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

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20 **Abstract**

21 The objective of this study was to understand quantitatively the role that bile salts
22 play in the digestion of emulsified lipids. The behaviours of digestion by pancreatin (1.6
23 mg/mL) of sodium-caseinate-stabilized emulsions (0.5 wt% protein) and bile-extract-
24 stabilized emulsions (0.2–5 mg/mL) as influenced by the addition of aqueous bile extract
25 were studied under simulated intestinal conditions (37 °C; pH 7.5; 39 mM K₂HPO₄, 150 mM
26 NaCl; with continuous agitation at ~ 150 rev/min for 3 h). The droplet characteristics (size
27 and ζ -potential) of the sodium caseinate- and bile extract-stabilized droplets were evaluated
28 by light scattering techniques. The kinetics of the total fatty acids released by hydrolysis of
29 the emulsified lipids was monitored by the pH-stat method with or without the presence of
30 continuous phase bile extract. The results suggested that the presence of unadsorbed bile
31 extract markedly enhanced the rate and the extent of lipid digestion. This could be attributed
32 to considerable removal of lipolysis products (free fatty acids, mono- and/or di-acylglycerols)
33 in mixed micelles, which are known to inhibit lipid digestion, by the unadsorbed bile salts.
34 This study provides new insights for the lipid digestion of food formulations.

35

36 *Keywords:*

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

39

40 **1. Introduction**

41
42 The rising levels of obesity and overweight populations are some of the most serious
43 global public health challenges and are creating a huge healthcare cost burden. For this
44 reason, there is a need for the development of effective microstructural strategies to delay the
45 digestion of energy-dense lipids, suppressing appetite and thereby reducing subsequent
46 calorie intake. Consequently, understanding the fundamental aspects of the digestion of
47 emulsified lipids under conditions that simulate the human gastrointestinal tract is of
48 paramount importance to gain insights into the physicochemical and biochemical processes in
49 the physiological milieu that further bioengineer the initial food structure (Golding, et al.,
50 2011; Mackie & Macierzanka, 2010; Sarkar, et al., 2015; Singh & Sarkar, 2011; Singh, Ye, &
51 Horne, 2009). In the last few years, a significant level of understanding on the gastrointestinal
52 structuring of emulsions after consumption, which typically includes different extents and
53 types of droplet flocculation and coalescence, adsorption/desorption of emulsifiers and
54 binding of metabolites, has been gained (Golding, et al., 2011; Hur, Decker, & McClements,
55 2009; Julia Maldonado-Valderrama, et al., 2008; Sarkar, Goh, & Singh, 2009; Sarkar, Goh,
56 Singh, & Singh, 2009; Sarkar, Horne, & Singh, 2010a, 2010b; Torcello-Gomez, Maldonado-
57 Valderrama, Martin-Rodriguez, & McClements, 2011).

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

66 crucial role in lipid digestion by pushing initial adsorbed materials from the interface and
67 permitting lipase/colipase complexes to act on the bile-coated oil droplets. Recent research
68 has focused mainly on bile-salt-mediated displacement studies, in which an understanding of
69 the orogenic mechanism of this displacement (Julia Maldonado-Valderrama, et al., 2008) and
70 the important role of the initial charge (Sarkar, Horne, et al., 2010a) and the type of protein
71 layer (Bellesi, Pizones Ruiz-Henestrosa, & Pilosof, 2014) in determining the kinetics of the
72 sequential adsorption or displacement of the adsorbed layer by intestinal bile salts have been
73 revealed. On the other end, the aggregation and self-assembly behaviour of bile salt solutions
74 and their role in absorption/transport have been well established (Holm, Müllertz, & Mu,
75 2013; Madenci & Egelhaaf, 2010). The aggregation of bile salts in solution is due to
76 hydrophobic interactions and hydrogen bonds between the polar hydroxyl and carboxylate
77 groups (Madenci, et al., 2010). Bile salts are believed to facilitate the solubilisation of lipid
78 digestion products into lamellar phase or mixed micelles. This solubilisation results in the
79 removal of digestion products, such as free fatty acids, mono and diacylglycerols from lipid
80 droplets and accelerates further digestion and absorption of lipidic excipients (Small, Cabral,
81 Cistola, Parks, & Hamilton, 1984). However, there is scant information available on the
82 quantitative role of aqueous (unadsorbed) bile salts in the lipid digestion and subsequent fatty
83 acid release as compared to that of the adsorbed phase.

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

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

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

102

103 **2. Materials and methods**

104

105 *2.1. Materials*

106

107 Sodium caseinate (Nacas) was obtained from Fonterra Co-operative Group Ltd,
108 Auckland, New Zealand. Porcine bile extract B8631 and porcine pancreatin (P1750, 4 ×
109 USP) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).
110 Porcine BE used in this study had a total bile salt content of 49 wt%, of which the majority of
111 the bile acid species were glycodeoxycholic acid (10–15 wt%) followed by taurodeoxycholic
112 acid (3–9 wt%) and deoxycholic acid (0.5–7 wt%) (Zangenberg, Müllertz, Kristensen, &
113 Hovgaard, 2001). The key phospholipid was phosphatidyl choline (6 wt%) and the content of
114 Ca²⁺ was less than 0.06% (w%). Based on the phospholipid/ bile acid ratio, it can be
115 suggested that the phospholipid was present as mixed micelles in conjunction with bile salt
116 (Wickham, et al., 1998). Commercial soy oil (refined, bleached and deodorized) was obtained
117 from Davis Trading Company, Palmerston North, New Zealand. All other chemicals were of

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

122

123 2.2. *Preparation of emulsions*

124

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

136

137 2.3. *Droplet size determination*

138

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

144 the surface-weighted mean diameter $d_{3,2}$ (μm) and were calculated using the equation $d_{3,2} =$
145 $\frac{\sum n_i d_i^3}{\sum n_i d_i^2}$, where n_i is the number of particles and d_i is the diameter of emulsion droplets.

146

147 2.4. ζ -Potential measurements

148

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

156

157 2.5. Preparation of simulated intestinal fluid (SIF) and mixing of emulsions with SIF

158

159 The SIF contained 39 mM K_2HPO_4 and 150 mM NaCl and the pH was maintained at
160 7.5 (Convention, 1995). For *in vitro* intestinal digestion with SIF, freshly prepared emulsions
161 were diluted with SIF buffer (without added pancreatin) and water at a ratio of 1:4, which
162 resulted in a final oil concentration of 4 wt%. Post dilution, the final concentrations of BE in
163 the BE-stabilized emulsions (0.1, 0.5 and 1.0 wt%) were 0.2, 1 and 2 mg/mL respectively.
164 The mixture of BE emulsion or Nacas emulsion with SIF was digested by the addition of
165 pancreatin (1.6 mg/mL) in powdered form in the presence of 0.2, 1, 2 or 5 mg/mL of BE
166 during digestion. In some experiments involving the digestion of Nacas emulsions, the
167 aqueous phase bile salts were removed by centrifugation at 48,000 g for 30 min before
168 addition of the pancreatin. During the digestion of the emulsions, small aliquots were
169 withdrawn periodically for analysis.

170

171 2.6. *Free fatty acid release*

172

173 The free fatty acids (FFAs) generated from emulsified lipids during the digestion of
174 the emulsions in SIF were measured by auto-titration. The emulsion–SIF mixture (4 wt% oil)
175 was mixed in a flask in a water bath at 37 °C. The system was then adjusted to pH 7.5 using
176 NaOH or HCl solution, followed by the addition of pancreatin powder (48 mg of powder to
177 30 mL of diluted sample). The intestinal digestion was carried out over 3 h while maintaining
178 the pH at 7.5 by the addition of 0.05 M NaOH, using a pH-stat automatic titration unit
179 (TitraLab 856, Radiometer Analytical SAS, Lyon, France), to neutralize the FFAs generated
180 by lipolysis. The volume of 0.05 M NaOH consumed was recorded and calculated as the
181 amount of FFAs hydrolysed from the emulsions.

182 A series of standard oleic acid solutions containing 0–800 μmol of oleic acid was
183 prepared and titrated with 0.05 M NaOH to create a standard curve. Briefly, a stock standard
184 oleic acid solution containing 5.9 mM oleic acid (molecular weight = 282.47 g/mol) was
185 prepared by dissolving oleic acid in methanol that was pre-adjusted to pH 7.5 using 0.05 M
186 NaOH. Different volumes of the stock standard oleic acid solution were then mixed with the
187 pre-adjusted methanol (pH 7.5) and titrated with 0.05 M NaOH to pH 7.5. The amounts of
188 NaOH consumed were plotted as a function of oleic acid concentration to create the standard
189 curve, which was expressed as the molarity of oleic acid (μM) versus the amount of 0.05 M
190 NaOH consumed (mL). The volume of NaOH consumed was converted to the amount of
191 FFAs (as μM FFAs/mL emulsion) based on the standard curve and was then plotted as a
192 function of the digestion time. The percentage of FFA released was calculated from the
193 number of moles of 0.05 M NaOH required to neutralize the FFA that could be produced
194 from the triacylglycerols if they were all digested (assuming the generation of 2 FFAs per
195 triacylglycerol molecule by the action of lipase action) using Equation 1 (Li & McClements,
196 2010):

197

$$198 \quad \%FFA = 100 \times \left(\frac{V_{NaOH} \times M_{NaOH} \times M_{W Lipid}}{2 \times W_{Lipid}} \right) \quad (1)$$

199

200 where, V_{NaOH} is the volume (mL) of sodium hydroxide, M_{NaOH} is the molarity of the
 201 sodium hydroxide solution used (0.05 M), MW_{Lipid} is the average molecular weight of soy oil
 202 (0.874 kg mol⁻¹, (Ionescu, 2005)) and W_{Lipid} is weight of lipid initially present in the reaction
 203 vessel. In many if not most emulsions, the fatty acid released (Φ) gradually increases with
 204 time t , potentially attaining the total release (Φ_{max}). The kinetic parameters for the initial
 205 FFA release were calculated using Equation (2) (Ye, Cui, Zhu, & Singh, 2013):

206

207

$$208 \quad \ln[(\Phi_{max} - \Phi_t) / \Phi_{max}] = -kt \quad (2)$$

209

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

212 **2.7. Statistical analyses**

213

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

218

219 **3. Results and discussion**

220

221 **3.1. Droplet characteristics of Nacas and BE emulsions**

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

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

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

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

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

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

267 3.2. *Effect of aqueous BE on FFA release during digestion of Nacas emulsions*

268
269 Fig. 3A shows the total FFA release from emulsions stabilized by Nacas in the
270 presence of 1.6 mg/mL of pancreatin with or without the addition of BE. In the absence of
271 BE, adsorbed Nacas appeared to restrict hydrolysis of the emulsified lipids by pancreatin and
272 the FFA release was < 5 μ M/mL ($\sim 7.9\%$ FFA release, data not shown) over 150 min of
273 digestion. This is in agreement with a previous study that showed that lipase can adsorb to
274 oil/water interfaces in the absence and presence of bile salts, but that the rate and the extent of

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

278 Linear relationships for FFA release with time were obtained using Equation (1) (Fig.
279 3B). In the absence of BE, the rate constant for the emulsion stabilized by Nacas was
280 extremely low ($0.91 \times 10^{-3} \text{ s}^{-1}$). The rate constant showed a fourfold increase in the emulsion
281 containing 1 mg/mL of aqueous BE. As the concentration of BE increased, the kinetics of
282 FFA release accelerated, gradually initially (0–0.2 mg/mL) and then more dramatically (1–5
283 mg/mL). In particular, the presence of 5 mg/mL of BE, which is in line with the physiological
284 concentration (Wickham, et al., 1998), led to a marked increase in the FFA release to 22.5
285 $\mu\text{M/mL}$, consistent with previous work (Ye, et al., 2013). Release of 22.5 $\mu\text{M/mL}$ FFA was
286 equivalent to 42.4% FFA release (data not shown), which is in line with the value obtained
287 in a previous study on digestion of protein-stabilized corn oil emulsion using similar pH stat
288 technique (Li, et al., 2010). As expected, the apparent rate constant (k) was almost 15 times
289 higher ($13.1 \times 10^{-3} \text{ s}^{-1}$) in the presence of the physiological concentration of bile salts (5
290 mg/mL) than in the absence of bile salts ($0.91 \times 10^{-3} \text{ s}^{-1}$). The addition of 0.2 mg/mL of
291 aqueous BE was not sufficient to incur any change in the rate of FFA release ($p > 0.05$). The
292 marked increase in the initial rate of lipid digestion in the presence of ≥ 1 mg/mL of BE (Fig.
293 3B) suggested that BE promoted the action of lipase on the lipid droplets. Interestingly, the
294 presence of aqueous BE had a more prominent influence on the magnitude of Φ_{max} than on k ,
295 which suggested that BE may have prevented the accumulation of inhibitory lipolysis
296 products (i.e. long fatty acids and monoacylglycerols) on the interface (Porter, Trevaskis, &
297 Charman, 2007; Sek, Porter, Kaukonen, & Charman, 2002), thus further enabling the
298 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 $\mu\text{M}/\text{mL}$ 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 $\mu\text{M}/\text{mL}$ 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,

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

337

338 3.3. *Effect of aqueous BE on FFA release during digestion of BE emulsions*

339

340 The lipid digestion kinetics of BE-stabilized emulsions was studied as the release of
341 FFAs in the presence of various concentrations of aqueous BE. Fig. 6 shows the rate and the
342 extent of FFA release when Nacas emulsion (0.5 wt% protein) or a BE emulsion (0.2 mg/mL
343 of BE) was digested with 1.6 mg/mL of pancreatin in the absence of added aqueous BE.
344 Interestingly, both emulsions had similar k (data not shown), Φ_{max} and $t_{1/2}$ values ($p > 0.05$)
345 with low levels of FFA release of $< 5 \mu\text{M/mL}$. This suggested that the presence of 0.2 mg/mL
346 of BE at the droplet surface had similar restrictive effects on both the binding of lipase on to
347 the emulsified lipid substrate and the continuing lipolysis to those seen in the Nacas
348 emulsion. Interestingly, BE emulsions that were stabilized by higher concentrations of BE
349 (2–5 mg/mL) had significantly enhanced rates of FFA release (Figs. 7A and 7B). The k

350 values of the BE emulsions were comparable with those of the Nacas emulsions on the
351 addition of equivalent amounts of aqueous BE extract ($p > 0.05$) (Fig. 3B), which highlighted
352 the influence of aqueous BE on lipolysis, irrespective of the emulsifier type. This suggests
353 that the adsorbed bile salts of 0.2 mg/mL of BE at the surface of droplets had a relatively less
354 influence on the degree and kinetics of lipolysis. One might argue that the emulsion was
355 stabilised by the very low concentration of BE (0.2 mg/ml), and thus almost entire quantity of
356 bile salts must have adsorbed on to the oil-water interface, with negligible amounts of
357 aqueous phase BE available, and later appears to be the key driving factor for lipid digestion.
358 However, it should be noted that such enhancement of FFA release in in case of 5mg/mL BE-
359 stabilized emulsion may have arisen from the combined effects of the presence of BE in the
360 aqueous phase and/or the adsorbed phase. To investigate this further, the emulsion stabilized
361 by 0.2 mg/mL of BE, which had the slowest and the least FFA release, was treated with
362 increasing concentrations of aqueous BE. It is evident from Figs. 8A and 8B that unadsorbed
363 bile salts played a crucial role in promoting lipid digestion, with the k value increasing to 14.2
364 $\times 10^{-3} \text{ s}^{-1}$ and Φ_{max} increasing to $24 \mu\text{M/mL}$, ($\sim 45.5\%$ FFA release, data not shown) at 5
365 mg/mL of aqueous BE. Hence, considering the generation of two FFAs and one
366 monoacylglycerol per triglyceride molecule during pH stat digestion, our value suggests that
367 the lipid digestion was nearing completion in presence of aqueous 5 mg/mL BE. Previous
368 authors have reported that some bile salts adsorb irreversibly while others can desorb to a
369 certain extent following buffer rinsing (Maldonado-Valderrama, Muros-Cobos, Holgado-
370 Terriza, Cabrerizo-Vílchez, 2014, Parker, Rigby, Ridout, Gunning, Wilde, 2014). The BE
371 used in our study contains phospholipids, which can also adsorb onto surface and stabilise the
372 emulsion. Hence, in order to understand the individual effect of pure bile salt being adsorbed
373 at oil-water surface, lipid digestion of emulsions stabilized by $0.1 \text{ wt}\%$ sodium deoxycholate
374 (NaDC) was conducted in presence and absence of aqueous BE. The emulsion droplets

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

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

400

401 **4. Conclusions**

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

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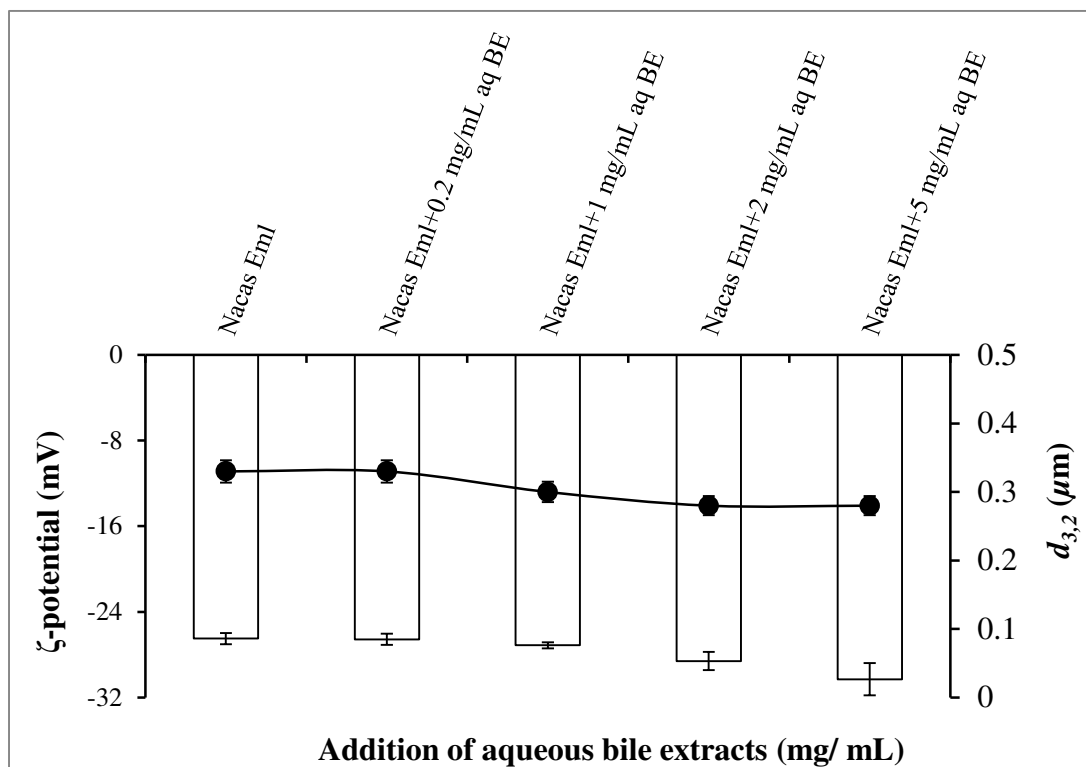
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520 **Figure 1**

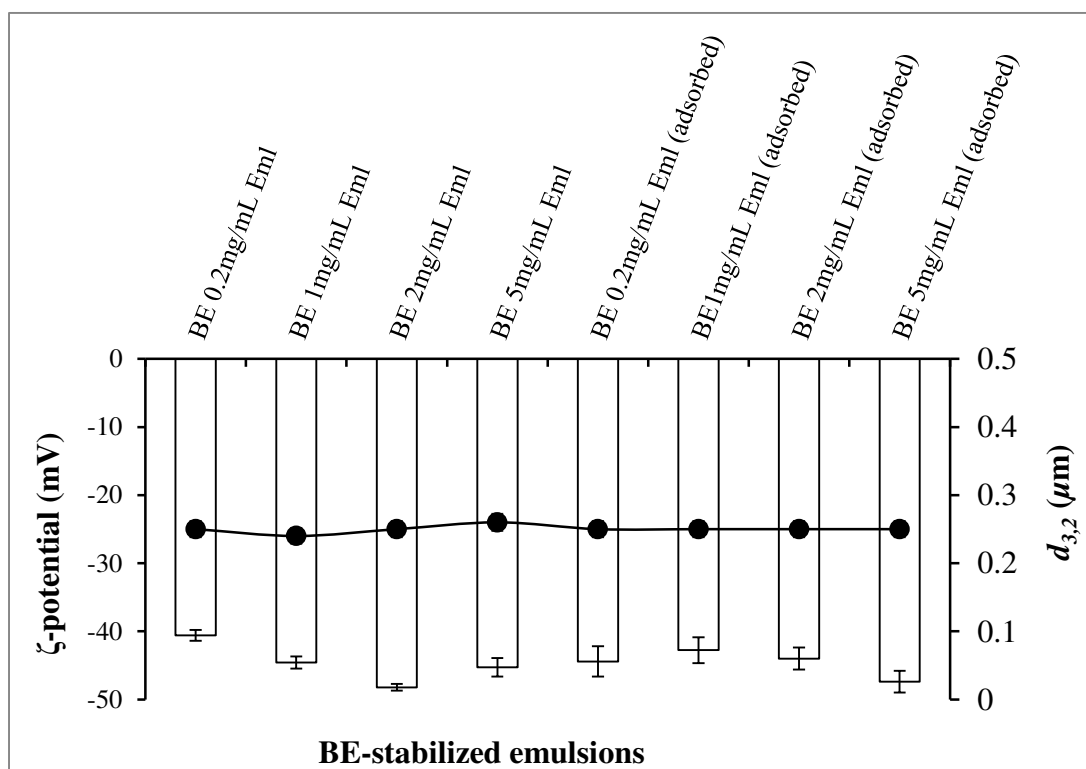


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524 **Figure 2**



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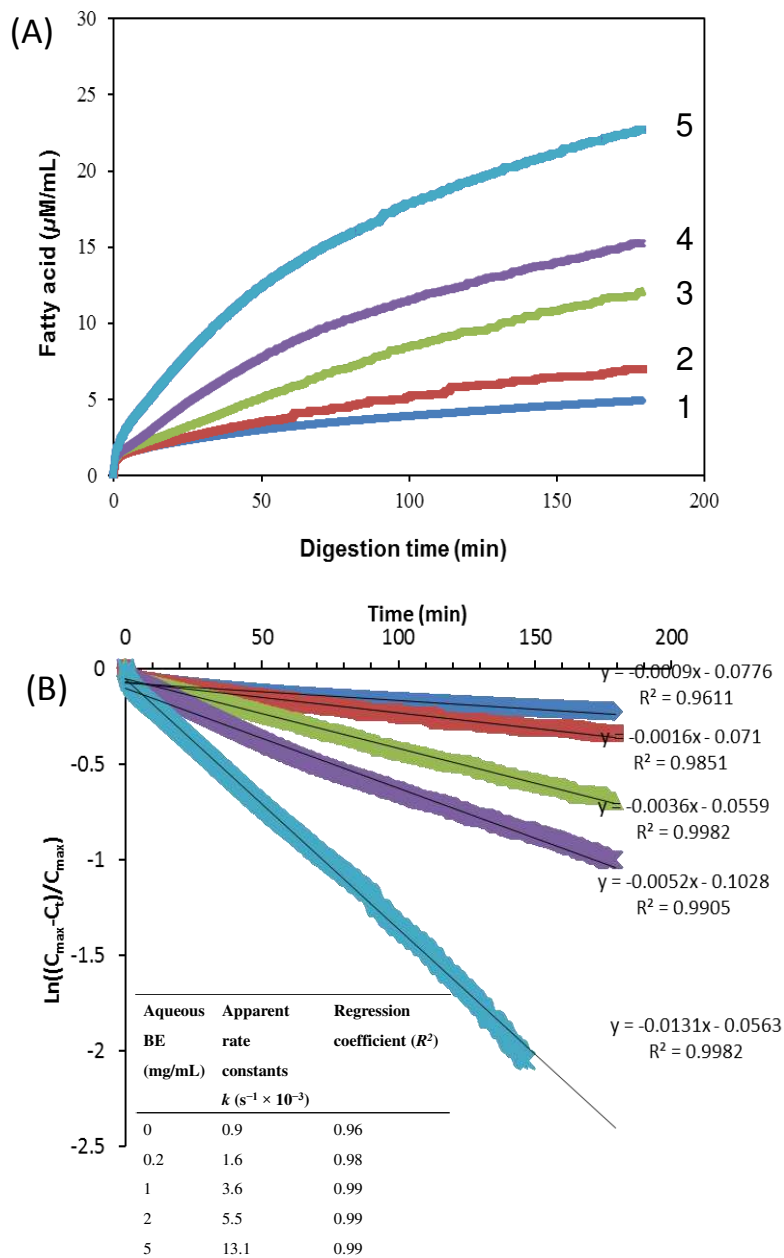
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528 **Figure 3**

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532 **Figure 4**

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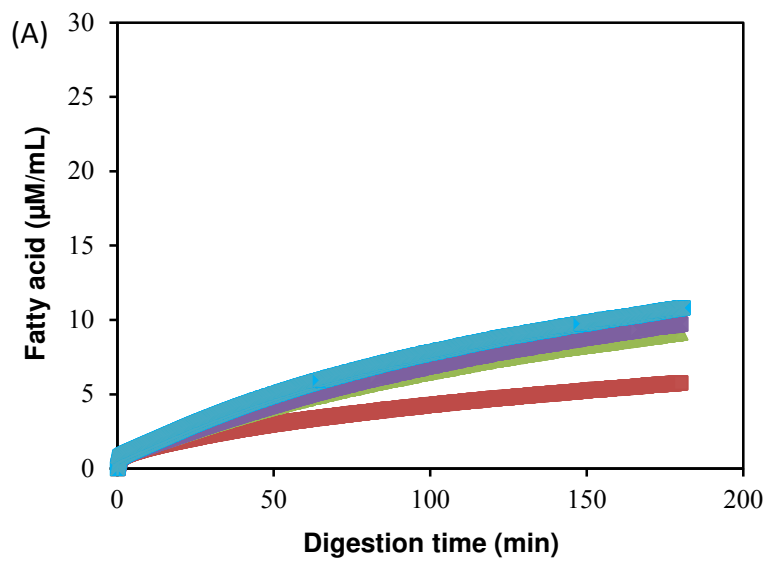
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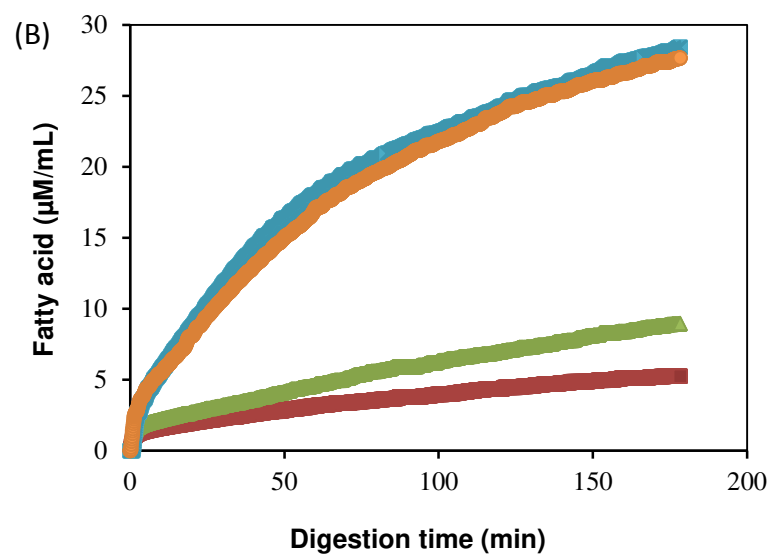
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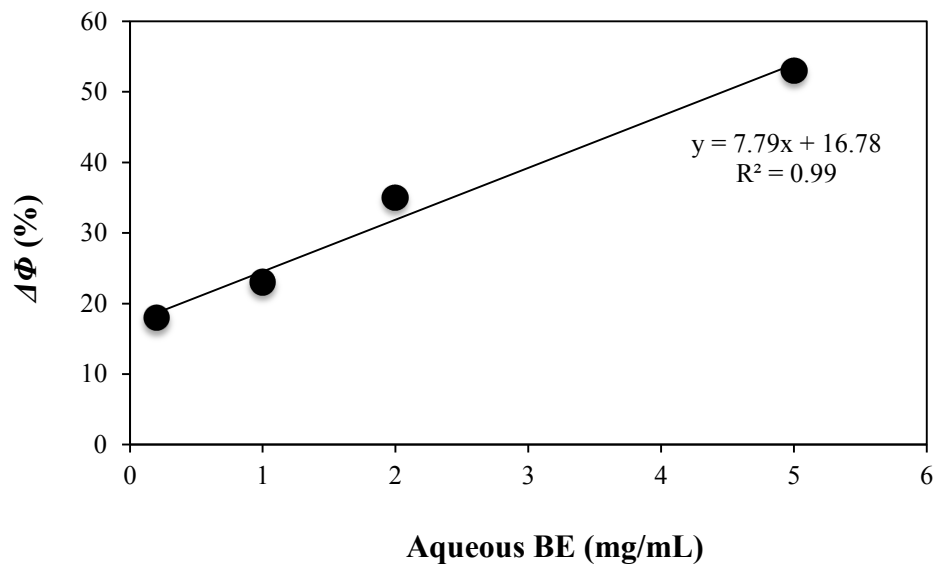
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553 **Figure 5**



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557 **Figure 6.**

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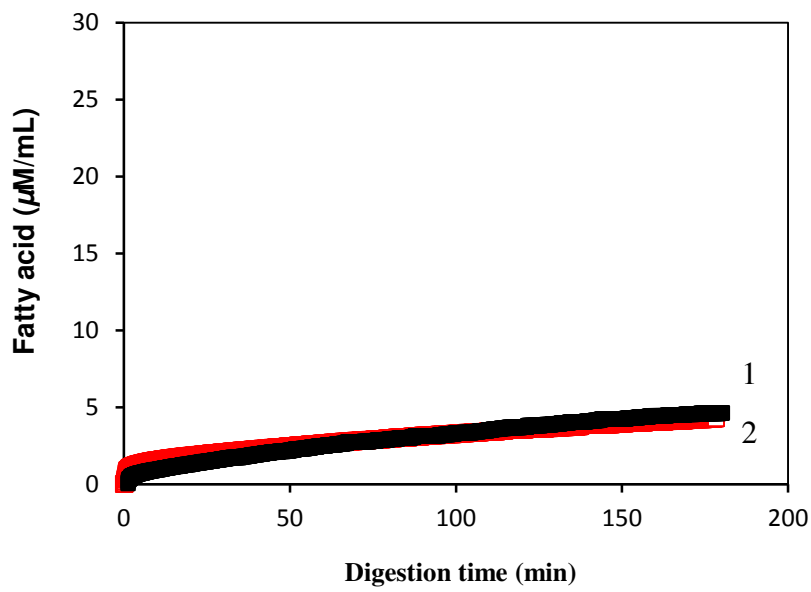
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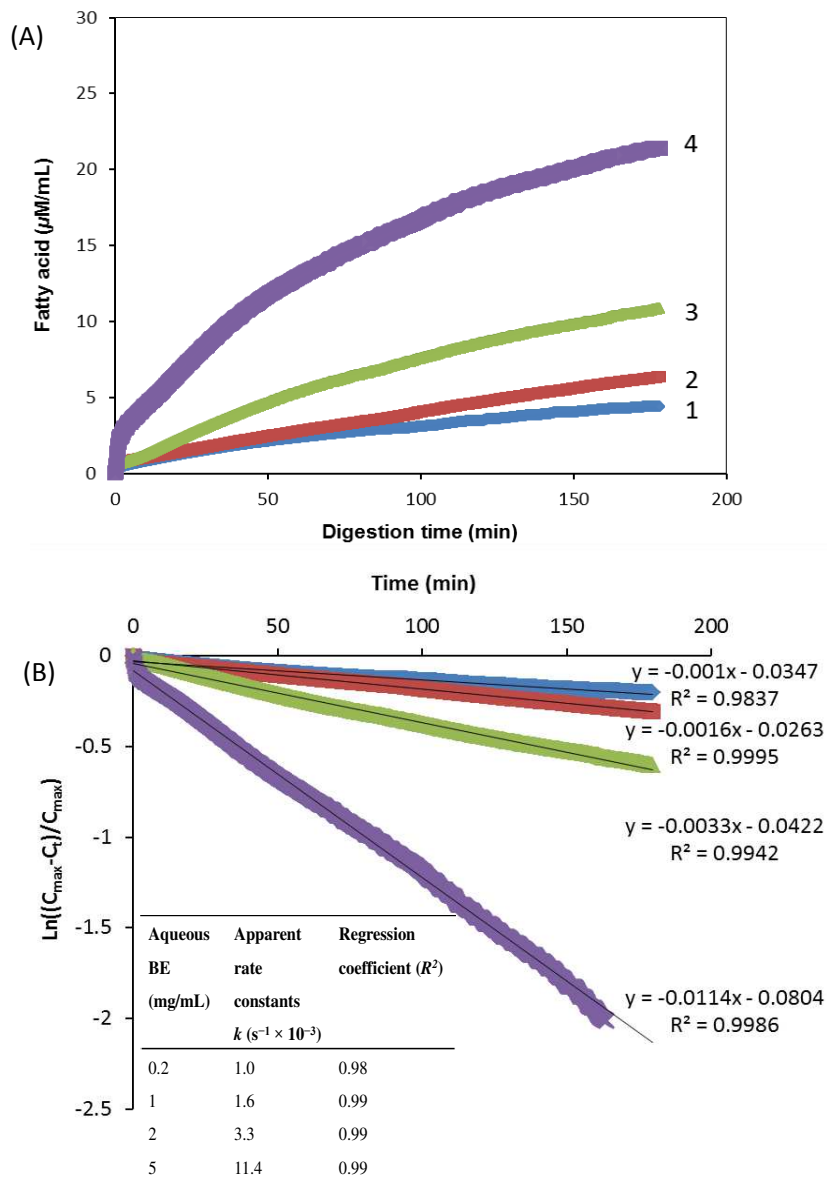
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581 **Figure 7**

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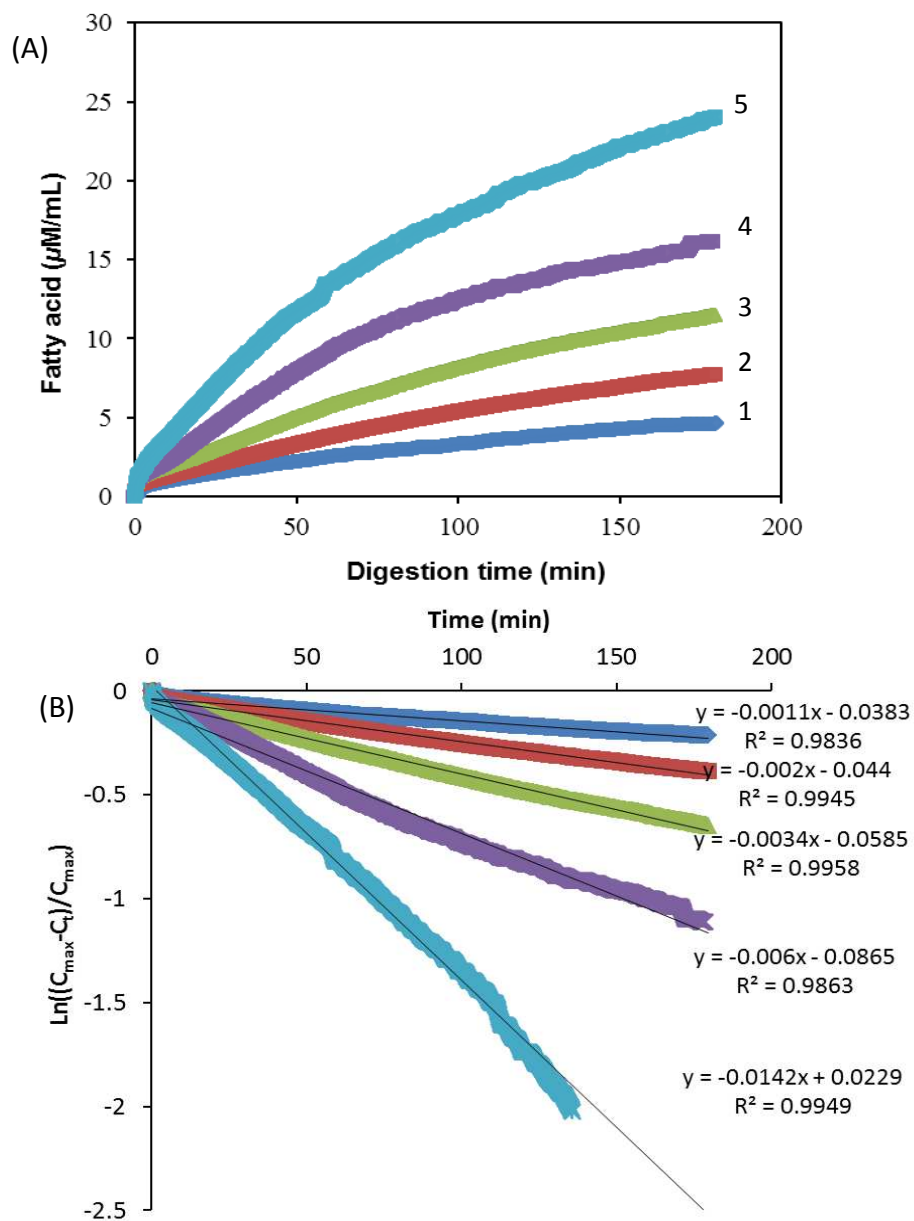
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585 **Figure 8**

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588 **Figure S1.**

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