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1 **Title: BILE SALTS IN DIGESTION AND TRANSPORT OF LIPIDS**

2 **Authors:** Adam Macierzanka^{1,2}, Amelia Torcello-Gómez³, Christian Jungnickel¹ and Julia
3 Maldonado-Valderrama^{4,5} *

4 **Addresses**

5 ¹Department of Colloid and Lipid Science, Gdańsk University of Technology, Narutowicza
6 11/12, 80-233 Gdańsk, Poland

7 ²STLO, UMR 1253, INRA, Agrocampus Ouest, 35000 Rennes, France

8 ³School of Food Science and Nutrition, University of Leeds, Leeds LS2 9JT, United Kingdom

9 ⁴Department of Applied Physics, University of Granada, 18071 Granada, Spain

10 ⁵Excellence Research Unit “Modeling Nature” (MNat), University of Granada, Granada, Spain

11

12 *Corresponding author: Julia Maldonado-Valderrama (julia@ugr.es)

13

14 **Five keywords for indexing**

15 Bile salt, lipid digestion, bulk interactions, oil-water interface, colloidal transport

16

17 **Abstract**

18 Because of their unusual chemical structure, bile salts (BS) play a fundamental role in intestinal
19 lipid digestion and transport. BS have a planar arrangement of hydrophobic and hydrophilic
20 moieties, which enables the BS molecules to form peculiar self-assembled structures in
21 aqueous solutions. This molecular arrangement also has an influence on specific interactions
22 of BS with lipid molecules and other compounds of ingested food and digestive media. Those
23 comprise the complex scenario in which lipolysis occurs. In this review, we discuss the BS
24 synthesis, composition, bulk interactions and mode of action during lipid digestion and
25 transport. We look specifically into surfactant-related functions of BS that affect lipolysis, such
26 as interactions with dietary fibre and emulsifiers, the interfacial activity in facilitating lipase
27 and colipase anchoring to the lipid substrate interface, and finally the role of BS in the
28 intestinal transport of lipids. Unravelling the roles of BS in the processing of lipids in the
29 gastrointestinal tract requires a detailed analysis of their interactions with different
30 compounds. We provide an update on the most recent findings concerning two areas of BS
31 involvement: lipolysis and intestinal transport. We first explore the interactions of BS with
32 various dietary fibres and food emulsifiers in bulk and at interfaces, as these appear to be key
33 aspects for understanding interactions with digestive media. Next, we explore the interactions
34 of BS with components of the intestinal digestion environment, and the role of BS in displacing
35 material from the oil-water interface and facilitating adsorption of lipase. We look into the
36 process of desorption, solubilisation of lipolysis products and formation of mixed micelles.
37 Finally, the BS-driven interactions of colloidal particles with the small intestinal mucus layer are
38 considered, providing new findings for the overall assessment of the role of BS in lipid
39 digestion and intestinal transport. This review offers a unique compilation of well-established
40 and most recent studies dealing with the interactions of BS with food emulsifiers,
41 nanoparticles and dietary fibre, as well as with the luminal compounds of the gut, such as
42 lipase-colipase, triglycerides and intestinal mucus. The combined analysis of these complex
43 interactions may provide crucial information on the pattern and extent of lipid digestion. Such
44 knowledge is important for controlling the uptake of dietary lipids or lipophilic
45 pharmaceuticals in the gastrointestinal tract through the engineering of novel food structures
46 or colloidal drug-delivery systems.

47

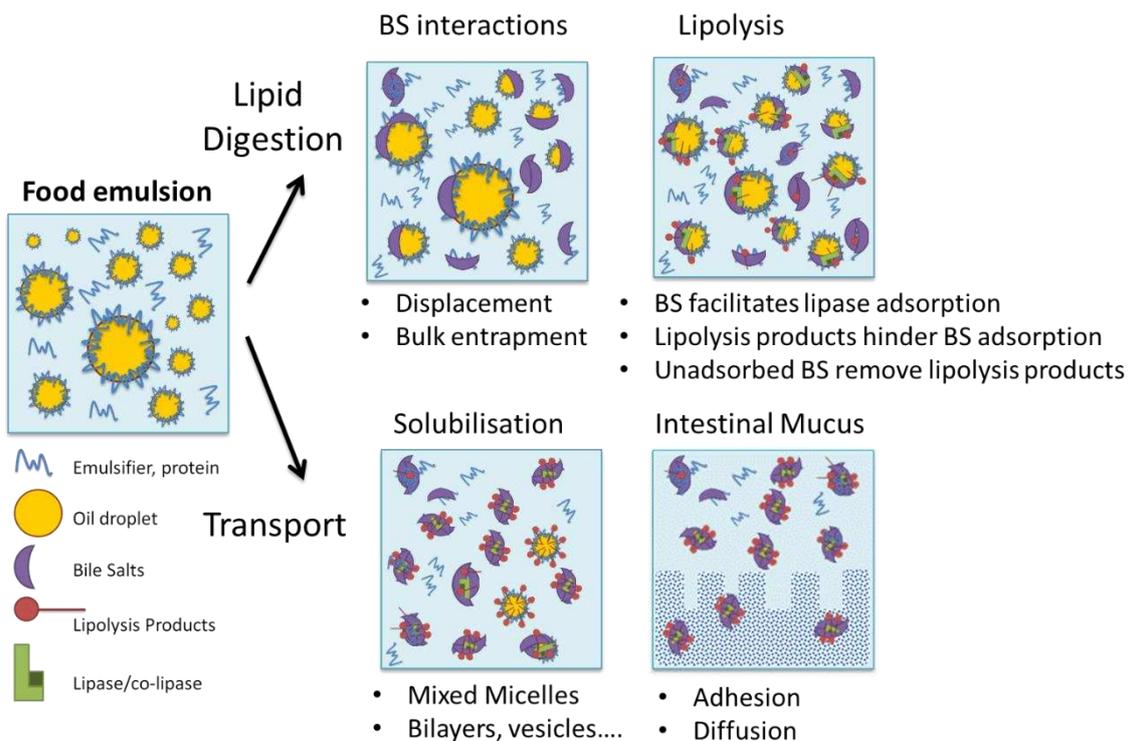
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66	List of Abbreviations
67	Beta-lactoglobulin: BLG
68	Bile Acids: BA
69	Cholic acid (CA)
70	Chenodeoxycholic acid (CDCA)
71	Glycocholic acid (GCA)
72	Glycochenodeoxycholic acid (GCDCA)
73	Taurocholic acid (TCA)
74	Taurochenodeoxycholic acid (TCDCA)
75	Bile extract: BE
76	Bile salts: BS
77	Cholate (C)
78	Deoxycholate (DC)
79	Chenodeoxycholates (CDC)
80	Glycodeoxycholates (GDC)
81	Taurocholates (TC)
82	Cellulose nanocrystals: CNC
83	Critical micelle concentration: CMC
84	Dietary fibre (DF)
85	Methylcellulose (MC)
86	Hydroxypropylcellulose (HPC)
87	Hydroxypropylmethylcellulose (HPMC)
88	Diffusion coefficient (effective diffusivity, D_{eff})
89	Dynamic light scattering (DLS)
90	Fatty acids (FA)
91	Free fatty acids (FFA)
92	Gastrointestinal (GI)
93	Mean-square displacement (MSD)
94	Nanoparticles (NP)
95	Nuclear magnetic resonance (NMR)
96	Phospholipid (PL)
97	Phosphatidylcholine (PC)
98	Small-angle X-ray scattering (SAXS)
99	Whey Protein Isolate (WPI)
100	

101 **1. General introduction to BS**

102

103 Bile is a very diverse physiological fluid, with a wide range of functions. It was recognised as
 104 early as 200 AD by Aelius Galen as one of the four humours of the body [1]. However, the
 105 plethora of functions of bile has only recently been identified. Bile is a complex mixture of
 106 many compounds, and some of them, e.g. BS, are surface active. In this article, we will review
 107 the synthesis, composition and path/fate of bile and the BS in the human body. However, the
 108 focus will be on the surfactant-related functions of bile salts in the gastrointestinal (GI) tract,
 109 such as interactions with food emulsifiers and dietary fibre, facilitating of the interfacial
 110 adsorption of lipase and colipase to the lipid substrate interface, and finally the role in
 111 intestinal transport of lipids through the participation in formation of mixed micelles and
 112 furthering colloidal transport in the intestinal mucus. These processes are illustrated
 113 schematically in Figure 1.



114

115 **Figure 1.** Schematic representation of the roles of bile salts (BS) in the intestinal digestion and
 116 transport of lipids. Only the main interactions, important for surfactant-related functions of BS,
 117 are shown.

118

119

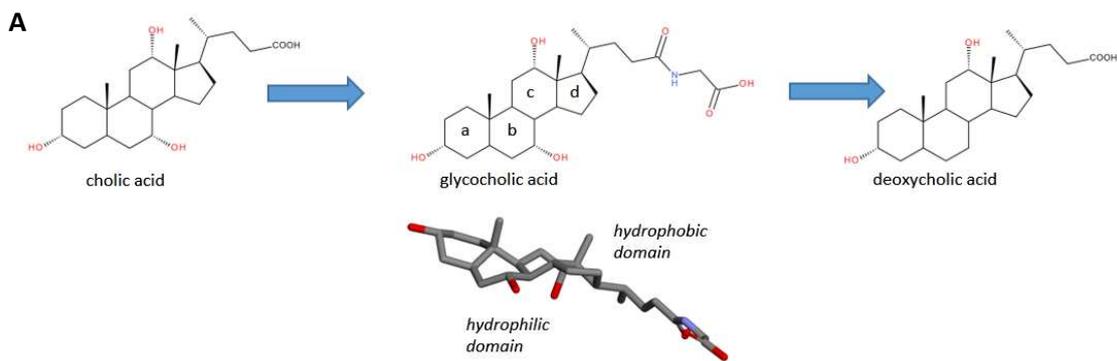
120 **1.1. Chemical structure**

121 Bile is comprised of 95% water, in which there is a number of inorganic and organic
 122 constituents, including bile acids (40 mM), cholesterol (3 mM), sodium and potassium (145
 123 mM and 4 mM, respectively), and phospholipids (7 mM), with a pH of 7.5-8.0 [2]. The colour of
 124 bile is determined by the concentration of bilirubin. Bile acids (BA) are physiologically planar
 125 surface active molecules, initially synthesised from cholesterol in the liver [3], as shown in
 126 Figure 2A. Those synthesised from cholesterol are generally known as primary BA, and in
 127 humans these are cholic acid (CA) and chenodeoxycholic acid (CDCA). The primary synthesis

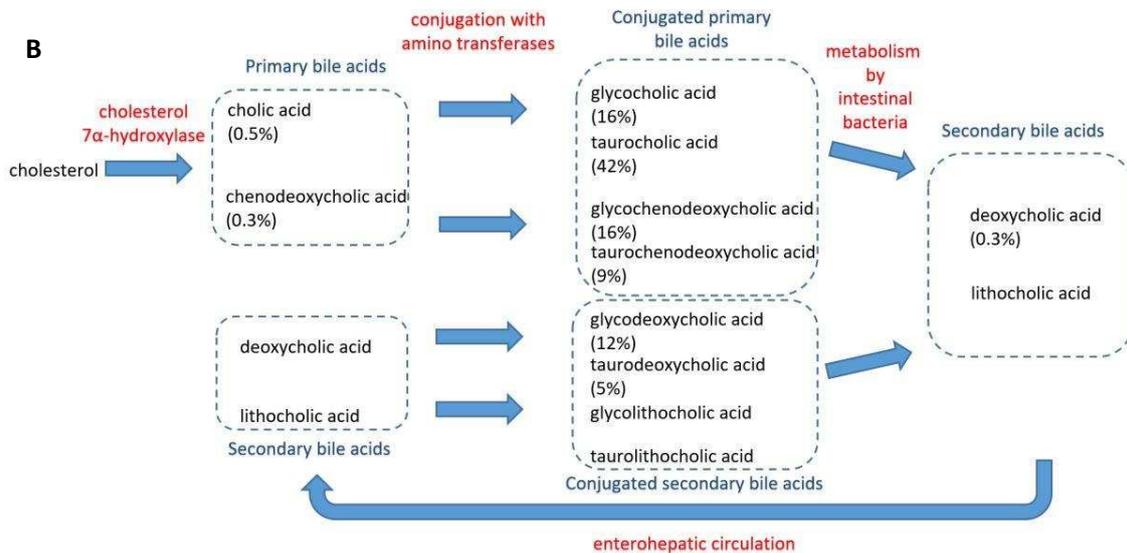
128 begins with the hydroxylation of cholesterol with cholesterol 7 α -hydroxylase, also known as
 129 the neutral (or classic) pathway [4], as shown in Figure 2B.

130 Most of the BA, before leaving the hepatocytes, are conjugated with glycine or taurine. These
 131 conjugated BA are glycocholic acid (GCA), taurocholic acid (TCA), glycochenodeoxycholic acid
 132 (GCDCA), and taurochenodeoxycholic acid (TCDCA). The conjugated BA are also known as BS,
 133 because the pK_a of conjugated BA is lower than the unconjugated form (pK_a of 3.9 for glycine
 134 conjugated vs 5.0 for unconjugated BA), and the BA exist in their deprotonated form in the
 135 duodenum [5]. For the purpose of this review, BA, when they have left the ampulla of Vater
 136 and entered the duodenum, are termed BS.

137



138



139

140 **Figure 2.** (A) Exemplary structures of primary, conjugated primary, and secondary bile acids (BA). The BA
 141 is comprised of three six-carbon rings (a, b, and c), as well as a five-carbon ring (d), with a flat structure
 142 depending on the cis- or trans- conformation between the first two rings (a and b). The concave side of
 143 the BA is hydrophilic due to the presence of the hydroxyl groups, whereas the convex side of the steroid
 144 skeleton is hydrophobic with a number of methyl groups. (B) Schematic representation of the path of
 145 formation of various primary, conjugated primary, secondary and conjugated secondary BA, including
 146 the recycling of the bile salts (BS) via enterohepatic circulation. The approximated duodenal BS
 147 composition is given in percentage (where available) [6].

148

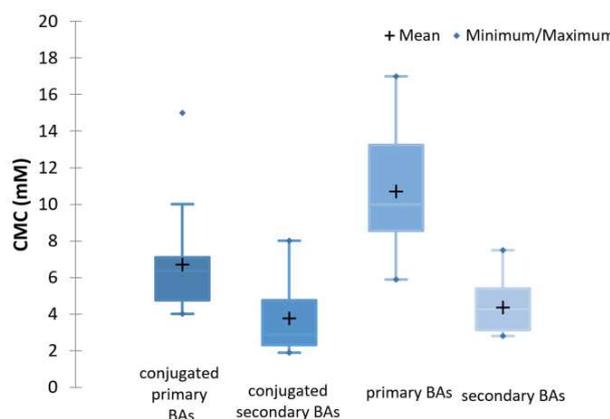
149 The conjugation takes place within the hepatocytes with BA CoA-ligase, and BA-CoA:amino
 150 acid N-acyltransferase [7], occurring in both the endoplasmic reticulum and peroxisomes of
 151 the cells [8]. The process of conjugation decreases the hydrophobicity of BA, as shown in Table
 152 1. A change in the ratio of glycine and taurine (normally 3:1) conjugation may be considered an
 153 indicator of liver disease [9,10], however it may also change with diet [11]. The decreased
 154 hydrophobicity, as well as the lower pK_a of the BA, limits re-adsorption of the BA (Boyer, 2013).
 155 The BA leave the hepatocytes by several ATP-driven export pumps into the canaliculus, a small
 156 space formed between adjoining hepatocytes. The BA then flow into bile ductules, and
 157 eventually into the common bile duct [5]. Once excreted, the BA become part of mixed
 158 micelles, which include also phospholipids (PLs); predominantly phosphatidylcholine (PC), and
 159 sterols (mostly cholesterol) [12]. As the BA travel through the biliary system, they pass by
 160 cholangiocytes. These release bicarbonate into the bile, thereby making the bile more alkaline.
 161 Finally, the bile is stored in the gallbladder [13]. The gallbladder will contract, after a meal is
 162 consumed, resulting in the release of bile into the duodenum.

163 Table 1 Changing hydrophobicity of various primary, conjugated primary and secondary bile
 164 acids. Data from Heumann et al. [14]

Bile acid	Hydrophobicity
Secondary conjugated lithocholic acid	↑
Secondary unconjugated deoxycholic acid	
Secondary conjugated deoxycholic acid	
Primary unconjugated chenodeoxycholic acid	
Primary conjugated chenodeoxycholic acid	
Unconjugated cholic acid	
Conjugated cholic acid	

165

166 BA have a wide range of critical micellar concentrations (CMCs) in water. The CMC of
 167 primary BA ranges between 5.9 and 17 mM, with a mean of 10.7 mM [15–19], whereas the
 168 CMC of conjugated primary BA ranges between 4 and 15 mM [20–22] with a mean of 6.7 mM,
 169 and that for secondary and conjugated secondary BA ranges between 2.8–7.5 mM and 1.9–8
 170 mM, respectively, with means of 4.4 mM and 3.8 mM [20,23,24]. The data are presented in
 171 Figure 3.



172

173 **Figure 3.** Comparison of the critical micelle concentration (CMC) values of various groups of bile acids
 174 (BA). Shown are the mean, minimum and maximum; and the bands represent the first and third
 175 quartiles of the CMCs. A total of 112 CMC values were collated.

176 On average, primary BA display a significantly higher average CMC than either conjugated
177 primary, secondary, or conjugated secondary BA. The BS micelles have a very small aggregate
178 number of 2 to 12 BA units [18]. Additionally, unlike traditional surfactants, the planar BA,
179 have a low potential for solubilisation of hydrophobic molecules [25]. The solubilising potential
180 of the micelles is enhanced by up to three times if the BA are mixed with PLs [26].

181

182 *1.2. Physiological function of BS*

183 BS and BA have a wide range of functions, from emulsification of dietary lipids, and facilitating
184 their digestion and absorption, to signal transduction in cell proliferation, metabolism, and
185 differentiation [27]. As a direct mode of action, BS form mixed micelles, which aids in the
186 absorption of lipolysis products and lipid-soluble vitamins. Further details on the function of BS
187 in lipid digestion will be discussed in the next sections. Indirectly, BA regulate their own
188 synthesis through the farnesoid receptor X. This receptor is located through the biliary system,
189 as well as in the ileal enterocytes. Activation of the farnesoid receptor X inhibits cholesterol
190 7 α -hydroxylase. Impairment of BA formation results in a range of cholestatic diseases. These
191 can lead to cirrhosis, fibrosis, liver failure, hepatocellular carcinoma, as well as
192 cholangiocarcinoma. Lower than normal concentrations of BA in bile can result in
193 supersaturation of cholesterol, which may lead to cholelithiasis [28].

194 At any given point in time, ca. 85-90% of the bile is in the small intestine, less than 1% is in the
195 liver, and ca. 10-15% are stored in the gallbladder [13]. Intestinal BS are mostly conjugated (as
196 shown in Figure 2B), and confer antimicrobial properties, by changing or disrupting the
197 bacterial membrane, or by denaturing membrane proteins. This enables to control microbial
198 overgrowth in the intestine [2,29]. Bacteria in the ileum and colon metabolise BS. Primary BA
199 are first deconjugated and 7 α -dehydroxylase transform them into secondary deoxycholic and
200 lithocholic acids. A small amount of tertiary ursodeoxycholic acid is also produced by 7 β -
201 hydroxysteroid dehydrogenase [13]. This deconjugation of BS reduces their solubility and
202 increases toxicity. However, about 95% of BS are reconstituted and reabsorbed by enterocytes
203 in the ileum. From there, the BA enter the portal blood, and are recycled in the liver. The
204 remaining 5% (or ca. 0.5 g of the total BA) are removed from the body by faecal excretion.

205

206 **2. Bulk interactions of bile salts with emulsifiers and dietary fibres**

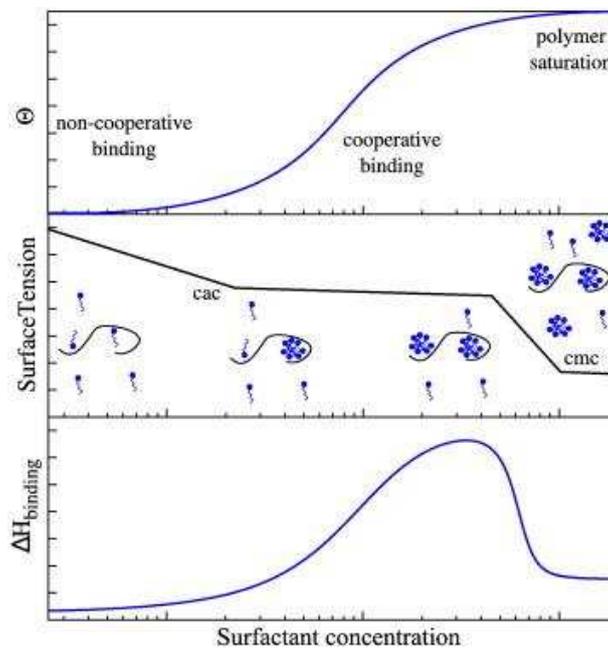
207 Once in the small intestine, BS have a number of functions to aid digestion. This section
208 focuses on reviewing interactions between BS and a broad range of food emulsifiers and
209 stabilisers in bulk. These interactions are relevant since they may interfere with the role played
210 by BS in food digestion, and thus have an impact on the hydrolysis, transport and absorption of
211 nutrients such as lipids and amino acids, which will be addressed specifically later on. The
212 mechanisms of interactions will certainly depend on the nature of the molecules involved and
213 the level of study (mesoscopic or molecular). At a meso-scale, physical entrapment of BS could
214 be expected, for instance, if dealing with water-soluble high-molecular weight stabilisers that
215 provide relatively high bulk viscosities. On the other hand, at molecular level, electrostatic
216 and/or hydrophobic interactions may occur if ionic and/or non-ionic amphiphilic
217 emulsifiers/stabilisers are present. In addition, these will depend on changes in solution and
218 environmental conditions such as pH, ionic strength, solvent composition and temperature. All

219 these parameters will be taken into account when reviewing interactions between BS and
220 individual dietary fibres, proteins and low-molecular weight surfactants of lipidic and non-
221 lipidic nature. *In vitro* studies mimicking the physiological environment in the small intestine
222 (pH, ionic strength, temperature, etc.) are of major interest here since *in vivo* studies usually
223 only report on the BS content of excreted or serum samples, from which little or no details on
224 the intermediate mechanisms of interactions can be elucidated [30,31]. In addition, defining
225 interactions between BS and food emulsifiers/stabilisers in animal or human gastrointestinal
226 (GI) contents is not trivial due to the complexity of the media [32]. Therefore, *in vitro* models
227 seem appropriate to contribute building up the comprehensive knowledge on the role of BS in
228 digestion.

229 2.1. BS and dietary fibres

230 The term dietary fibre (DF) was first postulated as indigestible carbohydrates of plant origin.
231 This term was then restricted to indigestible plant carbohydrates with associated health
232 benefits, comprising non-starch polysaccharides, lignin and other analogous polysaccharides.
233 Later on, the definition has expanded to include oligosaccharides with properties similar as
234 water-soluble DF, and indigestible resistant starches behaving as DF in the large intestine [33].
235 A broader definition also considers fibres of animal origin, such as chitosan, and modified or
236 synthetic carbohydrate polymers. The interactions between BS and DF have increasingly
237 received interest in the last decade due to the health benefits associated with the passage of
238 the latter through the GI tract, despite their indigestible nature [33,34]. The physiologic effects
239 of a particular type of DF within the GI tract are attributed to its physicochemical properties
240 such as solubility, degree of viscosity, and fermentation in the large intestine [33]. Dietary
241 fibres which are water-soluble, hereinafter termed as soluble DF, and either viscous or gel-
242 forming under GI conditions, may delay gastric emptying and small bowel transit and retard
243 the transport and absorption of glucose, triglycerides and cholesterol from the small intestine
244 [34]. This delaying effect is reduced as the molecular weight of DF, and thus viscosity,
245 decreases. Soluble DFs are also very accessible to bacterial enzymes, and therefore very
246 rapidly fermented in the proximal colon, tending to have less impact on colonic transit. On the
247 other hand, insoluble DFs do not affect the viscosity of gastric and intestinal contents, tending
248 to accelerate small bowel transit, and have more marked laxative effect contributing to faecal
249 bulking [35]. The healthy attributes of DFs are associated with their physiological effects within
250 the GI tract. The reduction of blood cholesterol and hyperlipidaemia is thought to be linked
251 with the interactions with BS, amongst other mechanisms. *In vivo* studies in humans have
252 shown that 2- or 3-day-diet with increased content of DF induces increased bile excretion
253 within the 24 h upon consumption [31,36]. One of the possible reasons for this is that DF has a
254 bile sequestering capacity, leading to bile excretion. An alternative, but non-exclusive,
255 explanation is that DF decreases the permeability of the intestinal mucus layer lining the
256 surface of epithelium, and thus hindering bile absorption [37]. These two combined
257 mechanisms may alter the bile reabsorption in the distal small intestine (ileum) into the
258 enterohepatic circulation [38]. Therefore, more BA need to be synthesised *de novo* from
259 cholesterol in blood in order to restore the bile pool. On the other hand, interactions between
260 DF and BS may also lower the rate of lipid digestion and uptake, with subsequent reduced lipid
261 concentration in plasma.

262 In general, direct interactions at a molecular level and/or reduction of bile diffusion by
 263 entrapment in polymer network are the two plausible mechanisms of interactions between DF
 264 and BS. Most of the investigations on the binding of BA to DF are based on centrifugation or
 265 dialysis methods, where the free bile content is measured in the supernatant or dialysate,
 266 respectively, and/or the bound bile is assayed in the pellet or dialysis sample, respectively [39–
 267 41]. Nevertheless, to date, scarce information is available in literature regarding specific
 268 molecular mechanisms of interactions between BS and DF to help elucidating the pathway or
 269 cascade of events related with the healthy attributes of dietary fibre. DFs occur in isolated,
 270 more or less soluble form or as a part of the more or less intact complex cell wall architecture
 271 in the diet, making it difficult to find the mechanisms behind their physiological effects [39].
 272 Studies on DF retaining an intact cell wall structure from fruits, vegetables and cereals did not
 273 provide conclusive results on the specificity of interactions with BA or the effect of the
 274 composition (e.g. soluble versus insoluble) that could contribute to the extent of binding with
 275 BS [39]. For all these reasons, we will focus on the interactions of isolated soluble DF and BS.
 276 Insoluble DF has rather low cholesterol-reducing effect as compared to soluble DF [40], which
 277 may be linked to its rather different physiological effect within the GI tract, as pointed out
 278 above. In addition, isolated insoluble DF such as cellulose do not exhibit BA binding properties.
 279 Dongowski observed that isolated microcrystalline cellulose do not bind BA *per se* [39], which
 280 may be explained by the hydrophilic nature of this fibre composed of linear chains of several
 281 thousand glucose units. Therefore, the author attributes its role in BA binding when it
 282 stabilises the cell wall architecture after the digestion process.



283

284 **Figure 4.** General trend of (from top to bottom) a binding isotherm, surface tension and enthalpy of
 285 binding in polymer-surfactant systems, as a function of surfactant concentration. Reprinted from [42].

286

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287 The interactions of BS with isolated soluble DFs depend on the physicochemical characteristics
 288 of fibres. Soluble DFs are composed, in principle, of biopolymers, of which backbone is based
 289 mainly on carbohydrates. These vary in the molecular weight, branching, type of substitution
 290 groups, distribution of substitution groups, surface charge and hydrophobic pockets, etc.,

291 which in turn depend on the pH and ionic strength conditions. In general, the interactions
292 between BA and biopolymers can be described as a cooperative process and depend on the
293 concentration regime of BA, which is the same as for any other surfactant. An example of a
294 typical binding isotherm, trend in surface tension and binding enthalpy in surfactant/polymer
295 systems is shown in Figure 4.

296 *2.1.1. BS and cereal β -glucan/arabinoxylan*

297 Cereal β -glucan consists of a linear backbone of glucosyl units. The consumption of 3 g/day of
298 β -glucan present in fibre-rich meals from oats and barley has associated health benefits, such
299 as reducing or maintaining normal blood LDL-cholesterol levels, amongst others [43,44]. This
300 health claim may be more related to the interactions with BA, and not to the inhibition of
301 cholesterol absorption or synthesis, as BA have been shown to be excreted to larger extent
302 with the intake of high-molecular weight β -glucan [45]. Hydrophobic and electrostatic
303 interactions between BA molecules and β -glucan are not expected due to the hydrophilic and
304 non-ionic nature of the latter. Indeed, direct binding of BA onto β -glucan fractions was not
305 observed by nuclear magnetic resonance (NMR) under physiological conditions of pH in the
306 presence of GCA [46]. Therefore, other physical mechanisms, such as bile micelles entrapment
307 by viscous solution of β -glucan may occur with this soluble dietary fibre. Similar mechanism
308 has been hypothesised for arabinoxylan [39]. Arabinoxylan is a heteropolymer comprising a
309 xylopyranose backbone with substituents of α -L-arabinofuranose. Arabinoxylan present in
310 wheat and rye is thought to have similar cholesterol-reducing properties as β -glucan [47].
311 NMR-titration studies evidenced direct dynamic molecular contact between β -glucan and BS
312 micelles of NaTDCD under physiological duodenal pH, as reflected in consistent chemical shifts
313 in BS resonance in the presence of β -glucan, whereas no direct molecular interactions
314 occurred between arabinoxylan and the same BS [48]. The reduced intensity in BS signal in the
315 presence of arabinoxylan suggests that arabinoxylan forms local entangled polymer network
316 reducing the mobility of BS micelles. Both mechanisms may explain the delayed passage of
317 NaTDCD and porcine bile through dialysis membrane in the presence of the polysaccharides
318 [49], the effect being greater at higher polymer concentration, and thus viscosity. These
319 mechanisms of interaction between β -glucan or arabinoxylan and BS were further
320 corroborated with another BS, NaGCDC, and the whole porcine bile by NMR spectra and small-
321 angle X-ray scattering (SAXS), and were found to be combined depending on the source of the
322 soluble DF and its molecular weight [50]. Namely, high molecular weight barley and oat β -
323 glucan and low molecular weight wheat arabinoxylan and high molecular weight rye
324 arabinoxylan showed the two combined mechanisms of interaction with BS.

325 *2.1.2. BS and chitosan*

326 Chitosan is a biopolysaccharide that is positively charged under acidic conditions and is
327 frequently used in food processing. It is derived from chitin exoskeleton of arthropods by
328 alkaline or enzymatic deacetylation, so that the linear molecular structure is composed of
329 randomly distributed glucosamine and N-acetylglucosamine units. The interactions between
330 oppositely charged biopolymers and surfactants have shown pronounced synergisms that lead
331 to interesting assembly features [42]. Chitosan and the BS, NaTC, have been reported to bind
332 by electrostatic interactions at pH 3, at which chitosan is positively charged, and a wide range

333 of temperature (10-40 °C) [51]. The technique used was isothermal titration calorimetry. It is
334 likely that electrostatic interactions take place between the sulfated head-group of NaTC and
335 ammonium ion in chitosan. The effect of salt addition (NaCl), which screened the electrostatic
336 attraction between the BS and chitosan or the repulsion between bile molecules, supported
337 this as a major driving force for BS binding. Also, the temperature dependence of the binding
338 enthalpy at intermediate BS concentrations had the opposite trend to that of the
339 demicellization enthalpy. This suggested that the BS binds to chitosan forming micelle-like
340 clusters. Chitosan also possesses hydrophobic pockets along the backbone, which play a role in
341 interacting with amphiphiles, such as BS, through the apolar steroidal backbone. Indeed, these
342 interactions have led to the design of chitosan-BA structures for encapsulation of lipids [52]
343 and for responsive drug release upon stimuli applied: an increase of temperature and salt
344 concentration [53].

345 2.1.3. BS and cellulose ethers

346 Cellulose ethers are derived from cellulose by chemical modification. They are obtained by
347 partially reacting the hydroxyl groups in each glucose ring in the native cellulose backbone.
348 These cellulose derivatives are widely used in the food industry due to their functional
349 properties, such as surface activity and thickening efficiency. Several studies have investigated
350 the bulk interactions between cellulose ethers and BS, NaTDC and NaTC, by micro-differential
351 scanning calorimetry and rheology under neutral pH conditions [54,55]. Hydrophobic
352 association of BS and non-ionic cellulose ethers: methylcellulose (MC),
353 hydroxypropylmethylcellulose (HPMC) and hydroxypropylcellulose (HPC), was hypothesised
354 based on the bile-induced inhibition of the thermogelation properties of cellulose ethers. This
355 effect was observed from the shift of the thermal transition to higher temperatures and the
356 reduction of the transition enthalpy, as well as from the weakened gel strength in the presence
357 on BS, which depended on the degree of hydrophobicity of the BS and cellulose ether [55].
358 Namely, the more hydrophobic BS (NaTDC) hindered to a larger extent the thermal structuring
359 of all cellulose ethers tested, suggesting greater extent of adsorption onto fibre molecules and
360 reducing their hydrophobic association. Examination of surface tension has also shown
361 evidence of molecular association between the BS, NaDC, and ethylhydroxyethyl cellulose in
362 aqueous basic solutions. This was reflected in the observation of a first plateau in the surface
363 tension-log BS concentration curve, followed by a decrease in surface tension above the
364 concentration of cellulose ether saturation by NaDC, and a second plateau corresponding to
365 the formation of pure BS micelles [56]. This could be considered a typical trend, similar to that
366 shown in Figure 4 (middle panel) for mixtures of polymers and surfactants. Pizones Ruiz-
367 Henestrosa et al. also observed bulk interactions between HPMC of different molecular
368 weights and hydrophobicity with the whole porcine bile extract (BE) in simulated intestinal
369 media [57]. The association was assessed by measuring particle size and conductivity, and by
370 comparing cloud point temperature. The BE aggregates formed at a physiological
371 concentration dissociated in the presence of both types of HPMC. A more recent NMR study
372 has proved interactions between a model BS, NaTC, and HPMC in simulated intestinal
373 media[58]. Interactions between the BS and HPMC molecules were favoured in the simulated
374 intestinal media mimicking the fasted state (lower BS concentrations) and might be driven by
375 hydrophobic forces. On the contrary, BS self-association predominantly occurred in the
376 simulated intestinal media mimicking the fed state (higher BS concentrations). Ionic strength

377 influenced the interactions between HPMC and NaTC, probably by screening the repulsion
378 between BS molecules, but the ion type had a minimal effect.

379 2.1.4. BS and pectin

380 Pectin is a soluble fibre commonly present in fruits and vegetables and is used as a gelling
381 agent in the food industry. It is a linear and branched polysaccharide comprising a backbone of
382 linked galacturonic acid monomers and branches of monosaccharides: galactose, mannose,
383 glucose and xylose. Interactions between pectin and BE or pectin and the BS, NaTC, have been
384 reported [59,60]. It was found that BE promoted the aggregation of pectin, which was
385 visualised by turbidity and optical microscopy, and isothermal titration calorimetry suggested
386 the direct binding of bile onto pectin molecules. Electrostatic attraction between both anionic
387 components, pectin and BS, at pH 7 are not expected, but hydrophobic interactions between
388 the apolar steroidal backbone of BA and the methoxyl groups in the pectin molecules may
389 occur. Interestingly, an early study showed that contaminants in commercial pectin samples
390 were responsible for this binding, instead of the purified fraction of native high-molecular
391 weight pectin. The authors analysed those possible interactions by NMR spectroscopy and
392 dialysis experiments [61]. The BS NaTC also showed binding to complexes of pectin and ϵ -
393 polylysine [60], which is a cationic biopolymer frequently used in the food industry as
394 preservative. This is relevant since these kinds of complexes are likely to be present in real
395 food matrices, and thus enter the GI environment, and binding of pectin with BS may be
396 affected. However, these results showed the ability of a model BS to bind biopolymer
397 complexes formed by electrostatic interactions. On the other hand, Singh et al. have recently
398 modelled the interactions of several BA (unconjugated CA, DCA, CDCA, and their glyco-
399 conjugated counterparts) with pectin through molecular docking, and provided the estimated
400 strength of the binding energy [62]. They showed that GCA, GDCA and CA had a larger binding
401 affinity than DCA, CDCA, GCDCA. It seems surprising that the more hydrophilic GCA
402 (conjugated and trihydroxy BA) showed more favourable interactions than the more
403 hydrophobic unconjugated and dihydroxy BA (DCA and CDCA). Thus, other important factors,
404 such as hydrogen bonding, might be underestimated in molecular docking to accurately model
405 experimental conditions.

406 2.2. BS and emulsifiers

407 2.2.1. BS and Proteins

408 Less attention has been paid in scientific literature to interactions between proteins and BA,
409 [63,64], despite their relevance to determining not only the bioaccessibility and bioavailability
410 of amino acids, but also the ascribed cholesterol-lowering effect [65]. Indeed, interactions
411 between protein and BS can be a determining factor used in protein purification based on the
412 predominant role played by hydrophobic interactions [64].

413 *In vitro* studies on protein isolates are the focus here to account for the sole contribution of
414 peptides in the interactions with BS. Soy protein isolate and wheat gluten have been reported
415 to have a BA binding capacity of 17% and 12%, respectively, under duodenal pH conditions.
416 Cholestyramine, a BA binding resin, was considered a reference of 100% BA binding capacity in
417 this study [66]. The authors used a mixture of BA formulated with 75% tauro-conjugated and

418 25% glyco-conjugated C, CDC and DC BA. On the other hand, acid-soluble lupin protein isolate
419 and corresponding hydrolysates have been shown to bind different BA to a greater extent than
420 soy protein counterparts with no particular trend among different BA [67]. Protein
421 hydrolysates need to be considered as well since proteins are digested by GI enzymes,
422 although the extent of digestion will depend on the native protein molecular structure. No
423 differences between BA binding capacity of undigested protein isolate (91% purity) and
424 hydrolysates ($\leq 20\%$ degree of hydrolysis) were found in the latter study. This contrasts with
425 the decreased BA binding capacity of lentil protein hydrolysates as compared to the
426 undigested lentil protein concentrate [68]. The higher degree of hydrolysis in the latter study
427 ($>25\%$) may account for these differences, as well as the lower protein purity of the lentil
428 protein concentrate (ca. 80%). All these earlier studies were based on centrifugation technique
429 and the BA binding capacity was assayed from the free bile content in the supernatant.
430 Therefore, no details on molecular mechanisms can be elucidated from this approach.

431 The use of UV-Vis absorption, fluorescence and circular dichroism have shed light on the
432 interactions between zein and NaTC at submicellar BS concentration. The combination of
433 techniques showed that protein undergoes conformational changes in the secondary and
434 tertiary structure in the mixed protein-BS aggregate above certain NaTC concentration [69].
435 The experimental procedure was carried out at pH 4, at which electrostatic binding is likely to
436 occur between positively charged zein nanoparticles (NPs) (IP at pH 6.2) and negatively
437 charged NaTC. Nevertheless, hydrophobic binding may also contribute, and eventually have a
438 similar unfolding effect under neutral pH conditions, which are usually typical of the small
439 intestinal lumen. In addition, BS are often present at higher concentrations (above the CMC) in
440 the more realistic conditions within the small intestine, thus the effect observed in the latter
441 study at submicellar BS concentration may be also envisaged. The conformational changes in
442 the protein secondary structure induced by BS [70,71] may explain the enhancement of
443 protein digestion for some dietary proteins (β -lactoglobulin (BLG), bovine serum albumin,
444 myoglobin and a commercial dietary protein supplement) observed by Gass et al. in the
445 presence of a mixture of conjugated BA [72], and their antimicrobial effect [71]. Indeed, BS
446 may bind to the hydrophobic pockets of protein, inducing changes in the native structure and
447 exposing hidden cleavage sites for enzymatic action. Martos et al. also observed and increased
448 *in vitro* duodenal digestibility of hen's egg ovalbumin in the presence of BS [73]. In a more
449 realistic environment of the gut, endogenous PLs vesicles are also present, and have been
450 shown to affect duodenal proteolysis in the presence of BS in different ways. For instance,
451 ovalbumin digestion by pancreatic enzymes was increased to a larger extent [73], whereas BLG
452 was protected from the enzymatic degradation [74]. The phospholipids vesicles did not induce
453 any significant conformational change in the secondary structure of both proteins as observed
454 by circular dichroism [70,73], suggesting that specific interactions between protein and
455 phospholipids, such as short-range hydrophobic, are not involved. Nevertheless, non-specific
456 interactions between protein and PLs (e.g. long-range electrostatic) are not ruled out and BS
457 may also mix with the PLs vesicles reducing the amount of BS monomers available to interact
458 with the protein. The last mechanism only accounts for the protective effect against BS-
459 induced denaturation that can explain the observations for BLG but not for ovalbumin.
460 Therefore, other protein-specific mechanisms are likely to occur.

461 Recent work has also studied interactions between BLG and BE, by combining transmission
462 electron microscopy, dynamic light scattering (DLS), ζ -potential and conductivity
463 measurements [75]. The bile aggregates that form upon an excess in BE concentration, evolve
464 to a different supramolecular structure in the presence of low protein concentration, and
465 eventually dissociate in the presence of high protein concentration. Another study has focused
466 on the molecular interactions between β -casein micelles and two BS, NaC and NaDC (at
467 concentrations below the CMC) by using fluorescence spectroscopy techniques and DLS [76].
468 At submicellar concentrations and neutral pH, the more hydrophobic NaDC interacts to a
469 larger extent with β -casein micelles than NaC, forming larger mixed aggregates. This suggests
470 that the driving forces in the formation of these possible mixed micelles are hydrophobic and
471 hydrogen bonding.

472 *2.2.2. BS and low-molecular weight surfactants*

473 The role of BS in lipid digestion, and in particular in the formation of mixed micelles with lipids
474 to facilitate nutrient transport, is well known and it will be further discussed in Section 4.
475 Mixed micelles of BS and lipids involve endogenous lipids, secreted during GI digestion, such as
476 PLs, and dietary lipids from the lipolysis of triglycerides, such as monoglycerides and free fatty
477 acids (FFA). Many reports are available in literature regarding self-assembly of lipids derived
478 from triglycerides, but less on their mixtures with BA. One example is the recently published
479 work by Sadeghpour et al. [77], where SAXS was used to explore the molecular bulk
480 arrangement of a mixture of lipids (monoglycerides: monoolein/monolinolein) in excess of
481 water in the presence of two BS, NaC and NaDC. The biosurfactants coexisted with the lipids
482 reducing the negative curvature of lipid membrane upon increasing their concentration to the
483 point of transition from bicontinuous cubic lyotropic liquid crystals to lamellar vesicles with
484 positive curvature. This observation suggests that BS molecules tend to occupy the head-group
485 region of the lipid molecules inducing positive average interfacial curvature. The size of the
486 vesicles is reduced at higher BS concentrations, suggesting a more efficient disruption by BS.
487 This behaviour agrees well with that reported previously by Gustafsson et al., between
488 NaC/NaTC and monoolein in excess of water or 0.9 wt% NaCl [78]. BS molecules were inserted
489 perpendicular to the surface of lipid bilayers in lamellar phases. This was inferred from the
490 estimation of the average molecular interfacial area occupied by BS, cholate (C). They also
491 reported that the BS incorporation in small mixed micelles was rather different, namely they
492 were positioned flat on the micelle surface. In addition, Salentinig et al. studied by SAXS the
493 addition of a mixture of glyco- and tauro-conjugated BS, along with PC and cholesterol, to
494 mixed monoolein and FFA (oleic acid) in phosphate-buffered saline at pH 6.8 [79]. Increasing
495 the concentration of BS led to a decrease in the negative curvature of the self-assembled
496 structures, evolving from a coexistence of inverted hexagonal and bicontinuous cubic
497 structures to vesicles.

498 Vinarov et al. explored further the interactions between various types (i.e. ionic, non-ionic) of
499 surfactants and porcine BE or NaTDC to explain the different mechanisms of drug solubility and
500 the role of surfactant charge [80]. NMR and surface tension measurements indicated that ionic
501 surfactants interact to a larger extent with BS at a 1:1 ratio, forming mixed micelles of lower
502 solubilisation capacity. On the other hand, non-ionic surfactants interact to a lesser extent with
503 BS at a 1:1 ratio, forming surfactant-rich and BS-rich micelles coexisting together. The size of

504 non-ionic surfactant-rich micelles is larger than BS-rich micelles and mixed micelles of ionic
505 surfactant and BS, allowing a better solubilisation capacity. Patel et al. have investigated the
506 interactions of non-ionic Triton X-100 with the BS, NaC and NaDC, by DLS, small-angle neutron
507 scattering, turbidity and viscosity, and for varying parameters such as pH and salt
508 concentration [81]. Since pH is not static within the gastric or the intestinal compartment, one
509 could expect variations within the proximal small intestine (duodenum) immediately after
510 gastric emptying. These variations may be more pronounced for the late phase of gastric
511 processing of food, when the gastric pH has achieved low values. The incorporation of acidic,
512 gastric chyme into the duodenum will locally alter the neutral pH and this may certainly affect
513 the mixed micelle behaviour of anionic BS. At acidic pH values, BS are in a protonated form
514 (below their pK_a). This neutralises the effective negative charge and imparts more
515 hydrophobicity to BA molecules, promoting a growth of mixed micelles, as reported for Triton
516 and NaC/NaDC; the effect being greater in the case of the more hydrophobic NaDC. Namely,
517 the mixed micelles change from nearly spherical at neutral pH to prolate ellipsoidal at acidic
518 pH and the BS concentrations above the CMC.

519 Finally, interactions between polymeric surfactants from the family of block copolymers,
520 Pluronic, and BS have been also recently addressed. Pluronic F68 and F127, with a central
521 polypropylene oxide hydrophobic block and two lateral polyethylene oxide hydrophilic blocks,
522 are approved for oral intake by the US Food and Drug Administration, and research work has
523 demonstrated the ability of these emulsifiers to control the lipid digestion rate of oil-in-water
524 emulsion-based delivery systems *in vitro* [82] and *in vivo* in rats [83]. The effect was greater *in*
525 *vitro* for the emulsions stabilised with Pluronic of larger molecular weight F127 than the low-
526 molecular weight homologue F68 [84]. The attributed mechanisms, for the sustained lipolysis
527 induced by Pluronic, are not only interfacial, as it will be discussed below, but also bulk-
528 related [85]. Micro-differential scanning calorimetry was used to investigate the bulk binding
529 between these Pluronic and a model BS, NaTDC, based on how the micellization enthalpy of
530 Pluronic was decreased and the micellization temperature shifted to higher temperatures by
531 the increasing concentration of BS [86]. Hydrophobic association is the main driving force for
532 the interactions between non-ionic Pluronic and anionic BS to occur, corroborated by the
533 larger extent of interaction with the relatively more hydrophobic Pluronic F127, and additional
534 experiments performed under high ionic strength conditions, screening electrostatic
535 interactions of NaTDC [87]. Later studies performed with Pluronic P123 micelles and two BS,
536 NaDC and NaTC, have corroborated the greater intensity of the interactions with the more
537 hydrophobic NaDC [88]. In addition, the combination of DLS and spectroscopic techniques
538 used in this study shed light on how BS molecules penetrate into the core of the Pluronic
539 micelle, increasing the polarity at low BS concentrations. At higher BS concentrations, mixed
540 micelles are formed and two types of complexes are formed: the copolymer-rich or the BS-
541 rich. This behaviour at higher BS concentration resembles the one reported above for non-
542 ionic surfactants by Vinarov et al. [80], and for HPMC [58].

543 The bulk interactions of different compounds with BA contribute, in combination with
544 interfacial mechanisms, to delaying lipid digestion in emulsion-based systems, as described in
545 more detail below.

546

547 3. Interfacial Interactions of BS with emulsifiers

548 Lipids consumed in a diet are either already emulsified within a food product, by emulsifiers
549 used during the food manufacture (e.g. milk proteins, PLs, etc.), or undergo progressive
550 emulsification caused by the combined action of peristaltic motion and physiological
551 surfactants (e.g. BS and PLs secreted with bile, or monoglycerides produced from triglycerides
552 during the gastric and small intestinal lipolysis). The gastric environment can promote
553 significant structural reorganisation of emulsified lipids, involving flocculation or coalescence
554 of emulsion droplets, and the mechanism often depends on the susceptibility of the original
555 emulsifier to factors such as low pH or hydrolysis by gastric enzymes [89–91]. The gastric
556 lipolysis of ingested lipids accounts for the release of only 10-25% of FFA from the triglyceride
557 substrate [92]. Therefore, the majority of consumed lipids are hydrolysed in the small
558 intestine, which they enter after the gastric pre-treatment. Interactions between the intestinal
559 BS and emulsifiers at the oil-water interface can ultimately determine the interfacial
560 composition, which in turn can impact on the degree and rate of the small intestinal lipolysis.
561 Usually, in the first step, BS adsorb onto the oil droplet displacing existing -surface active
562 material and promoting the adsorption of pancreatic lipase and colipase. Then, the lipase
563 hydrolyses triglycerides, into FFA and monoglycerides, which remain at the interface,
564 competing with BS adsorption. Finally, BS facilitate the removal of the lipolysis products from
565 the interface by desorbing and forming mixed micelles, which solubilise those products [70].
566 There have been a number of studies dealing with the interfacial activity of BS and interactions
567 with other emulsifiers, as reviewed recently by Poulos [93]. This section will focus on the
568 adsorption of BS in order to prepare the foundations of the next section addressing specifically
569 interfacial lipolysis.

570 3.1. Interfacial properties of BS: adsorption, desorption, self-assembly

571 The investigation of interfacial activity of pure BS is a fundamental step in understanding the
572 role of BS in lipolysis. However, adsorption curves and interfacial dilatational rheology of pure
573 BS are still scarce in the literature, and only recent studies reveal differences between
574 different BS species at the interface. Scientific literature agrees on the fact that BS reduce the
575 interfacial tension rapidly but to a lesser extent than conventional surfactants [70,94]. This is
576 due to their peculiar molecular structure, containing a rigid steroid ring system instead of a
577 liquid-like linear hydrocarbon that is typical for conventional surfactants. This rigidity is
578 responsible for the directional and specific nature of the intermolecular hydrogen bonds
579 between several hydroxyl groups of the BS molecule. Accordingly, BS are very effective in
580 covering the free interfacial area but do not create a very cohesive network consistent with
581 the relatively high final interfacial tension attained [95]. Some types of BS reduce the
582 interfacial tension in a stepwise manner, suggesting the existence of conformational regimes
583 and preferential adsorption orientations. The orientation of BS upon adsorption has been
584 debated in the literature and recently addressed with computer simulations by Euston et al.
585 [94]. Results show a complex distribution of lateral tilt angles upon molecular adsorption
586 where BS can adopt different orientations from flat to upright depending on the nature of the
587 molecule and on the conditions (pH, ionic strength, concentration, etc.). In fact, Euston et al.
588 point out the possible relation of this dynamic adsorption state with the reported ability of BS
589 to displace adsorbed material, relevant to their biological role in lipid digestion.

590 Recent works also report data on the desorption profiles of pure BS, which are more
591 susceptible to the molecular differences between BS [95,96]. These data will later relate to
592 transport and solubilisation of lipolytic products. Maldonado-Valderrama et al. reported on the
593 adsorption–desorption profiles of two different BS at the air-water interface by means of
594 surface tension. The study included dilatational rheology experiments, and revealed
595 differences between NaTC and NaGDC, which originated from different complexation
596 properties, relevant to the digestion process [95]. NaTC presents faster adsorption rates and
597 various conformational regimes in contrast to NaGDC, which adsorbs slower and in a single
598 conformation. Desorption profiles reveal that NaGDC fully desorbs upon buffer rinsing
599 whereas NaTC adopts an irreversibly adsorbed form at high surface coverage. Along similar
600 lines, Parker et al. reported on the adsorption of six different BS (C, DC and CDC conjugated
601 with taurine and glycine) onto solid hydrophobic surfaces, using dual polarisation
602 interferometry and atomic force microscopy [96]. DC and CDC adsorbed more rapidly and
603 desorbed to a greater extent following buffer rinsing than C, whereas taurine/glycine did not
604 influence the behaviour.

605 This peculiar, bifacial amphiphilicity of BS plays also a significant role in the self-assembly of BS
606 and the structure and morphology of BS micelles. Recent molecular simulations have been
607 applied to identify these structures, and provided evidence of the formation of flexible and
608 disordered structures that corroborate two models based on experimental findings. Namely,
609 the formation of primary micelles, owing to hydrophobic interactions, and secondary micelles
610 through hydrogen bonding, and also the formation of disk-like structures, owing principally to
611 hydrophobic interactions between the hydrophobic faces of BS molecules oriented to the
612 centre of the micelle are all representative BS micelles [94]. The molecular interactions
613 between BS at interfaces, the conformational states, the interfacial orientations and the self-
614 assembly have an important role in understanding the molecular features that control the
615 physiology of BS, but those research areas still hold large knowledge gaps and open questions.

616 3.2 Interfacial interactions of BS: competitive adsorption and displacement

617 The interactions between emulsion components and BS, in the absence of lipase, can
618 determine the interfacial composition where lipase adsorbs, hence influencing the rate and
619 extent of lipolysis, as reviewed recently by Pilosof [93]. There are many experimental
620 evidences, which correlate the presence of a cohesive interfacial layer of emulsifier that resists
621 the action of BS with a reduction in the FFA release, measured for similar emulsion systems
622 upon lipolysis [97,98]. Accordingly, there are a number of works investigating interactions of
623 BS with surface active agents/emulsifiers in model systems that do not contain lipase, and
624 using various experimental approaches such as examining interfacial tension, surface coverage
625 of emulsion droplets (ζ -potential, droplet size) and microscopy.

626 Consider an interfacial layer of emulsifier, which forms an adsorbed network, with addition of
627 BS into the bulk solution will result in BS adsorbing into defects at the interfacial layer and
628 growing into clusters. Hence, BS adsorb at the interface compressing the network and displace
629 the adsorbed emulsifier by means of orogenic displacement. This was visualised for BLG
630 adsorbed layers at air-water and oil-water interfaces with Atomic Force Microscopy, and
631 measured with interfacial tension techniques [99]. The mechanism of orogenic displacement

632 from the air-water interface was also visualised for the milk phospholipid and
633 phospholipid-protein monolayers [100]. Sarkar et al. compared the interactions of different
634 protein-stabilised emulsions with BS, and corroborated the orogenic mechanism but obtained
635 less displacement for cationic-lactoferrin compared to that seen for anionic-BLG coated
636 droplets. In this case, electrostatic binding of BS to the lactoferrin interfacial layer induced
637 more adsorption of cationic lactoferrin. Hence, interaction of the protein-stabilized interfaces
638 with BS depends somehow on emulsifier type [101]. More recently, the impact of BS on
639 protein-coated gold NPs has also been described by the orogenic mechanism, where islands of
640 aggregated proteins appeared, surrounded by larger regions of BS [102]. He et al. provide new
641 details for the molecular level of the displacement mechanism by identifying a lag-burst
642 kinetics in the displacement of surfactants from liquid crystal-water interface, which is BS
643 specific, depending on the number and position of the hydroxyl group in the BS molecule
644 [103]. In particular, the lag time and burst rate are largely correlated with the BS
645 hydrophobicity. Hence, the more hydrophobic mono- and dihydroxy BS show shorter lag time
646 and faster burst rate as compared to the more hydrophilic trihydroxy BS. Furthermore, the
647 authors also visualized, with polarizing optical microscopy, a liquid crystal transition from
648 homeotropic to tilted interfacial orientation, which followed the displacement.

649 Interfacial tension offers an interesting platform for carrying out model studies and assessing
650 the interaction of BS with different emulsifiers. BS lower the interfacial tension, and the
651 dilatational modulus can be easily assessed *in vitro* as the BS penetrate into the adsorbed
652 layer, and hence promote displacement [99]. Using that experimental approach, Torcello-
653 Gómez et al. demonstrated that Pluronics (F68) are more resistant to displacement by BS than
654 other types of surfactants, such as lecithin [104]. Emulsion studies supported this trend as BS
655 destabilised emulsions of Pluronic F68 and lecithin above a critical BS concentration, which
656 was higher when the emulsifier was the Pluronic [105]. Similarly, Bellesi et al. demonstrated
657 that soy protein is more resistant than BLG to displacement by BS from the oil-water interface.
658 Soy protein displays a larger dilatational modulus proving the existence of favourable
659 molecular interactions, which promote partially resistance to BS displacement [106]. The
660 strong interactions occurring between BS and fibres in bulk can also reflect in alteration of the
661 interfacial activity of BS, and this has received increasing attention lately [54,57,93,106].
662 Authors agree about a strong resistance of HPMC against BS adsorption at the oil-water
663 interface due to binding of cellulose fibres to BS in bulk, which was discussed in detail in
664 Section 2. BS are sequestered by hydrophobic interactions with HPMC fibres, which can be
665 dependent on the methyl/hydroxypropyl ratio [57] and the type of BS [55,107]. Similar results
666 have been reported for BS micelles binding to pectin molecules, promoting aggregation
667 through hydrophobic interactions [59].

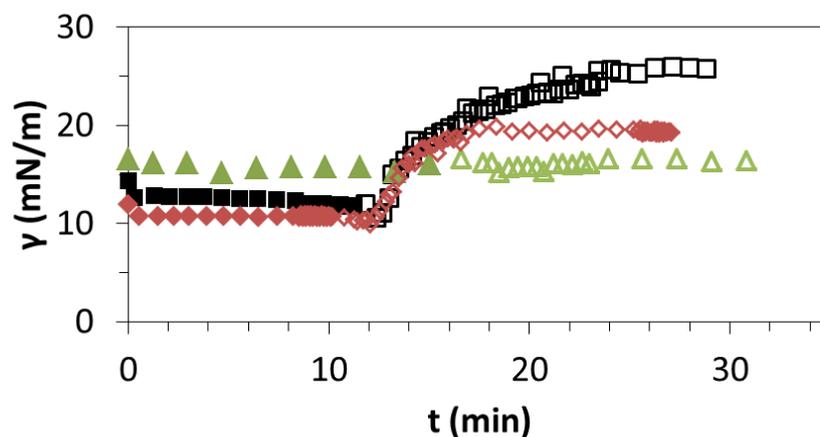
668 **4. Role of BS in small intestinal lipolysis**

669 In the GI tract, interactions of BS with digestive enzymes and products occur simultaneously
670 and take place both in bulk and at the interface of emulsions droplets. This comprises a
671 complex scenario and in order to understand all the different phenomena, lipid digestion is
672 mimicked in the literature *in vitro* by looking into isolated interfaces and into emulsified
673 systems and the encountered phenomena are analysed at different scales [93,108].
674 Unravelling the role of BS in lipid digestion at the interfacial level requires evaluating the role

675 of each of the major components of lipolysis. Accordingly, this section looks first into the
676 interaction of BS with digestive enzymes, and then explores more complex systems, composed
677 of BS, emulsifiers and lipase/colipase. Several studies on the desorption/displacement of
678 lipolysis products, leading to release of FFA and monoglycerides from emulsified systems, are
679 also summarised.

680 4.1. Interfacial interactions of BS with digestive enzymes

681 Scientific reports on BS and lipase interacting in the absence of other emulsifiers are scarce.
682 The interaction of BS with lipase at an oil-water interface readily complicates the system since
683 it includes the formation of FFA as the lipolysis proceeds. Accordingly, the interpretation of the
684 results at the interfacial level needs to establish a different interfacial response of each
685 compound [109]. Figure 5 shows a comparison of the adsorption/desorption profiles of BS,
686 lipase and BS+lipase mixtures at the olive oil-water interface in a duodenal buffer, highlighting
687 the differences encountered. BS lower the interfacial tension rapidly and practically recover
688 the value of the clean interface after desorption, i.e. 25-28 mN/m (Figure 5). The lipase
689 adsorbs irreversibly onto the olive oil-water interface as the interfacial tension remains
690 unchanged after the subphase exchange. The mixed BS+lipase system reduced the interfacial
691 tension to a larger extent than the individual components, suggesting the formation of a
692 complex interface composed of BS, lipase, monoglycerides, and FFA (the latter produced *in*
693 *situ*). Upon desorption, the value lies in between that of BS and lipase, owing to a possible
694 competition between the solubilisation of lipolysis products by BS and the presence of FFA
695 anchored at the interface (Figure 4). Analysis of the dilatational elasticity values of these
696 interfaces can show more detail on the composition and role of each compound in lipolysis
697 [109].



698

699 Figure 5: Adsorption (closed symbols) and desorption (open symbols) profiles of bile salts
700 (BS, black squares), lipase (green triangles) and BS+lipase (red diamonds) at the olive oil-water
701 interface. The desorption curves were recorded after the exchange of subphase with duodenal
702 media (37 °C, pH 7, NaCl 150 mM and CaCl₂ 10 mM). The BS is a mixture of 52.7% NaTC and
703 47.3% NaGDC to a total 1 mM and lipase 0.16 g L⁻¹. Values are means of three replicate
704 measurements, with SD < 2%. More experimental details can be found in [109].

705 Torcello-Gómez et al. have analysed the role of calcium in the interaction of BS and lipase at
706 the oil-water interface. In the absence of BS, calcium complexes with FFA at the oil-water
707 interface forming a relatively strong viscoelastic network of calcium soaps over time. The
708 presence of BS disrupts the network of calcium soaps promoting the formation of calcium soap
709 islands at the interface. As a result, Torcello-Gómez et al. proposed that the faster rate of
710 lipolysis in the presence of calcium is due to FFA complexation with calcium and desorption
711 rather than reduced electrostatic repulsion between lipase and BS. [110]. An interesting work
712 by Parker et al. addresses the adsorption/desorption profiles of mixed BS/colipase onto solid
713 hydrophobic surfaces [96]. When comparing different BS, simultaneous adsorption of colipase
714 shows a greater cooperativity with NaTC and less desorption upon buffer rinsing than with
715 NaGDC. The authors hypothesize that less desorption promotes the adsorption and residence
716 time of the colipase-lipase complex while more desorption favours the displacement and
717 solubilisation of lipolytic products from the interfacial region into the micellar phase [96]. This
718 is in agreement with the faster and more extensive desorption profile of individual GDC as
719 compared to C BS discussed above. This supports the idea that cooperative adsorption occurs
720 between certain BS and colipase to facilitate the adsorption and activity of pancreatic lipase.

721 4.2. Interfacial approaches to studying lipolysis: emulsion studies

722 We have already explained that interfacial lipolysis involves adsorption, conformational
723 changes, enzymatic processes and desorption of the lipolysis products. In this complex
724 scenario, the presence of emulsifiers can alter the process of lipolysis by either promoting or
725 decreasing the activity of lipases, as emulsifiers compete with lipases and BS for the interface
726 and interact with the enzymes and BS in the bulk solution. On the one hand, resisting
727 displacement by BS might alter lipolysis by preventing the access of lipase through competitive
728 adsorption [93]. The presence of lipolytic products at the interface also prevents further access
729 of lipase [111]. On the other hand, bulk interactions that entrap lipase promote hydrophobic
730 interactions, which reduce interfacial activity of lipase or directly inhibit the lipolytic site
731 through specific interactions [112]. Ionic surfactants can also denature lipase, resulting in the
732 reduced enzymatic activity, while non-ionic surfactants micelles can interact with lipase,
733 hindering adsorption of the enzyme. ref [113].

734 The specific role of interfacial coverage and lateral packing on lipolysis has been assessed by
735 means of monitoring changes in the interfacial tension of isolated interfaces [108], and
736 complemented with emulsion microstructure studies [93,97,98]. Polar lipids with larger head-
737 groups (galactolipids) provide a decreased lipolysis rate in emulsions accompanied by a
738 higher resistance to displacement by BS, which confirms the influence of interfacial molecular
739 packing of the lipids at the oil-water interface on lipolysis [114]. Similarly, the length of
740 hydrophilic/hydrophobic chains in Pluronics (F68 and F127) affects the rate of lipolysis [115]
741 Specifically, *in vitro* digestion occurring at oil water interfaces stabilised by Pluronic suggests
742 that in the presence of a steric barrier, the susceptibility to lipolysis depends on the interfacial
743 coverage as controlled by the interfacial tension, apart from the steric bulky layer remaining in
744 the aqueous phase. Pluronics can inhibit lipolysis owing to steric hindrance at the oil-water
745 interface caused by their hydrophilic tails, which hinders the adsorption of BS+lipase at the
746 droplet interface. [85]. Speranza et al. generalise this trend by stating that the bioaccessibility
747 of FFA increases with the hydrophobicity of the surfactant and decreases with the length of

748 aliphatic chain [116]. Lipolysis of multi-layered protein/pectin films also showed that the
749 combined action of BS and lipase is ruled by BS with lipase co-adsorbing and generating
750 lipolytic products [117]. This was further confirmed by Bellesi et al. who correlated the
751 susceptibility to BS displacement of different emulsifiers to FFA release upon lipolysis [118].
752 These findings have been recently corroborated by measuring the dynamics of the emulsion
753 interface during *in vitro* digestion by Fluorescence Resonant Energy Transfer [119]. This
754 technique allows measuring *in situ* structural changes of emulsion droplets stabilised by
755 lecithin upon simulated digestion providing changes in the intermolecular spacing between the
756 two different fluorescent probes labelled emulsifier. Results confirm that BS and lipase disrupt
757 the adsorbed layer. Furthermore, addition of a second adsorbed layer of chitosan or ϵ -
758 Polylysine reduces considerably the disruption of the interface and the release of FFA. The role
759 of BS in lipolysis with regard to the interfacial activity of BS is also highlighted in the studies
760 concerning the available interfacial area, where experimental results confirm the
761 intensification of lipolysis as the available interfacial area increases, that is the oil droplet size
762 is reduced [106,120–122].

763 Great attention has been directed lately to the use of NPs as emulsifiers and their impact on
764 lipolysis [98]. Most studies agree that NPs delay/impede lipid digestion owing to a strong an
765 irreversible adsorption which inhibits displacement by BS. Tzoumaki et al. report a lower
766 release of FFA from emulsions stabilised by chitin NPs compared to proteins [123]. Sarkar et
767 al., compared the barrier promoted by microgel particles and heat-treated fused microgel
768 particles, and found that the fused network hindered adsorption of BS, and hence delayed lipid
769 digestion [124]. The specific role of BS on lipolysis has been also addressed by Marefati et al.,
770 which compared physical stability of quinoa starch granules Pickering emulsions during *in vitro*
771 lipolysis, showing larger changes in samples in the presence of BS and less destabilization of
772 heated emulsions upon lipolysis [125]. Cellulose nano crystals (CNCs) are a novel ingredient,
773 which has been shown to impact on the microstructural stability of emulsions under *in vitro*
774 lipolysis. Sarkar et al. demonstrated that adding high concentrations of CNCs decreases the
775 rate and degree of lipolysis of protein-coated lipids. This is principally due to the binding of
776 CNCs to BS, which prevents BS adsorption to the oil-water interface, and hence the
777 displacement and later solubilisation [126]. However, as in the case of emulsions, the available
778 interfacial area could also be determinant here, as the presence of CNCs induces flocculation
779 of protein coated droplets reducing the overall area available to adsorption[126]. The pivotal
780 role played by the interfacial area in Pickering emulsions has been also noted by Shah et al.
781 [127]. A recent review deals specifically with Pickering emulsions, and analyses their stability to
782 various biophysical environments [98].

783 The type of oil can also alter the lipolysis profile of emulsified systems. In this regard, Marze et
784 al. established the importance of solubility of individual FA in the digestibility of different oils
785 [128]. Ye et al. corroborated the importance of oil composition by analysing duodenal
786 digestion of three representative oils emulsified with whey protein isolate (WPI), and ascribing
787 the different lipolysis degree to different FA compositions and triglyceride profiles. According
788 to the researchers, BS played a key role in displacing WPI and acting as a lipase activator and a
789 lipase-assisted adjuvant [129]. The interactions of BS and adsorbed protein depended on BS
790 structure, and hence on the hydrophobic forces acting between BS and protein and between
791 BS and lipid, and resulting in different abilities of BS to displace WPI from different oil phases.

792 The influence of the physical state of lipid was also assessed, by comparing lipolysis and β -
793 carotene transfer from canola oil emulsions and Solid Lipid NPs [130]. Nik et al. found a more
794 substantial degree of lipolysis and β -carotene transfer from canola oil emulsions, which
795 correlated with a greater surfactant displacement by BS from the liquid lipid surface [130].
796 Thus, as recently stated by Corsten et al., hindering adsorption of BS is one of the main
797 approaches to control lipolysis [131]. A recent work by Wilde et al. also highlights the
798 importance of food structure, apart from composition, in digestibility of oat-based food
799 matrices [132]. The presence of phytosterols affected the stability and physical-chemical
800 properties of mixed micelles promoting the disappearance of the smaller micelles following
801 digestion. Smaller micelles are likely comprised only by BS, which can exchange rapidly with
802 those in solution and adsorbed at the interface, removing lipolytic products. Their results
803 suggest that BS bound more effectively into larger complex structures, which may bind
804 lipophilic compounds hence reducing lipid hydrolysis.

805 The self-assembly structures formed during lipid digestion have only recently been studied by
806 scattering techniques, which showed that the type of self-assembly organisation depended
807 strongly on BS [79,133,134]. Salentinig et al. studied *in situ*, with time-resolved SAXS, the *in*
808 *vitro* digestion of a triolein emulsion by pancreatic enzymes in the presence of different
809 concentrations of BS mixture. In general, they observed that the original emulsion, with no
810 internal structure, transitioned during the lipolysis to an emulsified microemulsion, inverse
811 micellar cubic (Fd3m) liquid crystalline phase, inverted hexagonal liquid crystalline phase, and
812 finally lamellar vesicles at pH 7. The presence of higher BS concentrations accelerated the
813 formation of vesicles containing the lipolysis products. The use of depolarised DLS in
814 comparison to normal DLS and cryo-Transmission Electron Microscopy confirmed the presence
815 of anisotropic particles based on bilayer structures, such as bicelles (crossover between bilayer
816 and micelle). All these studies were focused on long-chain lipids, with more than 12 carbons in
817 the aliphatic chain. The formation of intermediate lyotropic liquid crystals cannot be usually
818 observed in medium-chain and short-chain lipid systems in the presence of simulated
819 intestinal juice containing BS, phosphatidylcholine and cholesterol, since there is a direct
820 transition to vesicles and micelles [79,135]. This is likely related to their higher solubility in
821 water, and associated with lower solubilisation capacity as compared to long-chain lipids. In
822 addition, these interactions will certainly depend on the pH, due to different protonation
823 states of lipids, and on the ionic strength, due to changes in electrostatic interactions. Namely,
824 higher pH and ionic strength accelerate structure formation and transitions. To our knowledge,
825 liquid crystalline phases, such as cubic or hexagonal, have not been reported in the limited *ex*
826 *vivo* studies available in the literature. The reason for this may be that these phases are
827 formed transiently at the surface of a triglyceride droplet being digested, and require surface-
828 specific techniques for their identification with increased time resolution [136]. Moreover, the
829 specific role of BS in the formation of these structures has not been addressed so far
830 systematically and literature works highlight some controversy. For example, Salentinig et al.
831 suggest vesicles as the dominating final structures of lipolysis [79], whereas Marze et al.
832 suggest that the major assembly formed is a mixed micelle with a lower proportion of vesicles
833 [133]. Interestingly, these works differ fundamentally in the concentration of BS used, which
834 might explain the discrepancy. Vesicles only appear at high concentrations of BS, whereas
835 inverse micelles or liquid crystalline phases appear at low concentration of BS or in their

836 absence. At the physiological concentrations, micelles and vesicles coexist, with the micelles
837 being the major type of assembly. According to Marze et al., the emulsifier used in emulsion
838 preparation seems to be less determinant than the type of oil in the self-assembly. However,
839 the influence of complex emulsifiers, NPs or fibres has not been addressed specifically with
840 this type of experiments yet.

841

842 **5. Role of bile salts in small intestinal transport**

843 5.1. Mixed micelles

844 As part of the digestion process, a range of physiological surfactants are secreted into the gut
845 lumen. These include PLs such as PC, which is present in the gastric compartment [137] and is
846 a significant component of the bile secreted into the proximal small intestine [138]. As
847 discussed in previous sections, bile also contains a range of charged BS [139]. In addition to
848 facilitating the interfacial lipolysis of triglycerides, BS also have the ability to solubilise the
849 products of lipolysis (FFA and monoglycerides) a process essential for effective absorption of
850 dietary lipids by the intestinal epithelium. The process of formation of mixed micelles often
851 involves incorporation of other lipids, such as lipid-soluble vitamins or cholesterol. The process
852 of solubilisation of cholesterol, is, in fact, the major mechanism of cholesterol removal from
853 the gut [26].

854 The relatively high surface activity of the lipolysis products means that their accumulation at
855 the oil-water interface during digestion can render the triglyceride substrate inaccessible to
856 pancreatic lipase. *In vitro* studies [140] showed that monoglycerides can be very efficient in
857 expelling the enzyme from the interface, although the FFA, which also formed during
858 digestion, did not have enough interfacial activity to exert a similar effect. Under physiological
859 conditions, the inhibition of digestion caused by interfacial accumulation of lipolysis products
860 is prevented by BS and PLs, which remove the products from the interface by solubilising them
861 into mixed micelles. Triglyceride oil droplets coexist with mixed micelles until the lipid
862 substrate is exhausted. Early light-scattering studies [26] showed that the size and structure of
863 micelles depend on PL (lecithin) to BS molar ratio (PL/BS). At $PL/BS < 0.6$, small mixed micelles
864 (hydrodynamic radius up to 3.5 nm) coexisted with simple BS micelles. Increasing the ratio
865 over 0.6 resulted in formation of mixed micelles only, with hydrodynamic radii up to 30 nm
866 and a disk-like shape, in which BS and PLs formed a mixed bilayer. This bilayer disk model [26]
867 has since superseded previous models [141].

868 As summarised in previous sections, modulating the adsorption of BS is one of the most
869 promising approaches to control the rate of lipolysis. However, apart from the physiological
870 role of adsorbed BS in displacing other material from the interface, a major importance of
871 unadsorbed BS has also been pointed out [111]. Sarkar et al. suggested that, compared to
872 adsorbed BS, the presence of unadsorbed BS had more impact on the lipolysis they studied.
873 Those BS present in mixed micelles might facilitate lipid digestion by an efficient removal of
874 the lipolysis products from the interface. The researches stated that structural information
875 about the mixed micelles formed in an aqueous phase would be needed in the future in order
876 to confirm that hypothesis [111].

877 The formation of mixed micelles of BS and surfactants can also affect the degree and rate of
878 lipolysis. Vinarov et al. studied the effect of surfactant concentration and charge on the
879 lipolysis of oil-in-water emulsions in the presence of porcine BE [142]. The researchers found
880 optimum solubilisation capacities of mixed micelles formed by bile and either non-ionic
881 (Tween 20 and Tween 80), cationic (cetyltrimethylammonium bromide) or anionic (α -
882 olefinsulfonate, and sodium lauryl ether sulfate) emulsifiers at various surfactant-to-bile molar
883 ratios. The maximum ratio at which the complete lipolysis was observed depended on the
884 nature of surfactant, and increased in the following order: non-ionic<cationic<anionic. This
885 experimental parameter was inversely related to the solubilisation capacity of the mixed
886 micelles to incorporate the lipolysis products. The lower the surfactant-to-bile molar ratio was
887 required to achieve complete lipolysis, the lower the amount of surfactant was needed at a
888 constant bile concentration. The non-ionic surfactants had the highest solubilisation capacity,
889 followed by cationic and then anionic. The different behaviours depended on the charge of the
890 surfactants, however additional research is needed to explain detailed molecular mechanisms
891 of interactions. The mechanisms of drug solubilisation reported for similar surfactants in the
892 presence of BS, previously discussed in Section 2, may help explain the different solubilisation
893 capacity of lipolysis products [80].

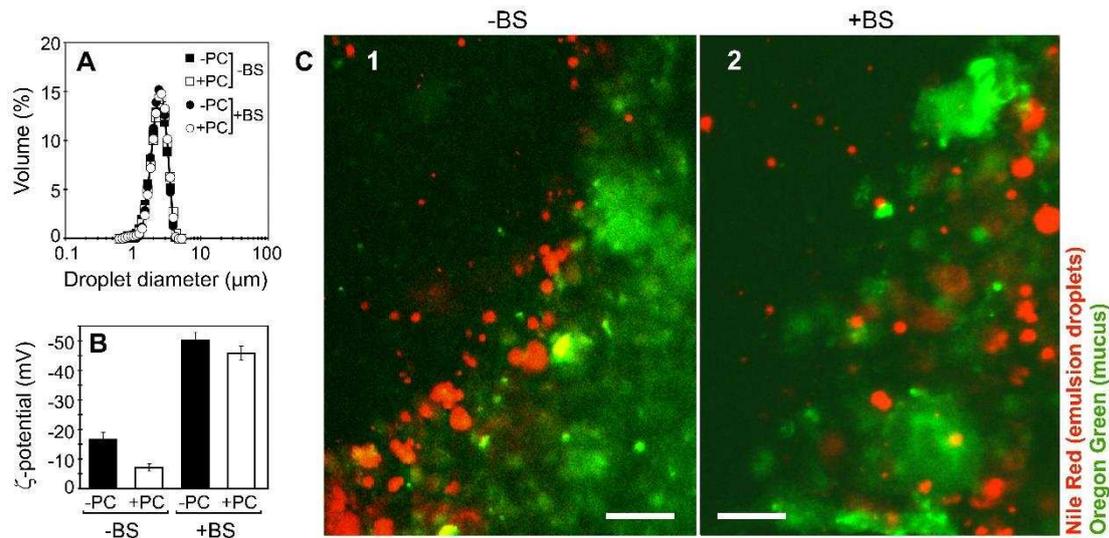
894 5.2. Transport through intestinal mucus

895 The formation of mixed micelles is usually described as the process completing lipid digestion,
896 which is then ultimately followed by absorption across enterocytes [143,144]. However, the
897 transport of solubilised FFA and monoglycerides, and any other lipid molecules incorporated
898 into the mixed micelles (e.g. cholesterol, lipid-soluble vitamins, hydrophobic pharmaceutical
899 substances, etc.), towards the epithelium requires the micelles to diffuse through the
900 protective barrier of intestinal mucus. The small intestinal mucus is a complex colloidal system
901 that coats the entire intestinal epithelium, thus protecting it from direct exposure to luminal
902 contents [145]. It acts as a natural sieve, allowing passage of nutrients, including the
903 solubilised products of lipolysis, so that they can reach enterocytes and get absorbed. At the
904 same time, the mucus prevents the epithelium from direct contact with pathogenic
905 microorganisms, etc. [146,147].

906 Intestinal mucus is a physiological, gelled secretion that is highly hydrated and contains a range
907 of organic compounds. Amongst them, there are two major, gel-forming biopolymers, MUC2
908 mucin glycoprotein [148] and extracellular DNA [149], which form a coherent network. The
909 mucin is actively secreted by goblet cells located in the intestinal epithelium, whereas the DNA
910 can originate from apoptotic epithelial cells [150], and its substantial amounts in the small
911 intestinal mucus have been proposed to result from the fast turnover of the intestinal
912 epithelium [149]. The mucus gel is produced through entanglement of the biopolymers and
913 forms an adherent, unstirred layer. The gel strength and viscoelasticity are determined by the
914 level of entanglement, the size of pores and “channels” in the network, and ultimately by the
915 concentration of mucin and DNA. Thickness of the mucus layer can thin in the presence of
916 shear, such as that induced by peristalsis. This creates a slippery plane, which has the ability to
917 lubricate the peristaltic motion. The thinned mucus can maintain the coherence of the
918 unstirred layer and its penetration is only possible by diffusion [146].

919 Particles in the GI tract lumen, including emulsified fat, cellular material and bacteria, are
920 exposed to physiological surfactants that can adsorb to the particle surface and change the
921 surface properties, and thus mucus interactions. This is one reason why the selective nature of
922 the intestinal mucus barrier is not well understood. Whilst there is a body of published work
923 looking at the micro-rheological properties of a range of different types of mucus [151–153],
924 evaluation of the effect of physiological surfactants on the ability of colloidal particles to
925 penetrate the mucus has not been extensively studied. We showed previously that interfacial
926 adsorption of BS to model particles, fluorescent latex beads (0.5 – 2 μm in diameter), allowed
927 them to penetrate into small intestinal mucus [154]. The BS imparted high negative surface
928 charge to the particles, but not the mucus. Adsorption of BS to the surface of particles was
929 assumed to significantly change their electrostatic interactions with the mucus network, which
930 was also negatively charged, and hence prevent mucoadhesion of particles. In contrast,
931 mucoadhesion of model bacteria (non-flagellated *Escherichia coli*) was observed despite the
932 presence of BS in the system. This was linked to the fact that BS did not modify the weak
933 negative charge of the bacterial cells, which might otherwise provide sufficient electrostatic
934 repulsion between the bacteria and the mucus, and allow penetration. Importantly, similar
935 studies were also conducted for model food emulsions that had been put through the *in vitro*
936 gastrointestinal proteolysis in the presence or absence of PC and BS [155]. The emulsions were
937 initially stabilised with a food-grade protein sodium caseinate, crosslinked by
938 transglutaminase. The protein was eventually displaced from the emulsion droplets by the
939 biosurfactants throughout the course of digestion. The BS adsorption to emulsion droplets
940 enhanced their negative surface charge under the simulated small intestinal conditions. This
941 was not expected for zwitterionic PC, and their co-adsorption did not significantly change the
942 electrostatic properties of droplets. As for the latex particles [154], the high negative charge
943 provided by adsorbed BS allowed the post-digestion emulsion droplets to penetrate into the
944 mucus (Figure 6). The results of those two studies imply that the ability to penetrate intestinal
945 mucus is not only limited to small molecules or nanometre-scale particles, such as mixed
946 micelles, but may also be expected for much larger particles under physiological conditions of
947 the gut. This, in turn, suggests that the lipolysis of fat droplets may not only take place in the
948 lumen but also much closer to the intestinal epithelium, after the droplets have penetrated
949 into the mucus.

950

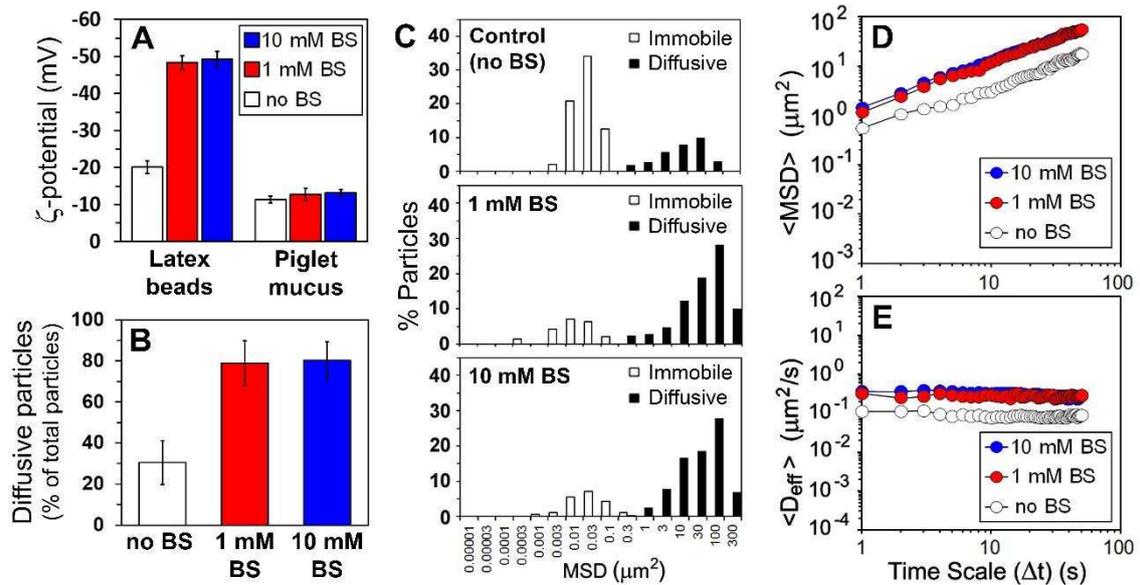


951

952 **Figure 6.** Post-digestion emulsion droplets and their interactions with porcine small intestinal mucus. (A)
 953 Size distributions and (B) ζ -potential values of the emulsion droplets used for experiments on diffusion
 954 in the intestinal mucus. The droplets were obtained after *in vitro* gastro-duodenal proteolysis (+/-
 955 phosphatidylcholine (PC) in the gastric phase of digestion; +/- bile salts (BS) in the duodenal phase of
 956 digestion) of transglutaminase-crosslinked, sodium caseinate-stabilised emulsions (Na-Cas 1mg/mL; 18%
 957 triglyceride oil, w/w). Subsequently, fine emulsion droplets (A) were separated from partially
 958 destabilised, post-digestion emulsions and fluorescently stained before diffusion experiments. (C)
 959 Confocal micrographs of the post-digestion emulsion droplets interacting with the *ex vivo* intestinal
 960 mucus in the (1) absence or (2) presence of BS adsorbed onto the surface of the droplets (the droplets
 961 were obtained from emulsions digested in the absence of PC and +/- BS). Top-left, dark areas in both
 962 images show diluted post-digestion emulsions where the diffusion of oil droplets towards the mucus
 963 layer took place from (15-min incubation at 37 °C). Image 1 shows droplets (red) accumulated at the
 964 surface of the mucus layer (green) and unable to penetrate into the mucus in the absence of BS. Image 2
 965 shows emulsion droplets penetrating into the mucus layer in the presence of BS. The scale bars
 966 correspond to 15 μ m. Adapted with permission from Macierzanka A. et al., *Langmuir* 2012;28:17349–62
 967 [155]. Copyright 2012 American Chemical Society.

968 The above examples of *in vitro* studies suggest that the presence of negatively-charged BS may
 969 play a vital role in determining passive transport of colloidal particles, including partially
 970 digested fat droplets, through the intestinal mucus under physiological conditions. However,
 971 the concentration of BS can vary significantly in the small intestine. In the postprandial adult
 972 human intestine, it can range from 5.2 to 14.5 mM [156,157] as the bile is secreted in response
 973 to meal consumption, whereas in the fasted state of digestion, the BS concentration is much
 974 lower, with a typical range of 0.8–5.5 mM [6,156,158]. Low concentrations of BS in the small
 975 intestinal lumen can also be found in human infants due to immaturity of the BS metabolism
 976 and turnover [159–161]. In new-born infants, decreased BS synthesis, degree of conjugation,
 977 and excretion rate have been reported [162–164], the latter caused by a lower gallbladder
 978 ability to concentrate bile, compared to adult humans. This is further accompanied by an
 979 impaired BS intestinal uptake [160]. The total BS concentration in the postprandial small
 980 intestine of full-term infants (3-15 days after birth) was found to range from 0.4 to 1.5 mM
 981 [165].

982



983

984 **Figure 7.** Effect of bile salt (BS) concentration on diffusion of 500-nm latex beads in the piglet small
 985 intestinal mucus gel, collected from jejunal mucosa. Impact of pre-incubation with different BS
 986 concentrations on (A) the electrostatic properties of particles and (B-E) the transport rates and
 987 distributions of latex beads in the mucus. (A) ζ -potential of the latex beads and the mucus dispersed in
 988 PBS buffer with/without BS (means \pm SD, $n = 5$). (B) Proportions of diffusive beads in the collected,
 989 undiluted mucus gel (means \pm SD, $n = 5$). (C) Distributions of mean-square displacement (MSD) values
 990 obtained for individual beads in the mucus gel at the time scale $\Delta t = 50$ s. (D) Ensemble mean-square
 991 displacements ($\langle \text{MSD} \rangle$) for diffusive fractions of beads in the mucus gel as a function of time scale (Δt),
 992 and (E) ensemble diffusivities ($\langle D_{\text{eff}} \rangle$) for diffusive fractions of beads in the mucus gel as a function of Δt
 993 ($n = 5$, with 100–150 beads per experiment). All measurements were done at 37 ± 0.1 °C. The data
 994 obtained for control samples (i.e. with no BS added) have been adapted from Reference [149].

995 We have investigated the extent to which changing BS concentration within the physiological
 996 range can impact on the permeability of the intestinal mucus to sub-micron sized particles,
 997 such as partially digested food particles or colloidal delivery systems, and their transport in the
 998 mucus. Fluorescent, carboxyl-modified, 500-nm latex beads were used as model particles in
 999 the experiment simulating the passage of particulate material from the lumen of the small
 1000 intestine into the mucus layer overlaying the mucosal epithelium. The results are presented in
 1001 Figure 7. The probe particles had been incubated with either 1 mM or 10 mM BS before they
 1002 were subjected to multiple-particle tracking experiments in the mucus in order to mimic
 1003 contrasting physiological concentrations of BS in the human small intestinal lumen [92,166]. A
 1004 mixture of two BS, comprising equimolar quantities of NaTC and NaGDC, was used. Control
 1005 samples, with no BS added, were also analysed. We used mucus collected from freshly excised,
 1006 proximal small intestines of 2-week old piglets as a substitute for human mucus. Incubation of
 1007 latex beads with BS caused a significant increase in their negative charge, from ca. -20 mV in
 1008 the absence of BS, to ca. -50 mV with BS (Figure 7A). However, there was almost no difference
 1009 in the ζ -potential value upon increasing the BS concentration from 1 mM to 10 mM. In
 1010 contrast, the presence of BS had almost no effect on the net charge of the piglet mucus, with
 1011 the ζ -potential values ranging from -11 mV to -13 mV before and after incubation with BS
 1012 (Figure 7A). This suggests that there was very limited interaction of the biosurfactant with the
 1013 mucus. In the next step, dispersions of latex beads were brought in contact with the mucus

1014 samples placed in an optical cell, and the particles allowed sufficient time to diffuse into the
1015 mucus. As revealed by multiple-particle tracking confocal microscopy, the pre-incubation with
1016 BS, and the resulting increase in the negative surface charge of the latex beads, had a
1017 profound effect on their diffusion in mucus. The number of particles able to diffuse in the
1018 piglet mucus increased to ca. 80% in the presence of 1 mM BS, from ca. 30% recorded for the
1019 control conditions (Figure 7B), where the particles had significantly reduced ζ -potential in the
1020 absence of BS. Thus for the latter, the majority of particles (ca. 70%) were found immobilised
1021 by the mucus structure over the time-scale of the experiment. However, increasing the BS
1022 concentration, from 1 mM to 10 mM, had no effect on the overall number of diffusive
1023 particles. As shown in Figure 7C, the distances travelled by individual diffusive beads varied
1024 largely after the 50-s time-scale examined, with mean-square displacement (MSD) values
1025 ranging from $0.3 \mu\text{m}^2$ to $300 \mu\text{m}^2$. This pattern was consistent across the different
1026 experimental conditions tested. The ensemble MSD ($\langle\text{MSD}\rangle$) for individual populations of
1027 diffusive particles in the function of time (Figure 7D) showed that the concentration of BS had
1028 almost no effect on the average distance travelled by the beads in mucus. The MSD data
1029 obtained from trajectories of individual beads have been converted to diffusion coefficients
1030 (effective diffusivities, D_{eff}) and ensemble diffusion coefficients ($\langle D_{\text{eff}} \rangle$) calculated for families
1031 of diffusive particles, using the procedure described before [149]. The diffusive particles
1032 showed free diffusion, expressed by constant $\langle D_{\text{eff}} \rangle$ values in time (Figure 7E). The diffusion
1033 rate did not seem to be affected by the BS concentration ($\langle D_{\text{eff}} \rangle = 0.27 \pm 0.03 \mu\text{m}^2 \text{s}^{-1}$ and 0.29
1034 $\pm 0.04 \mu\text{m}^2 \text{s}^{-1}$ for 1 mM and 10 mM BS, respectively). However, the pre-incubation of the latex
1035 beads with BS gave rise not only to higher numbers of diffusive particles as compared to the
1036 control conditions (Figure 7B) but also increased the rate of diffusion in mucus by a factor of
1037 ca. 3.5. The $\langle D_{\text{eff}} \rangle$ value recorded for the latex beads diffusing in the absence of BS was $0.08 \pm$
1038 $0.01 \mu\text{m}^2 \text{s}^{-1}$ (Figure 7E). Thus, although the presence of BS had a significant impact on the net
1039 surface charge of particles and enhanced their diffusion in the mucus, increasing the
1040 concentration from 1 mM to 10 mM had no effect. This might have been due to the molecular
1041 structure of BS and the flat conformation they adopt when adsorbed at interfaces, which
1042 allows a relatively large surface area to be occupied by a single BS molecule [70]. The similar
1043 electrostatic properties of the beads incubated at the two different BS concentrations suggest
1044 that the total surface area of the beads was fully saturated with BS already at the 1 mM
1045 concentration. If the mean molecular area of adsorbed BS at 37 °C is ca. 100 \AA^2 per molecule
1046 [167], then at 1 mM BS there was enough BS molecules available to cover an area three orders
1047 of magnitude larger than the total surface area of the latex beads used in the experimental
1048 set-up. Thus, increasing the concentration to 10 mM might not have significantly changed the
1049 BS surface density. Since the net surface charge of the mucus was almost unaffected by the
1050 variations in BS concentration, this can explain similar diffusion characteristics of latex beads
1051 for both BS concentrations tested.

1052

1053 **6. Conclusions**

1054 Fundamental understanding of the mechanisms underlying the role of BS in digestion and the
1055 transport of lipids will enable the development of strategies for improving food and
1056 pharmaceutical formulations with targeted digestibility. BS are key ingredients in the processes

1057 of assembling molecules into functional micro- and nanostructures, which transport lipophilic
1058 nutrients in the gut. However, there is a need for understanding the role of BS in biophysical
1059 transformations of lipids in the digestion process. The bulk and interfacial interactions of BS
1060 with lipids and the self-assembly are directly related to lipolysis, and the resulting
1061 solubilisation and transport. The interaction of BS with dietary fibre and food emulsifiers can
1062 influence those processes in several ways. Namely, physical entrapment of BS can hinder the
1063 BS adsorption and the hydrolysis of lipids. Also, electrostatic and/or hydrophobic interactions
1064 may occur depending on the nature of the emulsifier, altering the lipolysis rate. Additionally,
1065 there is a need to further explore the interactions of adsorbed BS with other physiological
1066 surfactants such as PC, FFA, cholesterol and unadsorbed BS. And finally, there is growing
1067 evidence concerning the crucial role of BS in the transport of lipids through intestinal mucus.
1068 However, in this area too, there is a need for more research that would address, for example, a
1069 possible role of BS in preventing mucoadhesion of other substances. Thus, unravelling all the
1070 roles that BS may play in the digestion and transport of nutrients/bioactives requires
1071 collaborative research through multidisciplinary approaches, in which fundamental colloidal
1072 studies are carried out to accurately reflect complex *in vivo* scenarios.

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1580 **List of Tables**

1581 **Table 1:** Table 1 Changing hydrophobicity of various primary, conjugated primary and
1582 secondary bile acids.

1583

1584 **List of Figures with reduced captions**

1585 **Figure 1.** Schematic representation of the roles of bile salts (BS) in the intestinal digestion and
1586 transport of lipids.

1587 **Figure 2.** (A) Exemplary structures of primary, conjugated primary, and secondary bile acids
1588 (BA) (B) Schematic representation of the path of formation of various primary, conjugated
1589 primary, secondary and conjugated secondary BA, including the recycling of the bile salts (BS)
1590 via enterohepatic circulation.

1591 **Figure 3.** Comparison of the critical micelle concentration (CMC) values of various groups of
1592 bile acids (BA).

1593 **Figure 4.** General trend of (from top to bottom) a binding isotherm, surface tension and
1594 enthalpy of binding in polymer-surfactant systems, as a function of surfactant concentration.
1595 Reprinted from Reference [42]

1596 **Figure 5:** Adsorption (closed symbols) and desorption (open symbols) profiles of bile salts (BS,
1597 black squares), lipase (green triangles) and BS+lipase (red diamonds) at the olive oil-water
1598 interface.

1599 **Figure 6.** Post-digestion emulsion droplets and their interactions with porcine small intestinal
1600 mucus. (A) Size distributions and (B) ζ -potential values of the emulsion droplets used for
1601 experiments on diffusion in the intestinal mucus.

1602 **Figure 7.** Effect of bile salt (BS) concentration on diffusion of 500-nm latex beads in the piglet
1603 small intestinal mucus gel, collected from jejunal mucosa. Impact of pre-incubation with
1604 different BS concentrations on (A) the electrostatic properties of particles and (B-E) the
1605 transport rates and distributions of latex beads in the mucus.