics of lipid digestion by pancreatin of Nacas emulsions and BE emulsions in the presence of various levels of aqueous BE would be driven by some factors other than surface area, as the droplet sizes were similar in all cases.

3.2. Effect of aqueous BE on FFA release during digestion of Nacas emulsions

Fig. 3A shows the total FFA release from emulsions stabilized by Nacas in the presence of 1.6 mg/mL of pancreatin with or without the addition of BE. In the absence of BE, adsorbed Nacas appeared to restrict hydrolysis of the emulsified lipids by pancreatin and the FFA release was < 5 μM/mL (~ 7.9% FFA release, data not shown) over 150 min of digestion. This is in agreement with a previous study that showed that lipase can adsorb to oil/water interfaces in the absence and presence of bile salts, but that the rate and the extent of

lipid digestion are highly dependent on the presence of bile salts (Gargouri, Julien, Bois, Verger, & Sarda, 1983). All levels of BE enhanced the rate and the degree of lipid digestion, compared with the emulsion without the addition of BE.

Linear relationships for FFA release with time were obtained using Equation (1) (Fig. 3B). In the absence of BE, the rate constant for the emulsion stabilized by Nacas was extremely low (0.91×10^{-3} s^{-1}). The rate constant showed a fourfold increase in the emulsion containing 1 mg/mL of aqueous BE. As the concentration of BE increased, the kinetics of FFA release accelerated, gradually initially (0–0.2 mg/mL) and then more dramatically (1–5 mg/mL). In particular, the presence of 5 mg/mL of BE, which is in line with the physiological concentration (Wickham, et al., 1998), led to a marked increase in the FFA release to 22.5 μM/mL, consistent with previous work (Ye, et al., 2013). Release of 22.5 μM/mL FFA was equivalent to 42.4% FFA release (data not shown), which is in line with the value obtained in a previous study on digestion of protein-stabilized corn oil emulsion using similar pH stat technique (Li, et al., 2010). As expected, the apparent rate constant (k) was almost 15 times higher (13.1×10^{-3} s^{-1}) in the presence of the physiological concentration of bile salts (5 mg/mL) than in the absence of bile salts (0.91×10^{-3} s^{-1}). The addition of 0.2 mg/mL of aqueous BE was not sufficient to incur any change in the rate of FFA release ($p > 0.05$). The marked increase in the initial rate of lipid digestion in the presence of ≥ 1 mg/mL of BE (Fig. 3B) suggested that BE promoted the action of lipase on the lipid droplets. Interestingly, the presence of aqueous BE had a more prominent influence on the magnitude of Φ_{max} than on k, which suggested that BE may have prevented the accumulation of inhibitory lipolysis products (i.e. long fatty acids and monoacylglycerols) on the interface (Porter, Trevaskis, & Charman, 2007; Sek, Porter, Kaukonen, & Charman, 2002), thus further enabling the continuation of lipid digestion. However, it was not clear whether this increase in the

299 digestion rate was related to the bile salts adsorbed at the droplet surface or to the unadsorbed
300 bile salts.

301 To understand this, the rates of FFA release were studied as a function of digestion
302 time in emulsion systems in which the excess, unadsorbed BE was removed by centrifugation
303 and replaced with Milli-Q water. Fig. 4A shows that both the rate and the extent of lipid
304 digestion decreased dramatically on removal of the continuous phase BE. For instance, Φ_{max}
305 decreased from 22.5 to 10.7 μM/mL and $t_{1/2}$ increased from 43 to 55 min when unadsorbed
306 BE was removed from emulsions to which 5 mg/mL of BE had been added initially. This
307 suggested that the unadsorbed BE made a significant contribution to the degree of total FFA
308 release, which has not been reported previously. Interestingly, these emulsions containing
309 Nacas and BE mixtures as the adsorbed layers (centrifuged and redispersed in MilliQ water
310 samples), were further treated with aqueous BE. The FFA release was increased to ~ 20
311 μM/mL within the initial 75 min of hydrolysis on further addition of aqueous BE (5 mg/mL),
312 indicating the importance of the presence of unadsorbed BE (Fig. 4B). To obtain a
313 quantitative understanding, the difference in the extent of fatty acid release ($\Delta\Phi_{max}$) on
314 removal of BE was calculated by the subtraction of FFA release in the centrifuged emulsions
315 dispersed in MilliQ water (Fig. 4A) from the FFA release of the emulsions containing the
316 aqueous phase BE at a particular BE concentration (Fig. 3A). The significant drop in FFA
317 release ($\Delta\Phi_{max}$) upon the removal of unadsorbed BE was markedly linearly correlated with
318 the initial concentration of BE present (Fig. 5). Quantitatively, the FFA levels dropped by
319 55% in the absence of unabsorbed BE at the highest level of initial BE addition (5 mg/mL of
320 BE).

321 It is worth noting that some proteins, such as β-lactoglobulin and bovine serum
322 albumin, undergo a significant increase in proteolytic digestion (both trypsin-mediated and
323 chymotrypsin-mediated digestion) in the presence of bile salts (Gass, Vora, Hofmann, Gray,

& Khosla, 2007). It has been suggested that bile acids can accelerate protein digestion, most probably by destabilizing the tertiary structures of dietary proteins, thereby making them more prone to attack by pancreatic endoproteases, such as trypsin and chymotrypsin. In our case, we used pancreatin, which is essentially a mixture of pancreatic lipase, proteases and amylases. As Nacas was used to stabilize the emulsions, it is possible that the unadsorbed BE may have interacted with Nacas and thus resulted in an increase in proteolysis of the interfacial layer. Such a plausible increase in proteolysis and the resulting peptides might simultaneously increase the access of lipase to the hydrophobic lipid core because of easy displacement of the interfacial remnants by bile salts, thus resulting in faster release of FFAs by lipolysis. To investigate further whether or not the contribution of unadsorbed bile salts to lipolysis was linked to a protein-specific mechanism, lipolytic studies with BE-stabilized emulsions were carried out. In this way, the interference of protein was avoided and the roles of adsorbed versus unadsorbed bile salts were revealed.

3.3. Effect of aqueous BE on FFA release during digestion of BE emulsions

The lipid digestion kinetics of BE-stabilized emulsions was studied as the release of FFAs in the presence of various concentrations of aqueous BE. Fig. 6 shows the rate and the extent of FFA release when Nacas emulsion (0.5 wt% protein) or a BE emulsion (0.2 mg/mL of BE) was digested with 1.6 mg/mL of pancreatin in the absence of added aqueous BE. Interestingly, both emulsions had similar k (data not shown), Φ_{max} and $t_{1/2}$ values ($p > 0.05$) with low levels of FFA release of < 5 μM/mL. This suggested that the presence of 0.2 mg/mL of BE at the droplet surface had similar restrictive effects on both the binding of lipase on to the emulsified lipid substrate and the continuing lipolysis to those seen in the Nacas emulsion. Interestingly, BE emulsions that were stabilized by higher concentrations of BE (2–5 mg/mL) had significantly enhanced rates of FFA release (Figs. 7A and 7B). The k

values of the BE emulsions were comparable with those of the Nacas emulsions on the addition of equivalent amounts of aqueous BE extract ($p > 0.05$) (Fig. 3B), which highlighted the influence of aqueous BE on lipolysis, irrespective of the emulsifier type. This suggests that the adsorbed bile salts of 0.2 mg/mL of BE at the surface of droplets had a relatively less influence on the degree and kinetics of lipolysis. One might argue that the emulsion was stabilised by the very low concentration of BE (0.2 mg/ml), and thus almost entire quantity of bile salts must have adsorbed on to the oil-water interface, with negligible amounts of aqueous phase BE available, and later appears to be the key driving factor for lipid digestion. However, it should be noted that such enhancement of FFA release in in case of 5mg/mL BE-stabilized emulsion may have arisen from the combined effects of the presence of BE in the aqueous phase and/or the adsorbed phase. To investigate this further, the emulsion stabilized by 0.2 mg/mL of BE, which had the slowest and the least FFA release, was treated with increasing concentrations of aqueous BE. It is evident from Figs. 8A and 8B that unadsorbed bile salts played a crucial role in promoting lipid digestion, with the k value increasing to 14.2 $\times 10^{-3}$ s^{-1} and Φ_{max} increasing to 24 μM/mL, (~ 45.5% FFA release, data not shown) at 5 mg/mL of aqueous BE. Hence, considering the generation of two FFAs and one monoacylglycerol per triglyceride molecule during pH stat digestion, our value suggests that the lipid digestion was nearing completion in presence of aqueous 5 mg/mL BE. Previous authors have reported that some bile salts adsorb irreversibly while others can desorb to a certain extent following buffer rinsing (Maldonado-Valderrama, Muros-Cobos, Holgado-Terriza, Cabrerizo-Vílchez, 2014, Parker, Rigby, Ridout, Gunning, Wilde, 2014). The BE used in our study contains phospholipids, which can also adsorb onto surface and stabilise the emulsion. Hence, in order to understand the individual effect of pure bile salt being adsorbed at oil-water surface, lipid digestion of emulsions stabilized by 0.1 wt% sodium deoxycholate (NaDC) was conducted in presence and absence of aqueous BE. The emulsion droplets

created with 0.1wt% NaDC were fine, uniformly dispersed (d_{32} < 0.5 μm) and carried high negative charge. These NaDC-stabilized emulsions also showed similar lipid digestion behaviour (Supplementary information, Figure S1) with limited FFA release in absence of aqueous BE, followed by enhanced FFA release on addition of 5 mg/mL of aqueous BE, when compared to BE -Stabilized emulsions (Figure 8). This further suggests the role of aqueous BE on the degree and rate of lipid digestion.

As summarized by Golding & Wooster (2010), the interfacial process of lipolysis involves essentially three key steps: binding of the bile salt–lipase/colipase complex to the oil/water interface, hydrolysis of the emulsified lipid to 2-monoacylglycerols and two FFAs and desorption of these lipolytic products to continue the digestion. Based on the results of this study, we suggest that unadsorbed bile salts contribute more significantly to the first and third steps whereas adsorbed bile salts dominate the second step. Furthermore, it is worth highlighting here that the porcine BE used in this study had a mainly glycodeoxycholic acid followed by taurodeoxycholic acids highlighted in the method section. The presence of a higher proportion of glycodeoxycholic acid than of taurodeoxycholic acid might have promoted solubilization of lipolytic products from the interfacial region into the micellar phase. The contribution to solubilization was more prominent than that to promotion of the bile salt adsorption and the residence time of the colipase/lipase complex by glycodeoxycholic acid; the latter is generally enhanced by the presence of taurodeoxycholic acid residues, as schematically discussed in a previous study (Parker, Rigby, Ridout, Gunning, & Wilde, 2014). Furthermore, at higher bile salt concentrations (5 mg/ mL), the continuous phase would consist of mixed micelles, monomeric bile salt as well as simple micelles of bile salts (Birru, et al., 2014; Wickham, et al., 1998). These mixed micelles of digested bile salts will have a higher capacity for solubilization of lipids and fat digestion products, which are generally inhibitory to lipolysis progress.

4. Conclusions

The present study showed that the impact of added BE on the kinetics of in vitro lipid digestion in emulsions was largely dependent on its presence in the unadsorbed phase rather than the adsorbed phase. Clearly, the bile salts adsorbed onto the interface had relatively less influence on lipolysis than the presence of bile salts in the aqueous phase. The rate and the extent of lipolysis appeared to be dominated mainly by the presence of aqueous bile salts. This may be attributed to the solubilization and removal of inhibitory digestion products (e.g. FFAs, mono- and/or di-acylglycerols), which may have been accumulated at the interface by the aqueous bile salts. Future work is needed to characterize the hydrolysis products (long chain FFA) generated in the bile-salt stabilized emulsions during digestion by pancreatic lipase in presence and absence of aqueous phase bile extracts using chromatographic techniques. Also, it would be interesting to provide structural information about the mixed micelle formed in the aqueous phase using small angle X-ray scattering.

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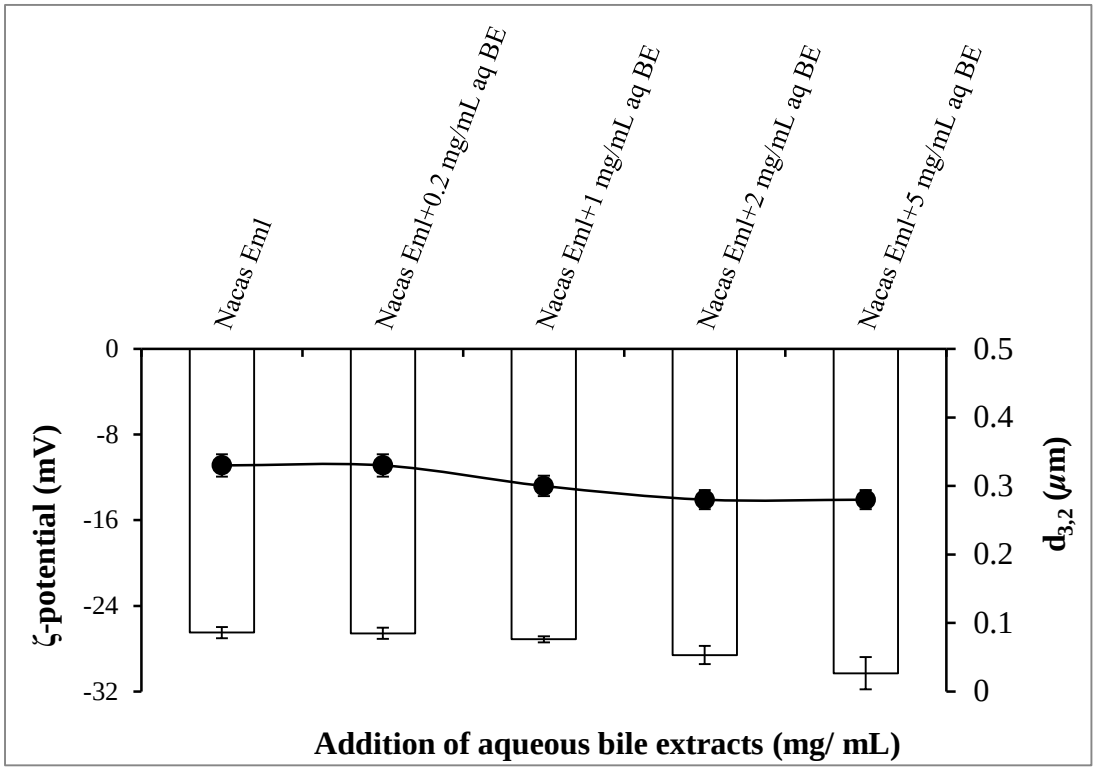
519

520 **Figure 1**



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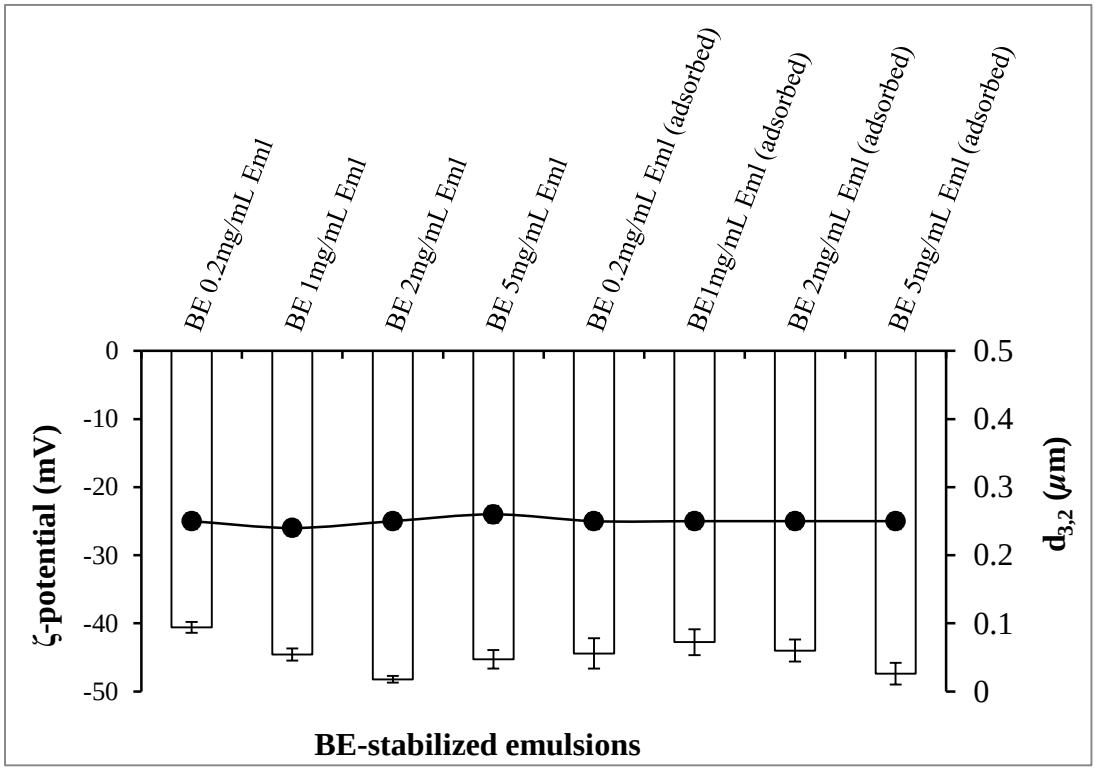
524 **Figure 2**



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528 **Figure 3**

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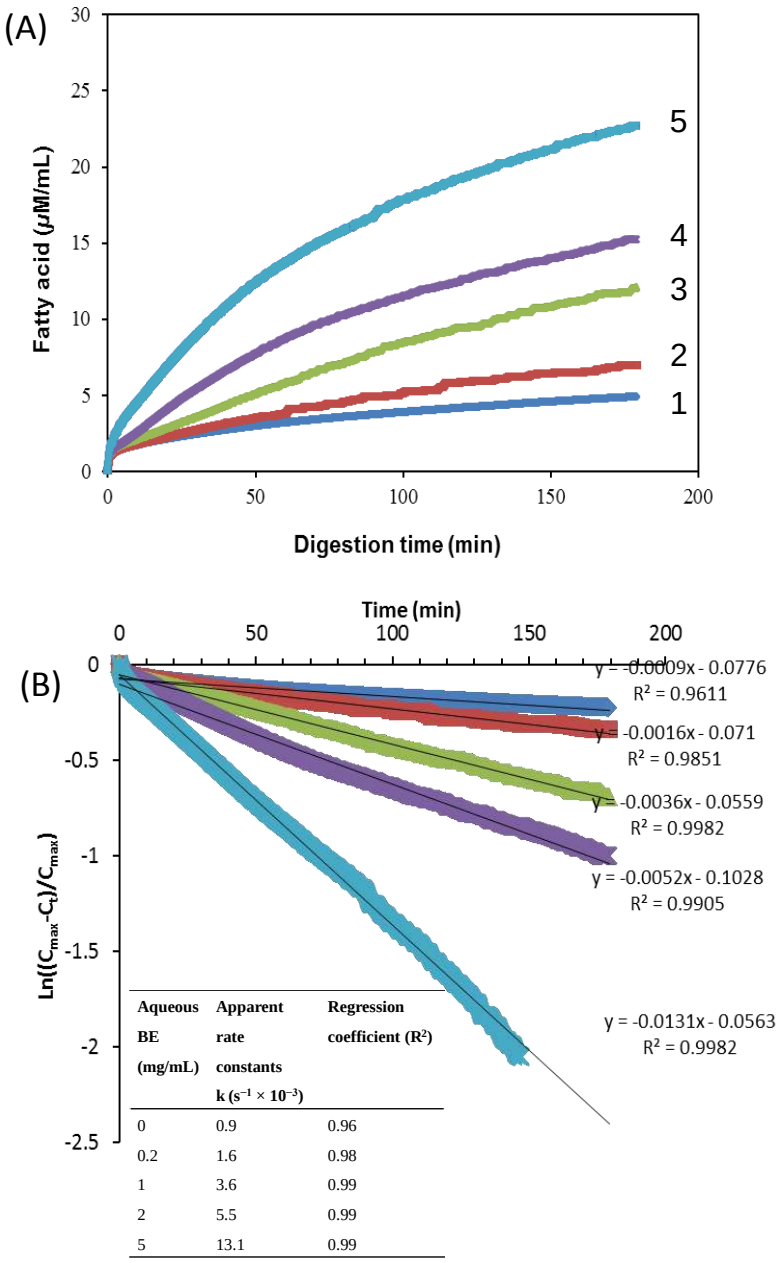


Aqueous BE (mg/mL)	Apparent rate constants k ($s^{-1} \times 10^{-3}$)	Regression coefficient (R^2)
0	0.9	0.96
0.2	1.6	0.98
1	3.6	0.99
2	5.5	0.99
5	13.1	0.99

Figure 4

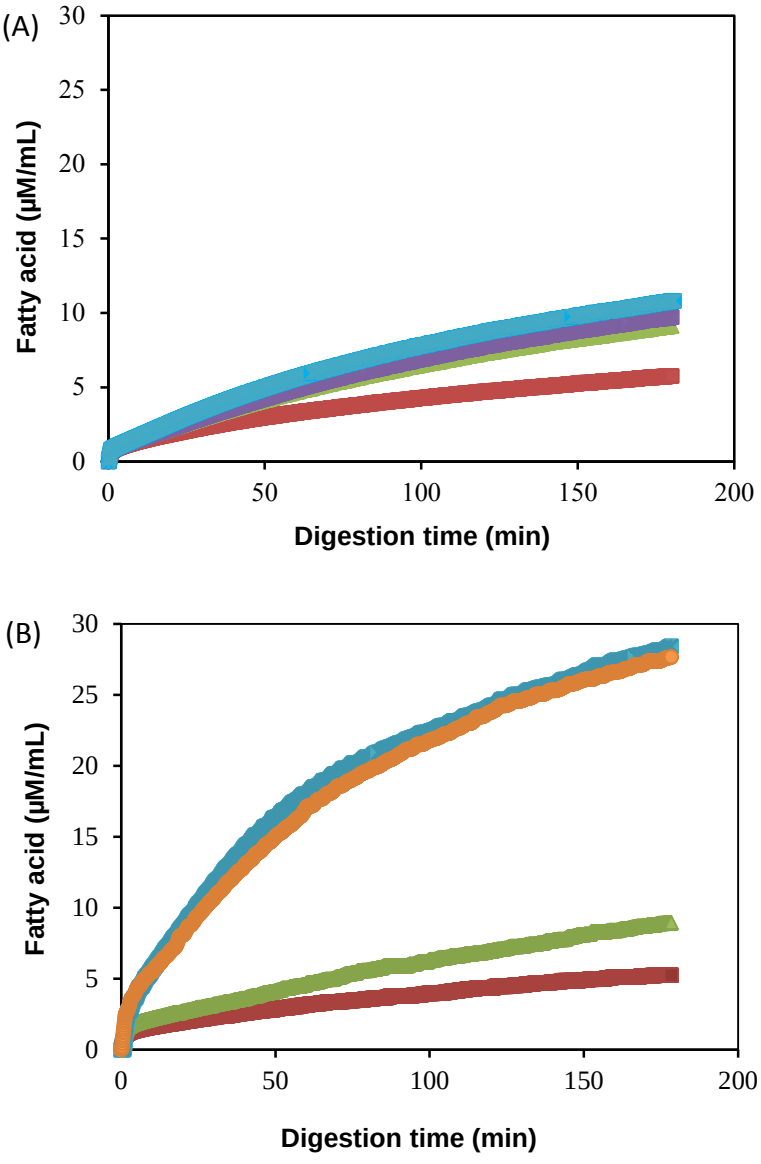

(A)
Fatty acid (μM/mL)
30
25
20
15
10
5
0
0
50
100
150
200
Digestion time (min)
(B)
Fatty acid (μM/mL)
30
25
20
15
10
5
0
0
50
100
150
200
Digestion time (min)

Figure 5

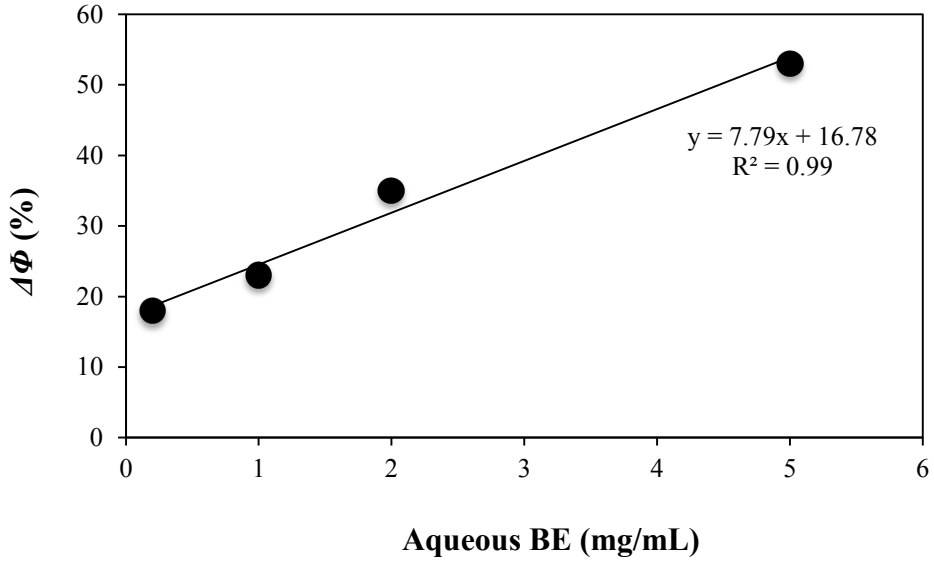
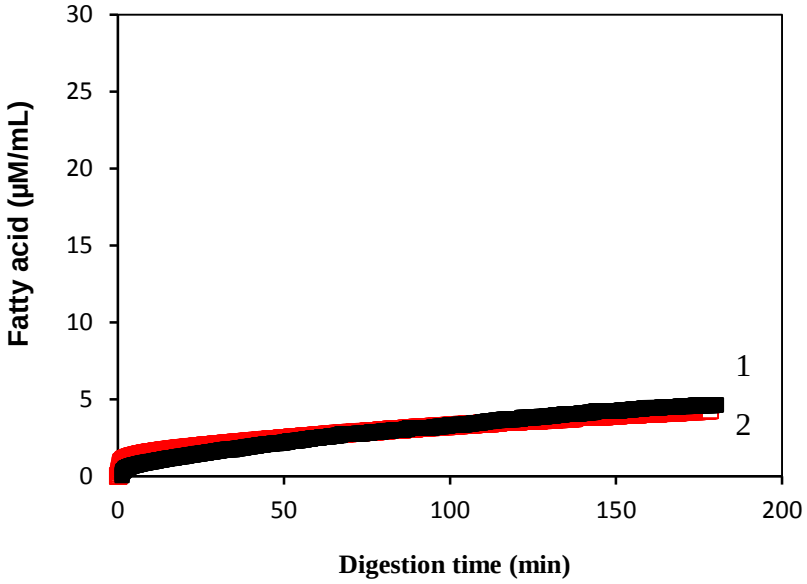


Figure 6.

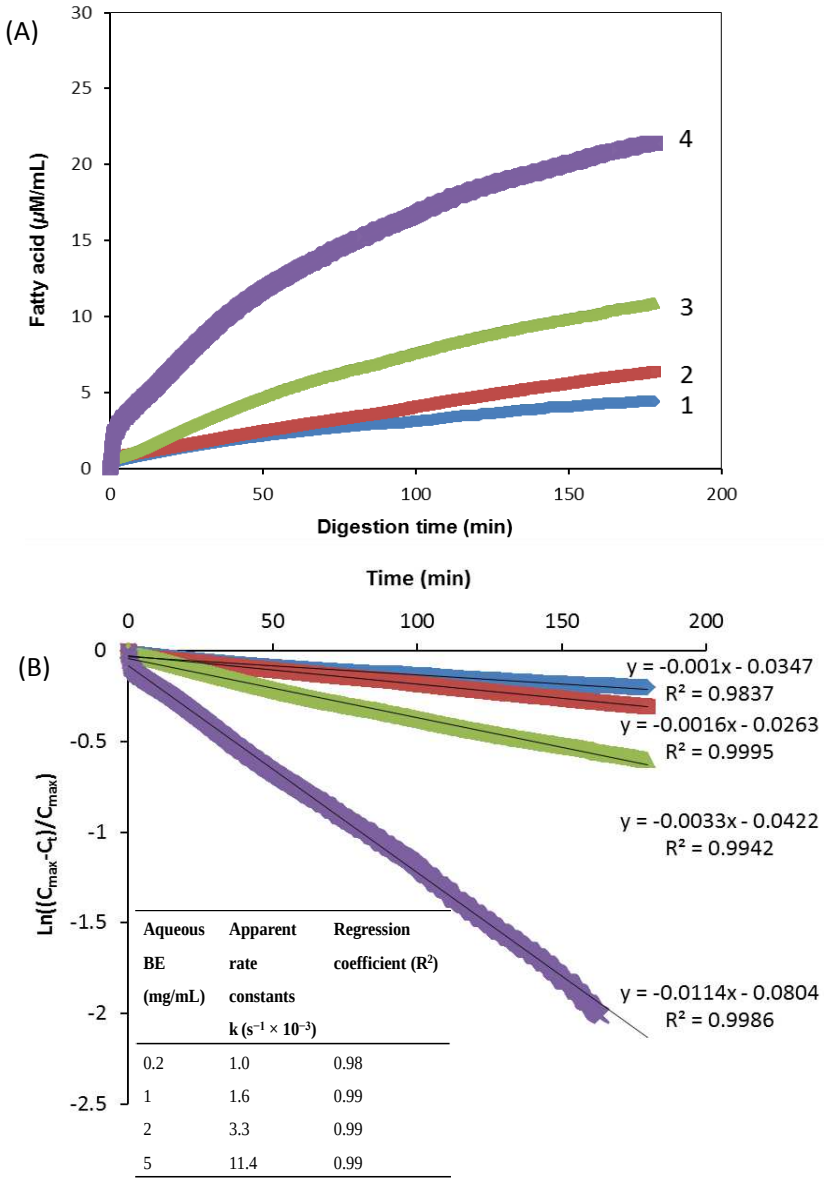

30
25
20
15
10
5
0
Fatty acid (µM/mL)
0
50
100
150
200
Digestion time (min)
1
2

581 **Figure 7**

582

583

584

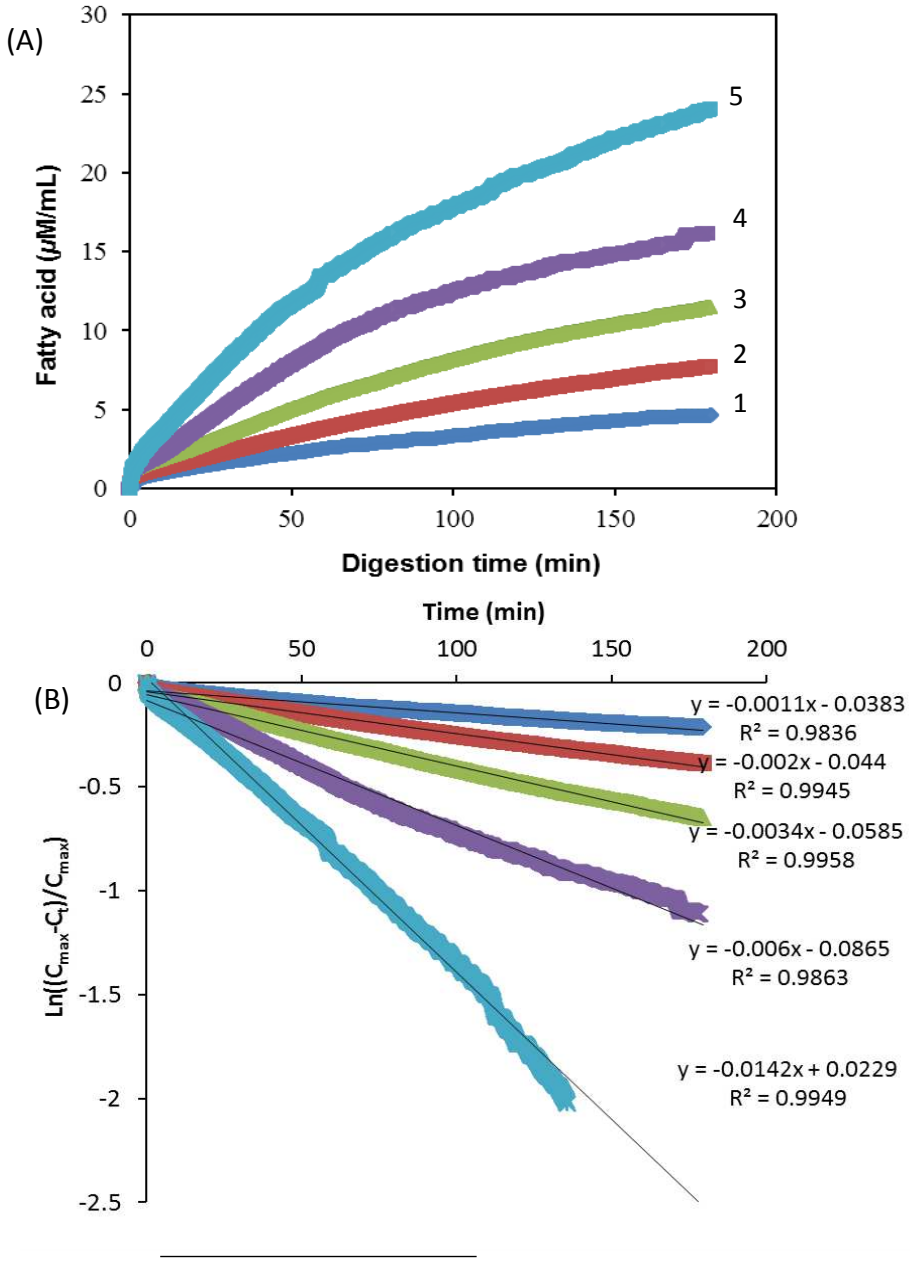


Aqueous BE (mg/mL)	Apparent rate constants k ($s^{-1} \times 10^{-3}$)	Regression coefficient (R^2)
0.2	1.0	0.98
1	1.6	0.99
2	3.3	0.99
5	11.4	0.99

585 **Figure 8**

586

587



588 **Figure S1.**

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