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The bile salt/phospholipid ratio determines the extent of *in vitro* intestinal lipolysis of triglycerides: Interfacial and emulsion studies

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Abbreviations:

βLg, β-lactoglobulin; BS, bile salt; DG, diglyceride; DLS, dynamic light scattering; E, dilatational modulus; E', storage modulus; E'', loss modulus; FFA, free fatty acid; GI(T), gastrointestinal (tract); IFT, interfacial tension; L, lipase; MG, monoglyceride; NaGDC, sodium glycodeoxycholate; NaTC, sodium taurocholate; PC, phosphatidylcholine; PL, phospholipid; PLA_2, phospholipase A2; SIF, simulated intestinal fluid; TG, triglyceride; WPI, whey protein isolate.

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Abstract

This study focused on the protein-stabilised triglyceride (TG)/water interfaces and oil-in-water emulsions, and explored the influence of varying molar ratios of bile salts (BSs) and phospholipids (PLs) on the intestinal lipolysis of TGs. The presence of these two major groups of biosurfactants delivered with human bile to the physiological environment of intestinal digestion was replicated in our experiments by using mixtures of individual BSs and PLs under *in vitro* small intestinal lipolysis conditions. Conducted initially, retrospective analysis of available scientific literature revealed that an average molar ratio of 9:4 for BSs to PLs (BS/PL) can be considered physiological in the postprandial adult human small intestine. Our experimental data showed that combining BSs and PLs synergistically enhanced interfacial activity, substantially reducing oil-water interfacial tension (IFT) during interfacial lipolysis experiments with pancreatic lipase, especially at the BS/PL–9:4 ratio. Other BS/PL molar proportions (BS/PL–6.5:6.5 and BS/PL–4:9) and an equimolar amount of BSs (BS–13) followed in IFT reduction efficiency, while using PLs alone as biosurfactants was the least efficient. In the following emulsion lipolysis experiments, BS/PL–9:4 outperformed other BS/PL mixtures in terms of enhancing the TG digestion extent. The degree of TG conversion and the desorption efficiency of interfacial material post-lipolysis correlated directly with the BS/PL ratio, decreasing as the PL proportion increased. In conclusion, this study highlights the crucial role of biliary PLs, alongside BSs, in replicating the physiological function of bile in intestinal lipolysis of emulsified TGs. Our results showed different contributions of PLs and BSs to lipolysis, strongly suggesting that any future *in vitro* studies aiming to simulate the human digestion conditions should take into account the impact of biliary PLs – not just BSs – to accurately mimic the physiological role of bile in intestinal lipolysis. This is particularly crucial given the fact that existing *in vitro* digestion protocols typically focus solely on applying specific concentrations and/or compositions of BSs to simulate the action of human bile during intestinal digestion, while overlooking the presence and concentration of biliary PLs under physiological gut conditions.

Keywords

Bile salts, dilatational rheology, interfacial tension, *in vitro* lipolysis, phospholipids, triglyceride digestion

1. Introduction

In the field of food colloids and human nutrition, understanding the interactions of various components at the molecular level is crucial for enhancing food functionality. This is particularly true for lipid digestion, a process deeply linked with the colloidal nature of food systems. Lipids are typically delivered in the diet in the form of emulsions, often stabilised by proteins such as whey proteins. Upon ingestion, they undergo complex transformations mediated by various interfacial interactions and biochemical reactions in the gastrointestinal tract (GIT). β-lactoglobulin (βLg), a major whey protein, is frequently used as a food emulsifier (De Wit, 1998), (Barbiroli, Iametti, & Bonomi, 2022). Its physicochemical behaviour at the oil-water interface and its high resistance to pepsin can be crucial for the efficiency of lipid digestion in the GIT (Dulko et al., 2021), (Heiden-Hecht & Drusch, 2022), (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009).

The digestion of lipids is a complex process, influenced by the physiological surface-active agents in the GIT, especially bile salts (BSs) and phospholipids (PLs). Bile, an essential exocrine secretion of the liver stored in the gallbladder, plays a key role in the physicochemical and biochemical transitions of dietary fats after ingestion. It comprises primarily water and organic constituents, with BSs and PLs forming the majority of its surface-active components (Vlahcevic, Bell, & Swell, 1970), (Boyer, 2013). Despite that, the roles of the two types of biliary surfactants in intestinal digestion have usually been studied independently in scientific research.

BSs are essential in efficient lipolysis of dietary triglycerides (TGs) into fatty acids (FAs), diglycerides (DGs) and monoglycerides (MGs) (Armand, 2007). The function of BSs extends beyond mere emulsification of dietary fats in the intestinal lumen; they facilitate the transport and absorption of lipolytic products across the intestinal brush border (Macierzanka, Torcello-Gómez, Jungnickel, & Maldonado-Valderrama, 2019). Therefore, BSs are often used in *in vitro* lipolysis studies as simple substitutes for human bile (Brodkorb et al., 2019), (Minekus et al., 2014). Previous research has further elucidated the role of BSs in altering the viscoelasticity and relaxation processes at the oil-water interface, disrupting stable emulsifier layers, and thereby enhancing the lipolysis of emulsified TGs (Mekkaoui et al., 2021), (Maldonado-Valderrama, del Castillo-Santaella, Gálvez-Ruiz, Holgado-Terriza, & Cabrerizo-Vílchez, 2021).

Although PLs share amphipathic qualities with BSs, their contribution to the lipolysis process has not been extensively studied. They have been recognised to play a crucial role in drug solubilisation, making them a common constituent in simulated intestinal fluids used in pharmaceutical studies (Jantratid, Janssen, Reppas, & Dressman, 2008), (Söderlind et al., 2010). Past studies have shown a complex relationship between PLs and lipolysis, with some suggesting a retarding effect of PLs on lipolysis, while others deny such inhibitory influences, suggesting instead the importance of the BS–PL interaction in determining lipolytic efficiency (Larsson & Erlanson-Albertsson, 1986), (Ferreira & Patton, 1990), (Lykidis, Avranas, & Arzoglou, 1997). This interaction is crucial under physiological conditions of the small intestine, where BS and PL molecules can form mixed micelles with lipolytic products, affecting the solubilisation of these products and their transport towards the intestinal epithelium before absorption by enterocytes (Macierzanka et al., 2019), (Pabois et al., 2019), (Phan et al., 2015), (Jantratid et al., 2008). The BS–PL interaction is also important under pathological conditions that affect the BS and PL contents of the bile delivered to the intestine (Sundaram, Bove, Lovell, & Sokol, 2008), (Cherraqi et al., 2023). These insights demonstrate the dynamic interplay of biochemical and physical factors in the digestion

process, which might substantially be influenced by the concentration and ratio of BSs and PLs.

In vitro models of digestion frequently utilise animal bile extracts, (e.g., porcine or bovine) to substitute human bile. This approach is predicated on the assumption that these extracts mirror the complexity of BSs in humans, with bovine bile, in particular, noted for its relatively close resemblance to the human bile (Capolino et al., 2011). Nevertheless, when adhering to established *in vitro* digestion guidelines, such as those outlined in the INFOGEST protocols (Brodkorb et al., 2019), (Minekus et al., 2014), (Menard et al., 2023), the emphasis tends to be on achieving a specific total BS concentration, often neglecting the presence and concentrations of other biosurfactants, like PLs, introduced through these animal bile preparations. Previous research on *in vitro* proteolysis, incorporating actual human bile, revealed that digestion outcomes hinge not only on the overall BS levels but also on the ratio of BSs to PLs (Dulko et al., 2021). That study also highlighted the ease and precision with which this BS/PL balance can be managed through the application of individual BSs and PLs. This suggests that, at least for *in vitro* proteolysis studies, accurately mimicking the complex BS profile of human bile may not be as critical for the successful simulation of intestinal digestion as previously thought.

The bile composition in humans exhibits significant inter-individual variation, encompassing differences in bile acid profiles and total BS concentrations (Rossi, Converse, & Hofmann, 1987). Furthermore, a range of health conditions, such as choledocholithiasis, cholestasis or malignant biliary obstructions, can markedly influence both the composition of bile and its flow from the bile duct into the small intestine (Dulko et al., 2021), (Li & Apte, 2015), (Costi, Gnocchi, Di Mario, & Sarli, 2014). In healthy adults, bile secretion into the small intestine varies between fasted and fed states, with additional factors such as age playing a role. For instance, BS concentrations in the intestinal lumen of adults have been reported to fluctuate between 0.57 mM and 6.0 mM during fasting periods, increasing to as much as 16 mM in the postprandial state. By contrast, in full-term infants, postprandial BS levels range from merely 0.4 mM to 1.5 mM (Bourlieu et al., 2014). Data on the concentrations of biliary PLs within the digestive tract are rarely reported. Therefore, we have conducted in this study a retrospective literature review aimed at estimating the average concentrations of BSs and PLs in human bile and in the small intestinal lumen.

As biliary surfactants, BSs and PLs are first introduced with bile into the duodenum and subsequently reach other parts of the small intestine (Boyer, 2013), where they play a crucial role in the digestion processes within the intestinal lumen. Consequently, this work has concentrated on studying the intestinal lipolysis, which constitutes the major hydrolysis of dietary lipids in adult humans (Acevedo-Fani & Singh, 2022), while gastric digestion has been excluded. For this reason, βLg-rich whey protein isolate was chosen as the emulsion stabiliser in our *in vitro* intestinal digestion experiments. As previously mentioned, βLg exhibits resistance to gastric pepsinolysis, a property observed to a certain degree even when the protein is adsorbed to the oil-water interface in emulsions (Macierzanka et al., 2009), (Sarkar, Goh, Singh, & Singh, 2009). However, this does not necessarily make it resistant to displacement from the interface by biliary surfactants when lipid emulsions move from gastric to duodenal conditions (Macierzanka et al., 2009). Therefore, we disregarded the impact of gastric proteolysis in this study and focused on the interactions between the adsorbed protein and the BSs and PLs directly under simulated intestinal conditions.

The initial part of our research aims to provide a detailed examination of the combined impact of physiologically relevant BSs and PLs on the evolution of interfacial tension and dilatational rheology during real-time lipolysis at model protein-stabilised oil-water interfaces. This fundamental approach will help in interpreting the efficiency of intestinal lipolysis when examined at a macro-scale for model food emulsions, in the final part of this study. We hypothesise that the combined effect of BSs and PLs is dependent on their ratio, particularly under conditions that aim to simulate the effects of real human bile.

Our study addresses an emerging need for understanding the complexity of intestinal lipid digestion, especially with regard to reliably mimicking this process using *in vitro* digestion models. It specifically focuses on how biosurfactants at the oil-water interface critically determine the efficiency and outcome of lipolysis. By focusing on the interaction between two major biliary biosurfactants (BSs and PLs) and their interaction with food proteins at the interface, this research contributes to the broader understanding of the rheological and structural characterisation of food colloids in the context of physicochemical (in)stability under dynamic digestion conditions.

This study is set to offer important insights into the lipid digestion process in physiologically relevant scenarios, potentially leading to improved *in vitro* digestion protocols as well as new food product designs and nutritional interventions targeting digestive efficiency and health.

2. Material and methods

2.1. Literature search of bile salt and phospholipid physiological concentrations

Scientific articles reporting on the physiological composition of adult human hepatic bile, gallbladder bile and the concentrations of biliary surfactants in the small intestinal lumen were collected using the Google Scholar and PubMed databases (Supplementary Material, S1.1). The concentrations of total bile salts (BSs) and phospholipids (PLs) were extracted into the MS Excel database and analysed. PL and BS data were selected according to the following filter attributes: (i) subject conditions (i.e., healthy adult individuals, fasted/fed state), (ii) sample collection point (i.e., gallbladder, main bile duct/hepatic duct, duodenal/small intestinal lumen), (iii) concentration unit (i.e., mM, mmol/L). The weighted arithmetic means were calculated for the BS and PL concentrations, taking into account the weight of the number of individuals for each mean extracted from relevant papers.

2.2. Bile salt solution and phospholipid dispersion

Simulated intestinal fluid (SIF, pH 7.0) was prepared following the INFOGEST protocol (Brodkorb et al., 2019). A stock solution consisting of two individual BSs was prepared using SIF, containing a 100 mM concentration with equimolar quantities of sodium glycodeoxycholate (NaGDC, 97% purity; Sigma-Aldrich, G9910) and sodium taurocholate (NaTC, 95% purity; Sigma-Aldrich, T4009). The two BSs were selected because they have often been used in other *in vitro* digestion and intestinal transport studies to mimic BSs of human bile (Mandalari, Adel-Patient, et al., 2009), (Dupont et al., 2009), (Chu et al., 2009), (Krupa et al., 2020), (Böttger et al., 2019), (Dulko et al., 2021). Additionally, a 65 mM stock dispersion of PLs was also prepared using SIF. The PL preparation used in the study was the egg yolk L-α-Phosphatidylcholine (Sigma-Aldrich, 61755). HPLC analysis showed the PL preparation contained phosphatidylcholine and phosphatidylethanolamine in approx. 4.1:1 (w/w) proportion. Given the fact that phosphatidylcholine is the most abundant PL amongst the human biliary PLs (Gilat & Sömjen, 1996), the source of PLs selected for this study seems to be justified for mimicking PLs in human bile. The PL stock dispersion was prepared using the sonication technique adopted from previous research (Silva, Ferreira, Little, & Cavaco-Paulo, 2010) and adjusted to achieve a nanosized average particle size. For this purpose, an ultrasound homogenizer equipped with a sonication probe was applied (VCX500, Sonics & Materials Inc., CT; 20 kHz, 500 W), and the sonication was carried out in two cycles (i.e., 2 x 3 min, 5 s/10 s pulse/pause, at 70% amplitude) with a 15-min break between the cycles, while keeping the PL dispersion in an ice/water/isopropanol bath (ca. 5 °C) (Silva et al., 2010). The mean particle size (Z-Average, n = 4) was determined from dynamic light scattering (DLS) measurements using a Zetasizer Nano-ZS system (Malvern Instruments, UK). The sample preparation for size determination was performed according to the Malvern manual (Malvern, 2013). Prior to further use in lipolysis experiments, aliquots of freshly prepared BS and PL stocks were mixed together (magnetic stirrer, 500 rpm, 20 min) in various proportions to achieve specific BS/PL ratios required for individual experiments (Table 1). The size of BS/PL aggregates produced that way was determined by DLS, and reported as the mean ± SD (n = 4) for each BS/PL ratio examined. The mean particle size in the BS solution was determined in the same way.

2.3. Oil phase preparation

Refined sunflower oil (Martins Polska S.A.) was mixed with Florisil (Sigma-Aldrich, 03286), 2:1 (w/w), for 120 min (150 rpm) at room temperature to remove any residual surface-active impurities. This was followed by removing the adsorbent by centrifugation (30 min, 9 000 rpm, 20 °C) and filtration under vacuum, using Millex filters (0.1 µm PDVF, Sigma-Aldrich). The oil was stored at 4 °C under nitrogen prior to further use.

2.4. Interfacial examination of lipolysis

The simulated intestinal lipolysis was examined with the subphase multi-exchange device, the OCTOPUS, controlled by Dinaten software. The device utilises a pendant drop technique and enables the droplet content to be exchanged in a controlled fashion during the experiment (Maldonado-Valderrama, Torcello-Gómez, Del Castillo-Santaella, Holgado-Terriza, & Cabrerizo-Vílchez, 2015), (Maldonado-Valderrama, del Castillo Santaella, Holgado-Terriza, & Cabrerizo-Vílchez, 2022). In the present study, the technique was used to monitor the evolution of the oil-water interfacial tension (IFT) during sequential adsorption/lipolysis/desorption measurements at 37.0 ± 0.1 °C. The experiments were carried out for various lipolysis conditions (Table 1). Measurements were done in triplicate (n = 3) for each set of conditions and data have been shown as the mean ± SD.

Step one – Adsorption: The protein adsorption was always the first step of experiment and done in the same way regardless of the conditions applied in the following lipolysis step. The sunflower oil was incubated at 37 °C in a glass cuvette (Hellma, OG, type 6030; 10 mm optical path length) with a coaxial double capillary (Patent ES 2 153 296 B1) immersed in the oil. The 0.5 wt% β-lactoglobulin (βLg; Sigma-Aldrich, L3908) stock solution was prepared by dissolving the protein in a saline (150 mM NaCl, 0.02 wt% NaN_3; pH 7.0). The freshly prepared stock was diluted 10× with the saline before being injected through the capillary to produce a 15 mm^2 pendant droplet in the oil. The protein was allowed 60 min to adsorb to the oil-water interface to achieve a stable IFT.

Subsequent to this, the dilatational rheology was measured. The droplet underwent deformation at a frequency of 0.1 Hz, with the deformation area consistently kept below 5% to prevent excessive disturbance of the interface. The measuring system captured the response of the IFT to the deformation of the interfacial area, yielding the dilatational modulus (E). E is a complex quantity, defined as $E = E' + iE'' = \varepsilon + iv\eta$, where E' is the real component (storage modulus), reflecting the interfacial elasticity (ε), and E'' is the imaginary part (loss modulus), indicative of the interfacial viscosity (η), with v representing the angular frequency of the applied oscillation (Maldonado-Valderrama et al., 2021), (del Castillo-Santaella & Maldonado-Valderrama, 2023). Following the deformation cycles, the interface was left undisturbed for 5 min. Prior to each experiment (i.e., before commencing the protein adsorption step), a control measurement of the sunflower oil/saline IFT was conducted. This yielded a consistent value of 23.5 ± 1.5 mN/m at 37.0 ± 0.1 °C.

Step two – Lipolysis: Following the protein adsorption step and dilatational rheology, the subphase exchange of the droplet content was carried out by a 10-fold exchange of the droplet volume. This was done using freshly prepared SIF solutions of porcine pancreatic lipase (Sigma-Aldrich, L3126; at a concentration required to achieve an enzyme activity of 2000 U/mL, (Brodkorb et al., 2019)) with BSs and/or PLs (at various BS/PL molar ratios, see Table 1). This intervention was done while keeping the original droplet area (i.e., 15 mm^2) constant. In similar studies, it has previously been observed that a 10-fold replacement of

droplet content ensures a thorough exchange of the subphase with a new fluid (Maldonado-Valderrama, Muros-Cobos, Holgado-Terriza, & Cabrerizo-Vílchez, 2014), (del Castillo-Santaella & Maldonado-Valderrama, 2023). The IFT was continuously monitored throughout the entire process of subphase exchange.

The lipolysis step was carried out for 60 min at 37.0 ± 0.1 °C. Control measurements for BS–13, PL–13 and BS/PL–9:4 (Table 1) were also conducted with no enzyme added. In order to evaluate any possible impact of the addition of colipase or phospholipase A2 (PLA_2) on the IFT, the measurements for the BS/PL–9:4 were performed in three different variants: (i) with the pancreatic lipase only, (ii) with the lipase and porcine pancreatic colipase (Sigma-Aldrich, C3028), and (iii) with the lipase and porcine pancreatic phospholipase A_2 (PLA_2; Sigma-Aldrich, P6534); see Table 1 for details. The lipolysis step was followed by measuring the dilatational rheology, according to the procedure given above for the protein adsorption step.

Step three – Desorption: Following the lipolysis step, a 10-fold exchange of the droplet content with SIF was performed. The evolution of IFT was monitored for another 60 min at 37.0 ± 0.1 °C. As in the previous two steps, the dilatational rheology was examined at the end of the desorption step by applying the deformation conditions described above.

2.5. Lipolysis in emulsion

The *in vitro* simulation of intestinal digestion was conducted according to the INFOGEST static protocol (Brodkorb et al., 2019).

2.5.1. Emulsion preparation

Oil-in-water emulsions were prepared using 20 wt% of the Florisil-cleaned sunflower oil, 0.5 wt% Whey Protein Isolate (WPI, 93% protein; BiPRO, Davisco Foods International Inc., Eden Prairie, MN), and 79.5 wt% saline (150 mM NaCl aqueous solution with 0.02 wt% NaN_3). WPI was dissolved in the saline (60 min, 720 rpm). The aqueous phase and the oil phase were combined in a glass vial and vortexed for 3 min. The emulsion premix was then sonicated for 3 min (5 s/10 s, pulse/pause) using a sonication probe operating at 80% amplitude (CVX 130 Vibra Cell Ultrasonicator, 20 kHz, 130 W, 13 W/cm^3). The glass vial with emulsion was immersed in an ice/water/isopropanol bath (ca. 5 ^{o}C) during the process.

The emulsion droplet size was measured from a DLS using a Nano-ZS Zetasizer (Malvern Instruments Ltd., Malvern, UK) operated in a size-measure mode and according to the Malvern manual (Malvern, 2013). The mean (± SD) size (Z-Average) was 2280 ± 140 nm (n = 27).

2.5.2. In vitro small intestinal digestion of emulsion

An aliquot of the freshly prepared emulsion was introduced to the SIF, and the intestinal digestion procedure followed that outlined in the INFOGEST protocol (Brodkorb et al., 2019), with the sole exception of the applied BS concentration. To ascertain the impact of both BS and PL concentrations and their ratios on emulsion lipolysis, the stock solution of individual BSs and/or the PL stock dispersion were utilised in amounts required to achieve the concentrations listed in Table 1 within the final digestion mix. This was followed by addition of porcine pancreatin (Sigma-Aldrich, P7545) prepared in SIF just before the experiment and used in quantities required to obtain 2,000 U/mL of pancreatic lipase activity in the digestion mixture. The lipolytic activity of pancreatin was assayed according to the INFOGEST recommendations (Grundy et al., 2021). The digestion experiment commenced with the addition of pancreatin.

Simulated intestinal digestion was conducted for 2 h at 37.0 ± 0.1 °C (pH 7) within a 20 mL conical glass vessel, stirred magnetically at 700 rpm. The free fatty acids (FFAs) liberated

from the emulsified triglyceride (TG) substrate (i.e., the sunflower oil), due to the action of pancreatic lipase, were neutralised by 0.1 N NaOH using a pH-stat micro-titrator (Cerko Lab Systems, Gdynia, Poland), controlled by Cerko Lab Solution software. The NaOH volumes (V_{NaOH}) administered to maintain constant pH were recorded every second. Blank experiments, replacing pancreatin with SIF, were also conducted, with any necessary NaOH titration monitored and the corresponding volumes deducted from those recorded during the active (i.e., with pancreatin) digestion experiments.

It was critical that for each digestion involving added PLs or BS/PL mixtures (Table 1), a respective control experiment was undertaken to evaluate any FFA release from PLs, possibly induced by pancreatin digestion (i.e., potentially through PLA_2 activity within the pancreatin). To ensure uniformity across experiments, control measurements were also executed for digestions in the presence of individual BSs, as well as in the absence of any biosurfactants. In all control experiments, the emulsion was substituted with SIF while maintaining the added PLs, BS/PL mixtures, or individual BSs consistent with their counterpart experiments on emulsion. These control experiments adhered to the identical methodology outlined above for the emulsion digestion experiments.

To assess the progression and extent of TG lipolysis in the emulsion (i.e., the exclusive release of FFAs from the TG substrate), V_{NaOH} values recorded for every time point during a control digestion experiment (i.e., in the absence of TG emulsion) were subtracted from those recorded for the counterpart emulsion digestion experiments (Supplementary Material, Fig. S2.1). The resultant V_{NaOH} values were used to calculate the percentage of FFAs released solely from the emulsified TGs, as follows:

$$FFA\ (\%) = \frac{(V_{NaOH} - V_{blank}) \cdot \mathrm{C} \cdot \mathrm{MW}}{\mathrm{m_{oil}} \cdot \mathrm{N} \cdot 1000} \cdot 100$$

where V_{NaOH} is the volume (mL) of NaOH needed to neutralise FFAs liberated from TGs, V_{blank} is the volume (mL) of NaOH required to neutralise a blank sample, C is the molar concentration of the titrant (NaOH, 0.1 M), MW is the average molecular weight of sunflower oil TGs (885.69 g/mol; as ascertained by GC-FID analysis of fatty acid composition (Kłosowska-Chomiczewska et al., unpublished results)), m_{oil} represents the amount (g) of oil incorporated into the digestion mixture via the emulsion, and N is the number of FFAs released from each TG molecule (i.e., N = 2, assuming two FFAs are released from one TG molecule by pancreatic lipase action).

All digestion experiments were conducted in triplicate (n = 3) for each set of conditions tested, and results are presented as mean (± SD), which is equally applicable to the control and blank experiments.

2.6. Statistical analysis

Statistical comparisons between two groups of data were conducted using a Student's *t*-test, while one-way ANOVA was employed for comparing three or more groups of data, with statistical significance set at a P value < 0.05.

3. Results and Discussion

3.1. Bile salt and phospholipid concentrations in the adult human upper gastrointestinal system: Retrospective analysis of scientific literature data

The experimental work had been preceded by a comprehensive literature review and data collection, which aimed to assess the extent of fluctuations in BS and PL average concentrations within the upper gastrointestinal (GI) system and determine the quantities of these biliary biosurfactants within the postprandial small intestine. The latter was vital for establishing the range of BS and PL concentrations essential for subsequent digestion experiments, which constituted the primary focus of our study.

Thirty-nine scientific publications, spanning the years 1968 to 2021, have been found to report on the physiological concentrations of BSs and PLs in both bile and the small intestinal lumen of adult humans. The data were categorised according to the GI locations, for which the total content of BSs and PLs had been documented in the scientific literature (see Fig. 1a). Physiological concentrations of BSs and PLs exhibit substantial variation among distinct GI segments in adult humans, including the gallbladder (the gallbladder bile), the hepatic duct (the hepatic bile), and the small intestinal lumen (Fig. 1b). Mean concentrations are lower in hepatic bile (38 ± 19 mM for BSs and 13 ± 7 mM for PLs; Fig. 1b), as evidenced by data extracted from 3 scientific reports (Supplementary Material, Table S1.1). In contrast, gallbladder bile exhibits much higher concentrations (135 ± 39 mM for BSs and 42 ± 14 mM for PLs; Fig. 1b), derived from information extracted from 13 scientific reports (Supplementary Material, Table S1.1). The highest quantities of these biosurfactants are in the gallbladder as this is the site for bile storage and concentration following hepatic production (Hofmann, 1999).

In the context of this study, the most crucial were the concentrations of BSs and PLs within the small intestinal lumen. During periods of fasting, the secretion of bile into the duodenum remains limited. However, following food ingestion, the liberated bile is delivered to the duodenum and subsequently diluted with other secretions and chyme present in the intestinal lumen (Janowitz et al., 1990). Consequently, the contents of intestinal BSs and PLs may fluctuate depending on the state of ingestion. Furthermore, BSs are exclusively delivered with bile, while PLs can originate from bile itself and, to some extent, from gastric secretions (Wenner, Gunnarsson, Graffner, & Lindell, 2000), as well as the ingested food. However, the latter aspect may significantly depend on the specific type of consumed food. To our knowledge, the contributions of biliary versus dietary and gastric PLs in the small intestinal lumen have not been extensively investigated so far. According to several early reports (Borgström, 1976), (Borgström, 1980), (Åkesson, 1982), the quantity of PLs secreted daily with bile in adult humans is approximately five times greater than the quantity of PLs consumed in the diet. This suggests that bile represents the primary source of PLs within the intestinal lumen.

Our analysis of the available literature data reveals that the mean concentrations of BSs and PLs in the fasted small intestine of healthy adult humans are relatively low, i.e., 3.7 ± 1.1 mM and 0.6 ± 0.5 mM, respectively (Fig. 1b, based on data extracted from 14 scientific reports; Supplementary Material, Table S1.1). This may be attributed to the aforementioned absence of ingestion-induced stimulation of bile flow into the duodenum. These concentrations increase in the fed small intestine. The data analysis returned a total BS concentration of 9.1 ± 2.7 mM and a total PL concentration of 4.0 ± 1.2 mM (Fig. 1b, based on data obtained from 14 sources; Supplementary Material, Table S1.1) in the postprandial small intestinal lumen of healthy adults. This indicates an approximately 2.3-fold, statistically significant (P<0.01) molar excess of BSs over PLs.

The calculated BS concentration aligns with the recommended 10 mM concentration in the *in vitro* static digestion protocol developed by the INFOGEST scientific network (Brodkorb et al., 2019), (Minekus et al., 2014), which is widely employed in various *in vitro* digestion studies worldwide (Zhou, Tan, & McClements, 2023). However, the protocol does not specify the PL concentration necessary to replicate the average postprandial small intestinal PL concentration. This omission may pose limitations concerning the physiological relevance of the protocol and similar *in vitro* methods for simulating conditions of food digestion. Numerous scientific studies have indicated that the presence and concentration of PLs can significantly influence the rate and extent of intestinal digestion, although a proteolysis has primarily been investigated in this context (Moreno, Mackie, & Mills, 2005), (Mandalari, Mackie, Rigby, Wickham, & Mills, 2009), (Macierzanka et al., 2009), (Macierzanka et al., 2012), (Böttger et al., 2019), (Dulko et al., 2021).

For the purpose of our current *in vitro* lipolysis studies, we approximated the mean BS concentration to 9 mM and considered a BS/PL molar ratio of 9:4 (BS/PL–9:4) as representative of the physiological conditions in the postprandial small intestinal lumen of adult humans. In addition to this physiologically relevant BS/PL–9:4 ratio, we also investigated the effects of other molar ratios of these biosurfactants, namely BS/PL–4:9 and BS/PL–6.5:6.5, as well as the sole presence of BSs (BS–13) or PLs (PL–13) (see Table 1). The combined total molarity of the two biosurfactants remained constant across different conditions, but varying proportions were employed to assess the influence of BSs versus PLs on the pattern and extent of lipolysis. Our objective was to determine whether deviations from the physiologically relevant BS/PL–9:4 ratio in the fed small intestine – whether due to conditions such as impaired bile production/secretion or the consumption of PL-rich foods – could impact the lipolysis of TGs under small intestinal conditions.

With the BS/PL proportions for investigation established in this study, we started the experimental work by determining the size (Z-Average) of PL liposomes produced according to Section 2.2 (i.e., for the PL–13 preparation). We also measured the sizes of BS/PL aggregates formed after mixing liposomes with BSs at varying BS/PL ratios, as described in Section 2.2 and outlined in Table 1.

Previous analysis of the colloidal fraction in aspirated, fed-state human intestinal fluid (Elvang et al., 2016) has revealed the presence of several types of supramolecular assemblies consisting of BSs and PLs. These assemblies primarily comprised mixed BS/PL micelles and PL vesicles with mean sizes ranging from 50 to 200 nm, coexisting with a smaller fraction of BS micelles and/or small mixed micelles (<20 nm) in the intestinal fluid. We replicated these physiological size characteristics using the preparation method detailed in Section 2.2. The mean particle sizes obtained for the various BS/PL preparations in SIF decreased as we incorporated BSs and increased their proportions, yielding the following results: 80.5 ± 0.6 nm for PL–13, 72.2 ± 1.6 nm for BS/PL–4:9, 64.2 ± 1.9 nm for BS/PL–6.5:6.5, and 59.6 ± 2.6 nm for BS/PL–9:4. In the case of BS–13, DLS examination returned a diameter of 5.6 ± 0.7 nm, indicating the formation of BS micelles.

3.2. Interfacial analysis of intestinal lipolysis at different BS/PL proportions

This part of the study aimed to characterise whether conducting lipolysis solely in the presence of individual BSs, PLs, or at various proportions of these biliary surfactants might yield differences in the evolution of the oil-water IFT. As previously mentioned, the lipolysis of TGs is an interfacial reaction that generates surface-active compounds (i.e., MGs, DGs and FFAs). Therefore, any discrepancies in the IFT patterns observed in lipolysis experiments conducted at different BS/PL ratios may imply BS/PL-dependent modifications in the rate of

lipolysis and/or alterations in the mechanisms of associated interfacial processes. Determining the extent of such BS/PL-dependent variations while monitoring lipolysis at the oil-water interface was deemed essential for comprehending the progression of TG digestion within emulsions, which constituted the final part of this study (Section 3.3).

The interfacial characterisation of intestinal lipolysis, occurring at the interface of an aqueous droplet suspended in sunflower oil, was conducted *in vitro* using the OCTOPUS setup (Maldonado-Valderrama, Terriza, Torcello-Gómez, & Cabrerizo-Vílchez, 2013). The experiment comprised three consecutive steps: (1) protein adsorption, (2) lipolysis, and (3) desorption. In the initial step, i.e., βLg was allowed sufficient time to adsorb to the interface until the IFT reached a stable value (60 min). This led to the formation of a protein-stabilised oil-water interface, mimicking the interfacial protein membrane of a single droplet in a protein-stabilised emulsion (Fig. 2a). The protein adsorption procedure remained consistent across all interfacial experiments, resulting in a similar reduction of the oil-water IFT. Initially, the IFT was 23.5 ± 1.5 mN/m for the bare oil-water interface before adsorption. After 60 min of incubation with βLg at 37 °C, the IFT decreased to 12.5 ± 1.6 mN/m (Fig. 3a).

The subsequent lipolysis step was initiated by replacing the content of pendant drop with SIF containing individual BSs and/or PLs, along with digestive enzyme(s) (Fig. 2b), as detailed in Section 2.4. The concentrations of BSs and/or PLs, calculated according to Section 3.1, had to be reduced by a factor of 10 in the interfacial experiments. This adjustment became necessary because using the original concentrations in the pendant droplet setup resulted in a rapid decrease in the IFT to below 2 mN/m, leading to the detachment of the droplet from the capillary. A similar approach has been employed in previous studies (Maldonado-Valderrama et al., 2015), (Del Castillo-Santaella et al., 2015), (Maldonado-Valderrama et al., 2013). Nonetheless, we investigated all the BS/PL molar proportions identified in Section 3.1 (see Table 1).

The introduction of BSs and PLs can induce the rearrangement of proteins at the interface, resulting in their partial or complete displacement. The extent of this effect depends on various factors, such as the type and quantity of adsorbed protein or the concentration of biosurfactants (Bellesi, Pizones Ruiz-Henestrosa, & Pilosof, 2014). Consequently, it can lead to a reduction in IFT. Previous research has demonstrated the capability of both BSs and PLs to displace proteins, including βLg, from the oil-water interface (Maldonado-Valderrama et al., 2008), (Macierzanka et al., 2009), (Macierzanka et al., 2012). Furthermore, it has been established that the introduction of lipase, followed by its adsorption at the interface, initiates the interfacial hydrolysis of the TG substrate into surface-active products, resulting in a decrease in IFT (Janowitz et al., 1990). Substantial reduction in IFT was observed in our present lipolysis study. To establish experimental conditions, initial experiments were conducted for BS/PL–9:4 in the presence of lipase, with or without colipase or PLA_2.

The combined action of biosurfactants and enzymes gradually reduced the IFT to as low as 2–3 mN/m over the course of the 60-min lipolysis step, with a substantial decrease in IFT noticeable within the first few minutes (Fig. 3a). This strongly suggests the presence of biosurfactants at the interface, which eventually displace βLg from the interface. Importantly, this marked decrease in the interfacial tension (IFT) was noted during the lipolysis step conducted with lipase, irrespective of whether colipase or PLA_2 was present or absent (Fig. 3b). Consequently, all subsequent interfacial examinations of lipolysis were conducted using lipase alone, without the addition of colipase or PLA_2.

We also conducted control experiments (i.e., with no enzymes added) to investigate the specific contribution of BSs and/or PLs to the reduction in IFT and to compare the isolated effect of these biosurfactants to the effect observed when they act in conjunction with lipase. Both active (i.e., with lipase) and control experiments were conducted for BS–13, PL–13, and BS/PL–9:4 (Fig. 4).

In each case, substantial differences between the active experiments and their control counterparts were evident. In general, the sole use of BSs and/or PLs (i.e., in the absence of lipase) resulted in only a limited reduction in IFT. The most substantial decrease was observed for BS/PL–9:4, where the IFT decreased to 7.5 ± 0.7 mN/m after a 60-min exposure to BSs and PLs under control lipolysis conditions (Fig. 4c). However, to achieve a more significant reduction in IFT, it was essential to conduct the experiment with the addition of lipase. This suggests that the interfacial hydrolysis of TGs into MGs, DGs and FFAs played a crucial role in the observed decline in IFT under the experimental conditions. The surface-active lipolysis products generated *in situ* were likely responsible for the more substantial reduction in IFT compared to the control experiments. The magnitude of reduction was dependent on the biosurfactant used. During lipolysis in the presence of BS–13, the IFT decreased to 4.0 ± 0.3 mN/m (Fig. 4a), while a minimum of 7.0 ± 0.4 mN/m was reached when lipolysis was facilitated by PL–13 (Fig. 4b). Interestingly, the combined use of BSs and PLs (i.e., BS/PL–9:4) during digestion resulted in the lowest IFT (2.2 ± 0.1 mN/m, Fig. 4c). This aligns with the most efficient reduction in IFT observed when the biosurfactants were used in combination in the control experiment (without lipase, Fig. 4c), highlighting a synergistic effect in their interfacial activity.

To further investigate the combined effect of BSs and PLs, we conducted repeated lipolysis experiments using different molar proportions of the biosurfactants than BS/PL–9:4 (Table 1). Application of equimolar quantities of BSs and PLs (i.e., BS/PL–6.5:6.5) or even an excess of PLs in the biosurfactant mixture (i.e., BS/PL–4:9) resulted in an IFT of approximately 3 mN/m at the conclusion of the lipolysis step (i.e., 3.1 ± 0.3 mN/m and 3.2 ± 0.3 mN/m, respectively; Fig. 5). This value was higher than that recorded for BS/PL–9:4 (2.2 ± 0.1 mN/m) but still lower than when BSs were used as the sole biliary surfactants (i.e., 4.0 ± 0.3 mN/m for BS–13) (Fig. 5). This confirmed that the three different molar proportions of BSs and PLs employed during lipolysis could contribute more effectively to the reduction of IFT than BSs or PLs applied in isolation (i.e., BS–13 or PL–13).

However, it is essential to emphasise that the interfacial *in vitro* lipolysis experiments could not directly demonstrate whether and to what extent the BS/PL mixtures assisted in the enzymatic hydrolysis of TGs and the generation of surface-active lipolysis products. These experiments only illustrate the evolution of IFT during lipolysis due to the combined action of BSs and PLs, along with lipase, converting TGs to MGs, DGs, and FFAs. Nevertheless, it is noteworthy that every lipolysis experiment in the presence of BSs, BS/PL mixtures, or PLs resulted in a much more substantial reduction in IFT compared to the control lipolysis (i.e., with no biosurfactants added). In the control experiment, the sole use of the enzyme could only reduce the IFT to 12.0 ± 0.4 mN/m, indicating limited hydrolysis of TGs into surface-active products (Fig. 5). This control experiment also showed that any disturbance to the droplet content caused by the subphase exchange process prior to the lipolysis step did not substantially affect the interfacial film of βLg.

The interfacial experiments also included a desorption step, as it has been described in Section 2.4 and illustrated in Fig. 2c.. The subphase exchange depletes the droplet's aqueous environment of any excess surface-active material that may have been dissolved there, thereby promoting desorption from the interface. This process can result in an increase in the IFT (Maldonado-Valderrama et al., 2015), (Maldonado-Valderrama et al., 2022). The desorption step served as the final experimental phase, providing insights into the desorption capabilities of BSs, PLs, and/or the lipolytic products from the interface. The ease of washing away any of these surface-active compounds would be reflected in an increased IFT, indicating their limited affinity for the interface.

In the absence of lipase during the control lipolysis step with BS–13 (Fig. 4a), the subsequent subphase exchange led to a substantial increase in the IFT, rising from 9.7 ± 0.6 mN/m to 21.7 ± 1.2 mN/m. Considering that the IFT of the bare interface was 23.5 ± 1.5 mN/m (Section 2.4), this proves the applied individual BSs could have been relatively easily

and effectively desorbed from the interface due to the concentration gradient caused by the bulk concentration depletion. This observation aligns with previous studies on the interfacial behaviour of NaGDC and NaTC, where these two BSs were found to almost completely desorb from the air-water and oil-water interfaces upon exchanging the aqueous phase with pure water (Maldonado-Valderrama et al., 2014), (del Castillo-Santaella & Maldonado-Valderrama, 2023). When the control experiment was conducted exclusively with PLs in the present study (i.e., for PL–13, Fig. 4b), the desorption step led to a modest increase in IFT, indicating the presence of an irreversibly adsorbed layer of PLs, or possibly PLs in conjunction with βLg, at the interface. The desorption phase, carried out after the βLg adsorbed layer had been exposed to a combination BSs and PLs (BS/PL–9:4) under control conditions, resulted in slightly lower IFT (13.8 ± 1.2 mN/m, Fig. 4c), relative to when PL–13 was utilised (15.8 ± 1.0 mN/m, Fig. 4b). However, out of the two compared IFT values, only the one obtained for BS/PL–9:4 (13.8 ± 1.2 mN/m) was reached after a substantial increase from much lower value recorded at the conclusion of the control lipolysis step (i.e., from 7.5 ± 0.7 mN/m, Fig. 4c). This indicates a partial desorption of BSs. It further implies that the interfacial layer remaining post-application of BS/PL–9:4 comprised a complex arrangement of biosurfactants, capable of synergistically reducing the IFT.

As previously stated, the addition of lipase during the lipolysis step induced a more pronounced reduction of IFT compared to the control conditions (i.e., in the absence of the enzyme), Fig. 4. Additionally, the activity of lipase, presumably leading to the formation of surface-active lipolysis products, contributed to lower IFT values in the subsequent desorption step when compared with the control conditions. In systems containing PLs (Fig. 4b,c), the IFT exhibited a comparable trend to that seen without the presence of lipase; that is, the IFT increased by 2-5 mN/m following lipolysis and during the desorption phase in these systems. The most notable result in this set of active experiments (i.e., with enzyme) was that even in the BS–13 system (i.e., in the absence of PLs), the subphase exchange conducted after the lipolysis step led to a moderate increase in IFT during the desorption step (an increase of ca. 5 mN/m, Fig. 4a). This is in stark contrast to the substantial rise in IFT caused by desorption in the control BS–13 system (an increase of ca. 11 mN/m, Fig. 4a). This suggests that it was the lipolysis products, rather than the BSs, that partially remained attached to the interface under conditions that facilitated desorption. In this context, the behaviour of the lipolysis products appeared to be most similar between the BS–13 and BS/PL–9:4 experiments. Nonetheless, the highest IFT following desorption was recorded for BS–13 (9.9 ± 0.9 mN/m, Fig. 4a), followed by a lower value for PL–13 (8.1 ± 1.2 mN/m, Fig. 4b), and the lowest for BS/PL–9:4 (5.7 ± 0.7 mN/m, Fig. 4c). Again, the latter underscores the synergistic effect of the two types of biosurfactants.

The data presented in Fig. 5 provide additional insights into how the BS/PL ratio influenced post-lipolysis desorption. It is evident that there is only a limited increase in the IFT during the desorption step in systems containing PLs. To further underscore this effect and to evaluate the influence of the PL content, the IFT values recorded at the end of the lipolysis and desorption steps are illustrated in Fig. 6. Additionally, the difference between the respective IFT values (Δ_{IFT}) has been calculated for each experimental condition. The results indicate that this relative difference in IFT depends on the BS/PL proportion used, decreasing by approximately 6-fold between the experiments with BS–13 and PL–13 (Fig. 6). This hindered desorption of interfacial material in PL-rich systems may have implications for the efficiency of intestinal lipolysis of emulsified TGs. Further exploration of any such effects is presented in the *in vitro* emulsion lipolysis study detailed in Section 3.3.

The findings illustrated in Fig. 6 once again underscore the crucial role of BSs in the desorption and solubilisation of lipolysis products. In the scenario where only PLs were present, and BSs were absent (PL–13), the obtained low Δ_{IFT} value implied limited solubilisation of lipolysis products from the interface. In contrast, when only BSs were used as biliary surfactants during lipolysis (BS–13), the observed much larger Δ_{IFT} value suggested a more effective solubilisation of interfacial compounds. Experiments involving

both BSs and PLs showed intermediate Δ_{IFT} values. Together, these outcomes hint at several potential interactions at the interface: i) PLs may not be displaced by BSs, or only to a limited extent; ii) PLs could obstruct the desorption of BSs; iii) PLs might facilitate the formation of complexes with lipolysis products, which are harder to desorb/solubilise by BSs compared to the case without PLs. However, the latter may vary depending on the specific BS/PL ratios and BS concentrations.

In addition to examination of IFT, dilatational rheology measurements were also employed to assess variations in the elasticity and viscosity of the interfacial films formed by the biosurfactants used (i.e., BSs and/or PLs), as well as those produced *in situ* (i.e., lipolysis products). The dilatational viscoelastic moduli of the interfacial layers were measured after the completion of each experimental step (protein adsorption, lipolysis, and desorption) as described in Section 2.4.

As outlined in Section 2.4, the dilatational modulus (E) is defined as a complex quantity. It consists of a real component, known as the storage modulus (E'), and an imaginary component, referred to as the loss modulus (E'') (Maldonado-Valderrama et al., 2021), (del Castillo-Santaella & Maldonado-Valderrama, 2023). In this report, results are specifically presented for an oscillation frequency of 0.1 Hz (Fig. 7), as this frequency is believed to closely align with the peristaltic frequency observed in the small intestine during digestion (approximately 11.7 per minute in the duodenum (Kellow, Borody, Phillips, Tucker, & Haddad, 1986)). In all cases analysed, the real component substantially exceeded the imaginary component. Therefore, the interfaces were considered mainly elastic, and only the dilatational elasticity will be discussed here. The experimental results obtained for the dilatational viscosity are shown in Supplementary Material (Fig. S2.2).

The data presented in Fig. 7 offer further insights into the compositional differences of interfacial layers formed after lipolysis and, subsequently, after the desorption steps. These insights elucidate the influence of the employed BS/PL ratios on these layers. The E' value of the βLg interfacial layer, formed following the completion of the protein adsorption step, was 21.9 ± 2.9 mN/m. This is consistent with previous literature data, which demonstrated a comparable value for the dilatational modulus of adsorbed βLg films at olive oil-water interfaces (del Castillo-Santaella, Sanmartín, Cabrerizo-Vílchez, Arboleya, & Maldonado-Valderrama, 2014), (Maldonado-Valderrama et al., 2013). Conducting lipolysis in the subsequent experimental step, without the addition of biosurfactants, reduced E' to 5.5 ± 2.4 mN/m (Fig. 7). This indicates that the formation of a certain amount of surface-active lipolysis products has rendered the interfacial layer less elastic. This reduction confirms that lipolysis progressed in the absence of biosurfactants, despite only a very limited reduction in the IFT being observed between the protein adsorption and lipolysis steps under these control conditions (Fig. 5). The subsequent desorption step did not affect the rheological properties of the interfacial layer, as an E' of 4.9 ± 0.3 mN/m was recorded (Fig. 7). This finding aligns with the minor post-lipolysis change in the IFT (Δ_{IFT}) observed in the absence of biosurfactants (Fig. 6).

The inclusion of BSs and/or PLs during the lipolysis phase substantially altered the rheological characteristics of the interfacial layers formed in the presence of the enzyme. It is well-established that the planar configuration of BSs promotes their rapid adsorption at interfaces, leading to an immediate decrease in IFT (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011), (Macierzanka et al., 2019), (del Castillo-Santaella & Maldonado-Valderrama, 2023). When BSs are adsorbed to an interface, they can create a fluid-like layer, marked by low dilatational moduli (Maldonado-Valderrama et al., 2008), possibly due to weak lateral interactions among the BS molecules within the adsorbed films (Matubayasi, Kanzaki, Sugiyama, & Matuzawa, 1996). Our research has verified this behaviour for the BSs used (Fig. 7). The E' recorded for the film formed with BS–13 (i.e., at a

concentration of 1.3 mM BSs, Table 1) under control lipolysis conditions without the enzyme (-L) was only 3.0 ± 0.7 mN/m. This can likely be ascribed to the rapid interchange of BS molecules between the interface and the bulk solution during the oscillation phase (Maldonado-Valderrama et al., 2011). When active lipolysis conditions were introduced (i.e., with the presence of lipase, +L), the E' value was observed to be even lower (2.1 ± 0.2 mN/m, Fig. 7), indicating that the surface-active lipolysis products contributed to a further decrease in the elasticity of the interfacial layer. As previously noted, the *in situ* generation of lipolysis products was evidenced by the significant drop in IFT seen upon introducing the enzyme in the BS–13 lipolysis experiment (Fig. 4a).

A stepwise decrease in BS concentration alongside an increase in PL concentration, achieved using BS/PL–9:4, BS/PL–6.5:6.5, and BS/PL–4:9 (Table 1) for the interfacial lipolysis experiments, resulted in the creation of progressively more elastic interfacial films. This progression is reflected in the ascending E' values recorded after lipolysis for these three BS/PL combinations: 3.7 ± 0.3, 5.1 ± 0.8, and 7.2 ± 0.9 mN/m respectively (Fig. 7). These findings strongly imply a simultaneous presence of both BSs and PLs at the interface. Furthermore, measurements of the elastic modulus following the lipolysis phase with PL–13 (i.e., at a concentration of 1.3 mM PLs, Table 1) yielded an even higher value of 12.8 ± 1.7 mN/m (Fig. 7). This result is in close agreement with previous findings for a PL-stabilised olive oil-water interface exposed to lipase (Torcello-Gómez et al., 2011), suggesting limited lipolysis under these experimental conditions.

The described rheological characteristics can likely be traced back to the diminished mobility of BSs at the interface due to the presence of anchored PLs. When present alone, PLs (such as phosphatidylcholine, PC) are inclined to form dense and elastic interfacial films composed of irreversibly adsorbed molecules with strong interactions, attributed to the two hydrocarbon chains anchoring PLs, and leading to a tightly packed layer (De Vleeschauwer & Van der Meeren, 1999), (Torcello-Gómez et al., 2011). However, less compact and more disordered arrangement of BS-PL mixed films has been found in studies involving BSs and PLs, which aligned with a reduction in dilatational elasticity (Chu et al., 2010), (Torcello-Gómez, Jódar-Reyes, Maldonado-Valderrama, & Martín-Rodríguez, 2012), (Pabois et al., 2019).

Similar to the findings described above for BS–13, in the two experimental configurations involving PLs (i.e., BS/PL–9:4 and PL–13), the E' values recorded following active lipolysis (+L) were consistently lower than those measured after the control lipolysis without the enzyme (-L) (Fig. 7). This, coupled with the IFT findings illustrated in Fig. 4 b,c, further validates the generation of surface-active lipolysis products in the presence of the enzyme and suggests their role in reducing the elasticity of interfacial films formed during the intestinal digestion of TGs.

The rheological properties of the interfacial films created during lipolysis in the presence of BS/PL mixtures (BS/PL–9:4, BS/PL–6.5:6.5, and BS/PL–4:9) were further highlighted following the experimental desorption step. During this phase, partial removal of adsorbed substances led to a notable increase in the E', reaching approximately 11–16 mN/m (Fig. 7). This is in stark contrast to the reduced modulus observed after applying BS–13 (4.5 ± 1.3 mN/m, Fig. 7), further suggesting the critical role of PLs in enhancing the elasticity of the interfacial layer. The increase observed for the BS/PL systems could be ascribed to the selective desorption of BSs in comparison to PLs. The replacement of the subphase with pure SIF created an environment that facilitated the easier removal of BSs, consequently resulting in an interfacial layer predominantly composed of PLs, which likely exhibited more resistance to desorption. The heightened E' values (Fig. 7) alongside the relatively low IFT values (Figs. 5 and 6) for the three BS/PL combinations post-desorption imply the establishment of a dense molecular arrangement at the interface, propelled by robust

intermolecular forces that limit the mobility of BSs. The persistence of PLs against desorption is corroborated by the rheological characteristics of the interfacial layer formed with PL–13, where the E' value post-desorption remained elevated and virtually unaltered compared to its post-lipolysis measurement (11.9 ± 0.9 mN/m versus 12.8 ± 1.7 mN/m, Fig. 7). This suggests that, in this case, the interfacial PLs, along with lipolysis products that may have been produced, predominantly remained at the interface following the substitution of the subphase with SIF. Such traits have previously been documented in research focusing on dilatational rheology pertaining to the PC interfacial layer at the n-decane–water interface (Mekkaoui et al., 2021). Moreover, our static measurements also confirm this, demonstrating that the final IFT recorded during the lipolysis phase with PL–13 remained largely constant even after the desorption phase was completed (Fig. 6). The aforementioned research by Mekkaoui and co-workers (Mekkaoui et al., 2021) also indicated that interfaces with either BS (sodium cholate) alone or in conjunction with PC exhibited lower dilatational modulus values compared to interfaces where only PC was used.

Fig. 7 additionally displays the E' values measured after the desorption step for control experiments involving BS–13, BS/PL–9:4, and PL–13, but without the use of lipase (-L). Broadly, the pattern in the progression of interfacial rheology mirrors the outcomes noted after enzyme usage (+L). The major deviation is that the E' values post-desorption in the latter scenario are lower than those measured in the absence of lipase. This suggests that the lipolysis products were not entirely removed during the desorption phase, thereby diminishing the elasticity of the interface relative to the elasticity analysed under the control conditions when the enzyme was not included in the experiment (i.e., when the surface-active lipolysis products were not generated).

3.3. Intestinal lipolysis of model food emulsions at different BS/PL proportions

Following the interfacial studies, we employed porcine pancreatin to investigate the lipolysis of TGs in emulsion by monitoring the release of FFAs during simulated small intestinal digestion. This approach is commonly used in static *in vitro* digestion studies (Grundy et al., 2021), (Brodkorb et al., 2019), (Minekus et al., 2014), (Menard et al., 2023). However, pancreatin – primarily applied as a source of pancreatic lipase in our study – may have also exhibited some limited PLA_2 activity. This could have resulted in the hydrolysis of PLs and, consequently, the production of additional FFAs. To account for this potential source of FFAs released during the lipolysis experiments conducted with added PLs or BS/PL mixtures (Table 1), corresponding control digestion experiments were also carried out in the absence of emulsion (Section 2.5.2). To assess the quantities of FFAs liberated exclusively from the emulsified TGs, the volumes of titrant (NaOH) employed in a control experiment were subtracted from the volumes required during the corresponding emulsion experiment (Supplementary Material, Fig. S2.1). The titrant volumes, obtained in this manner, were ultimately used to calculate the percentage of FFAs liberated solely from the emulsified TGs (Fig. 8). To ensure consistency across all pH-stat lipolysis experiments, control measurements were similarly conducted for digestion studies involving solely individual BSs (BS–13), as well as under conditions devoid of any biosurfactants.

As determined in the interfacial experiments (Section 3.2.), the BS/PL proportion deemed physiologically relevant for the postprandial intestinal lumen (i.e., BS/PL–9:4) resulted in a significantly lower IFT during lipolysis compared to the IFT observed for lipolysis with BS–13, or with other BS/PL ratios (P<0.05, Fig. 6). This likely contributed to the highest conversion of TGs to FFAs in the emulsion study employing BS/PL–9:4 (Fig. 8a). However, the superiority of BS/PL–9:4 over other BS/PL ratios in enhancing TG to FFA conversion became more

apparent as the lipolysis time increased. After 900 s of digestion, BS/PL–9:4 was the only ratio where the amount of released FFAs was significantly higher (P<0.01) compared to PL–13 (Fig. 8b). This difference became even more pronounced (P<0.001) when the lipolysis experiments were extended to 1800 s (Fig. 8c). At this point, the quantity of FFAs released from TGs in the presence of BS/PL–9:4 was also significantly greater (P<0.05) than that observed with the other two BS/PL ratios (BS/PL–6.5:6.5 and BS/PL–4:9, Fig. 8c). Additionally, using BS/PL–9:4 proved more effective than solely using BSs (i.e., BS–13) in facilitating lipolysis, particularly within the initial 3600 s (Fig. 8a). However, the difference in FFA release between the BS/PL–9:4 and BS–13 experiments was not statistically significant over this period (Fig. 8b,c,d). Beyond 3600 s, the trends in FFA release between these two systems were very similar for the remainder of the digestion experiments (Fig. 8a,e). Ultimately, after 7200 s of digestion, 57.8 ± 2.2% FFAs were liberated from emulsified TGs in the presence of BS/PL–9:4 (Fig. 8e) – an increase of approximately 9% compared to other studied BS/PL ratios (P<0.001), and about 20% higher than in control lipolysis without biosurfactants (P<0.001).

The combined findings from the interfacial and emulsion studies outlined above imply that, in addition to lowering IFT, the ability of biliary surfactants to easily desorb from the oil-water interface plays a vital role in the overall progression of lipolysis. This desorption process likely involves the solubilisation of lipolysis products from the interface, thereby enabling further interfacial hydrolysis of TGs (Macierzanka et al., 2019), (Acevedo-Fani & Singh, 2022). In our *in vitro* study, the most efficient desorption of post-lipolysis interfacial material occurred in the BS–13 lipolysis/desorption experiment, as evidenced by the highest Δ_{IFT} value (Δ_{IFT} = 5.9 mN/m, Fig. 6). Incorporating PLs and increasing their concentration in BS/PL mixtures resulted in a decrease in Δ_{IFT} (Fig. 6), correlating with the efficiency of emulsion lipolysis. Generally, lower Δ_{IFT} values, derived from interfacial experiments, corresponded to reduced TG to FFA conversion in the emulsion lipolysis experiments (Fig. 9). This PL-dependent correlation, evident in Fig. 9, persisted despite the interfacial experiments being conducted in the presence of purified pancreatic lipase, whereas the emulsion lipolysis study employed pancreatin, which, aside from lipase, may have also contained some PLA_2 (as explained, any potential PLA_2 activity was accounted for). Notably, even the exclusive use of PLs (i.e., PL–13) in emulsion digestion experiments significantly enhanced TG conversion compared to control conditions without biosurfactants (Fig. 8).

However, it is important to note that the addition of a small quantity of PLs to BSs does not seem to diminish overall FFA generation. In our study, this was evident in experiments using BS/PL–9:4. In this particular case, the synergistic effect of BS/PL–9:4 in most effectively reducing the IFT during lipolysis (Figs. 5 and 6) even appeared to enhance the conversion of TGs to FFAs during the initial 3600 s of emulsion digestion (Fig. 8a), as previously mentioned. After 3600 s, the extent of FFA release was comparable in experiments using BS–13 and BS/PL–9:4 (Figs. 8a,e), even though the Δ_{IFT} value for BS/PL–9:4 was lower than that for BS–13 (3.5 vs 5.9, Fig. 9). This implies that under physiologically relevant conditions of intestinal lipolysis, which involve the BS/PL–9:4 ratio, the combined effects of these two types of biosurfactants in lowering the oil-water IFT and their relatively good desorption from the interface contribute to efficient digestion of TGs. Collectively, these findings highlight the superior efficiency of the physiologically relevant BS/PL–9:4 ratio in facilitating intestinal digestion of TGs, compared to other biosurfactant formulations that do not represent physiological scenarios.

Despite the presence of PLs together with BSs in the intestinal content under physiological conditions of digestion, there has been a very limited number of scientific studies specifically examining the effect of both types of biosurfactants on intestinal lipolysis. Lykidis et al.

(Lykidis et al., 1997) demonstrated that PLs can act, in conjunction with BSs, as activators of pancreatic lipase. However, the positive effect of PL addition was only observed for BS concentrations well above 5 mM and for a PL/BS ratio lower than approximately 0.6. In our emulsion lipolysis study, these conditions are met for the BS/PL–9:4 ratio, which showed the most substantial increase in the release of FFAs from digested TGs compared to the control lipolysis conditions in the absence of biosurfactants (Fig. 8). Nevertheless, the authors of the cited report did not actually examine the digestibility of TGs under simulated intestinal conditions, nor did they assess the sole effect of PLs on enzyme activity. In a recent study by Ahn and Imm (Ahn & Imm, 2023), a modest increase in the extent of *in vitro* intestinal lipolysis was observed for medium-chain TG (MCT) emulsions stabilised by milk proteins and PLs, compared to those produced without PLs. However, in that study, PLs were primarily used in small quantities as an emulsion co-stabiliser. Simulated intestinal lipolysis of MCT emulsions stabilised with PLs (soybean lecithin) was studied by Mekkaoui and co-workers (Mekkaoui et al., 2021). They found that including 5 mg/mL BS (sodium cholate) in the digestion medium significantly increased the extent of FFA release from TGs digested with pancreatic lipase, compared to control experiments without BS. The limited lipolysis under control conditions was explained to be attributed to the formation of a compact film of PL molecules at the surface of the emulsion droplets, restricting enzyme access to the TG substrate. According to the authors, the addition of BS could mitigate the restricting effect of PLs by forming a mixed BS-PL interfacial film that enhanced lipolysis. However, the study did not present results of digestion in the absence of both BS and PL, thus the sole effect of PLs (whether inhibitory or catalytic) on the digestion of emulsified TGs could not be concluded from that study.

4. Conclusions

This study marks an important advancement in the comprehension of colloidal and interfacial phenomena crucial to the field of food and nutrition science. It specifically sheds light on the digestion of emulsified TGs within the context of varying BS and PL ratios. The innovative and comprehensive nature of this research is highlighted in its detailed examination of the interplay between BSs and PLs at the oil-water interface and its impact on lipolysis.

A central discovery of this research is the synergistic effect in reducing the oil-water IFT observed when combining BSs and PLs. This effect is particularly prominent at the BS/PL–9:4 ratio, identified as physiologically relevant in the postprandial small intestine. This ratio not only demonstrates the most efficient reduction in IFT during lipolysis but also the highest conversion of TGs to FFAs in emulsion lipolysis experiments. These findings underscore the crucial role of appropriate proportions of BSs and PLs in mimicking the physiological function of bile in intestinal lipolysis of emulsified TGs.

To accurately analyse the role of each compound in lipolysis, it is essential to consider both the absolute values of the IFT after lipolysis and desorption and the dilatational response of the resulting layers. This combined approach is crucial in revealing a comprehensive and representative picture of the FFA release. Our results indicate that the efficiency of desorption of interfacial material post-lipolysis correlates directly with the BS/PL ratio, diminishing as the PL proportion increases. This suggests that the presence and concentration of PLs considerably influence the rate and, and consequently the extent, of intestinal digestion. The focus of this study has been on thoroughly analysing the extent of lipolysis.

Moreover, this study presents novel insights into the desorption capabilities of BSs, PLs, and the lipolytic products from the interface. These findings bring attention to the importance of the desorption process, involving the solubilisation of lipolysis products from the interface, thus enabling further interfacial hydrolysis of TGs. In systems with PLs alongside BSs, a complex interfacial layer forms, which is more resistant to desorption. This has implications for the efficiency of intestinal lipolysis of emulsified TGs, especially at higher PL ratios.

In conclusion, our research offers a novel and comprehensive understanding of the interfacial dynamics in the digestive process, particularly in relation to food emulsions. The findings highlight the critical role of the appropriate BS/PL ratio in efficient digestion and underscore the synergistic effects of these biliary biosurfactants in intestinal lipolysis. This work lays a groundwork for future research and potential applications in developing functional foods and dietary strategies to optimise the digestion process. This research also demonstrates that *in vitro* investigations designed to replicate human digestive processes should incorporate the influences of both BSs and PLs to accurately reflect the physiological contributions of bile in the colloidal and biochemical dynamics of intestinal lipolysis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at ……

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Figures

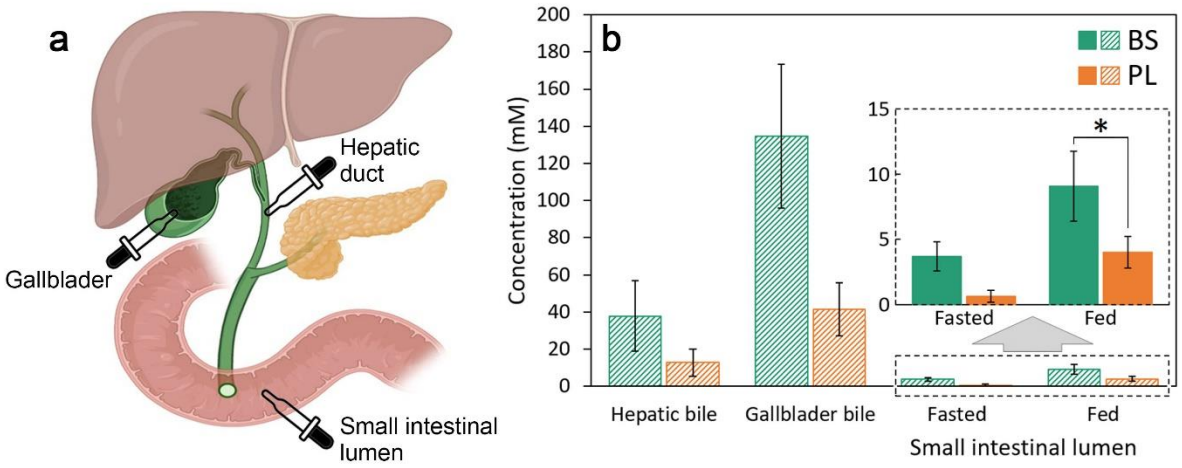


Fig. 1. Bile salt (BS) and phospholipid (PL) concentrations in the upper gastrointestinal (GI) system of adult humans. (a) Schematic representation of the upper GI system illustrating various locations (indicated by pipette symbols) for which concentrations of BSs and PLs in bile or intestinal content have been documented in the scientific literature. These locations encompass the gallbladder (the gallbladder bile), the hepatic duct (the hepatic bile), and the small intestinal lumen (image created with BioRender.com). (b) Analysis of scientific data compiled in accordance with Section 2.1; Total concentrations (means ± SD) of BSs and PLs in the hepatic bile, gallbladder bile, and the small intestinal lumen (in both fasted and fed states) of healthy adult humans. * P<0.01, by Student's t-test.

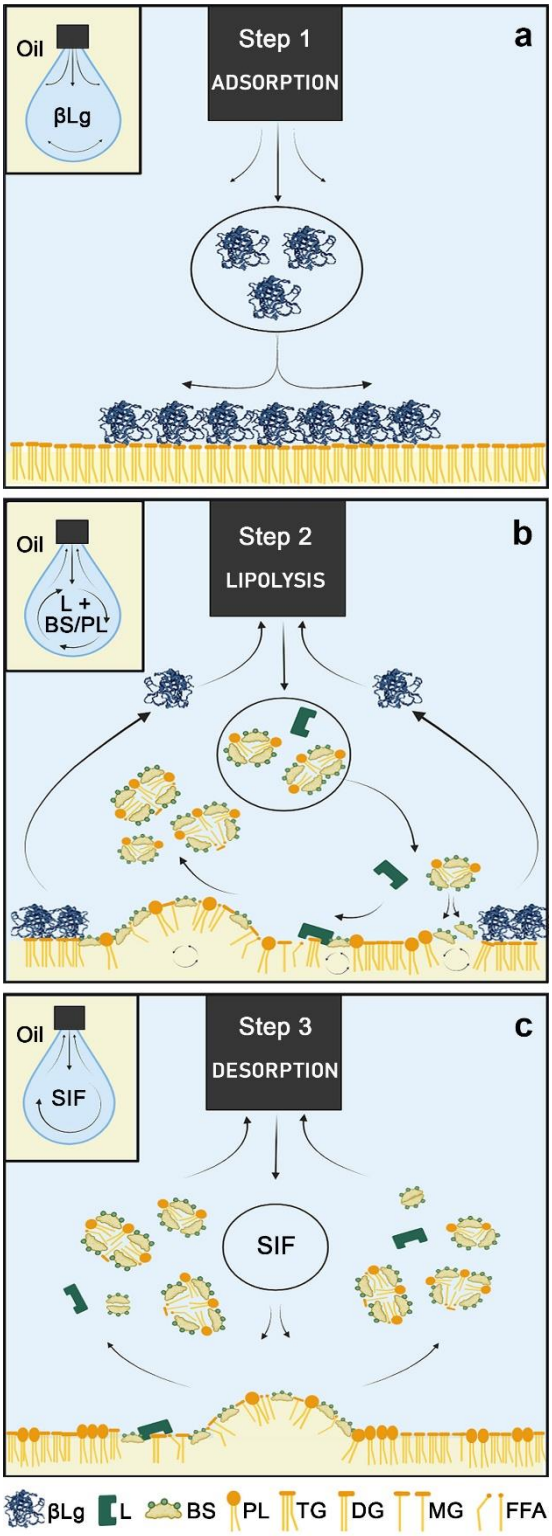


Fig. 2. The interfacial phenomena studied. Schematic representation of the sequential (a) protein adsorption, (b) lipolysis, and (c) desorption steps in the interfacial experiments carried out using the OCTOPUS – subphase multi-exchange pendant drop measuring system (Section 2.4). Schematic illustrations of interfacial phenomena pertaining to each experimental step are presented. Abbreviations: BS, bile salt; DG, diglyceride; FFA, free fatty acid; L, lipase; MG, monoglyceride; PL, phospholipid; SIF, simulated intestinal fluid; TG, triglyceride; βLg, β-lactoglobulin. Created with BioRender.com.

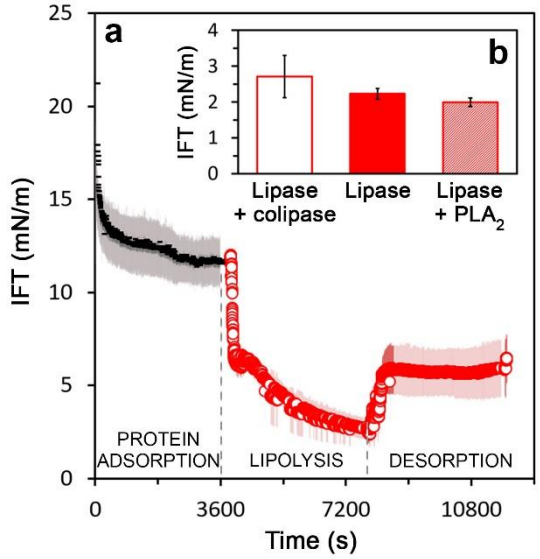


Fig. 3. Lipolytic enzymes in the interfacial experiments. (a) Evolution of the oil-water interfacial tension (IFT; mean ± SD) during the protein adsorption step (n = 18), the intestinal lipolysis step (n = 3), and the desorption step (n = 3); maintained at 37.0 ± 0.1 °C. The interfacial lipolysis experiment was conducted in the presence of pancreatic lipase and colipase, and using BS/PL–9:4 (Table 1). (b) Impact of intestinal lipolysis on the IFT; comparison of IFT values at the conclusion of the lipolysis step conducted for BS/PL–9:4 and in the presence of lipase and colipase ('Lipase+colipase'), lipase and PLA_2 ('Lipase+PLA_2'), or lipase only ('Lipase') (means ± SD, n = 3).

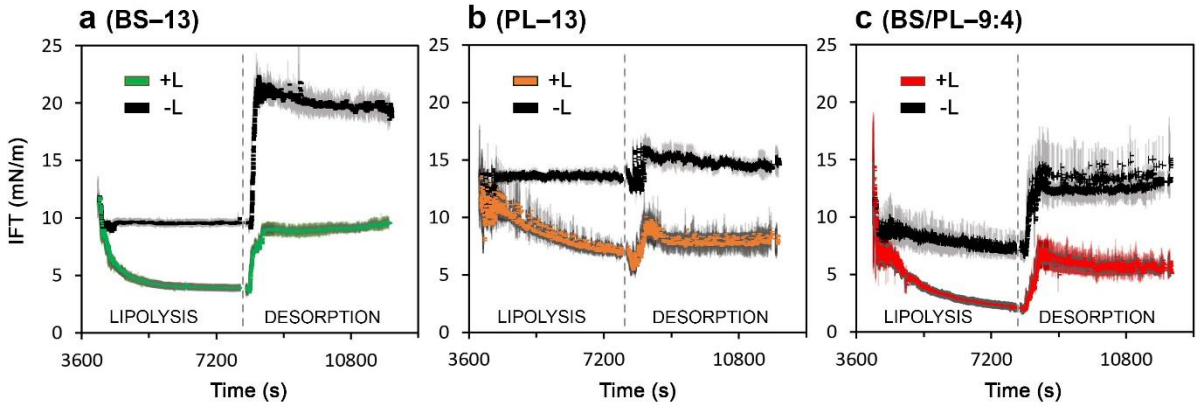


Fig. 4. Interfacial study: effect of the pancreatic lipase. Evolution of the oil-water interfacial tension (IFT) during the simulated intestinal lipolysis step conducted with pancreatic lipase (+L) or under control conditions without the enzyme (-L) (mean ± SD, n = 3 for each set of conditions). The lipolysis step was carried out in the presence of (a) BSs (BS–13), (b) PLs (PL–13), or (c) with both surfactants combined (BS/PL–9:4), as detailed in Table 1. Subsequently, the lipolysis step was followed by the desorption step (mean ± SD, n = 3 for each set of conditions). The initial step of the experiment – the βLg adsorption step – was consistently carried out, though it is not shown here; please see Fig. 3a for the protein adsorption results. All steps were conducted at 37.0 ± 0.1 °C.

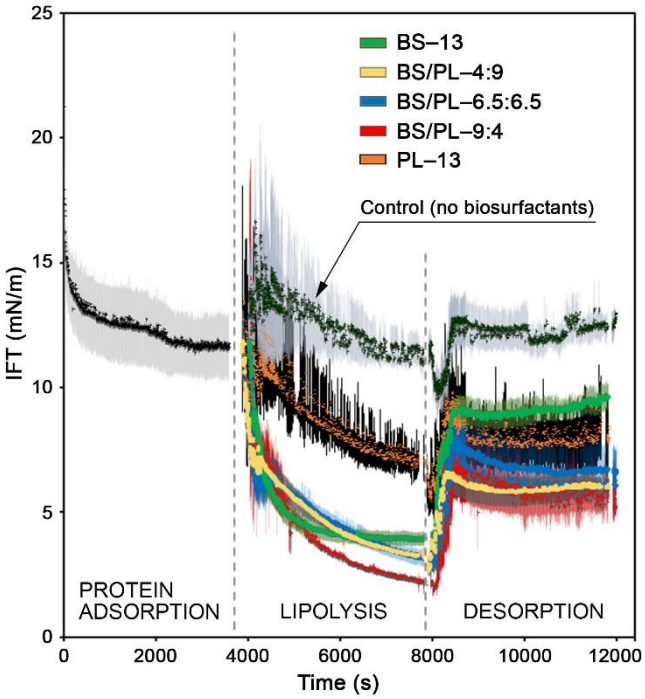


Fig. 5. Interfacial study: effect of the BS/PL ratio. Evolution of the oil-water interfacial tension (IFT; mean ± SD) during the βLg adsorption step (mean ± SD, n = 18), followed by the simulated intestinal lipolysis step with pancreatic lipase (mean ± SD, n = 3 for each condition), and the desorption step (mean ± SD, n = 3 for each condition). The lipolysis step was conducted in the presence of BSs or PLs (i.e., BS–13 or PL–13), at various BS/PL molar proportions (i.e., BS/PL–9:4, BS/PL–6.5:6.5, or BS/PL–4:9), or in the absence of the biosurfactants (Control), as detailed in Table 1. All experimental steps were conducted at 37.0 ± 0.1 °C.

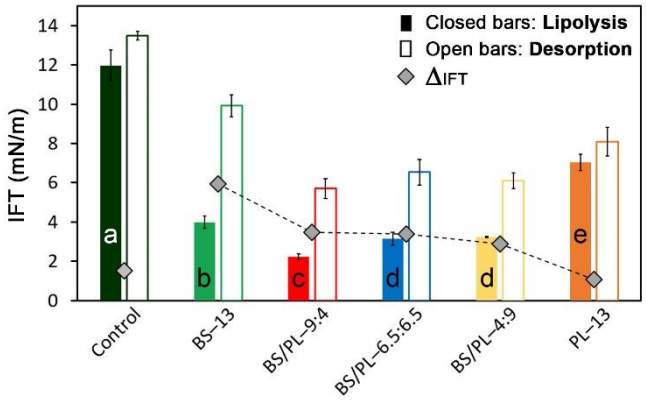
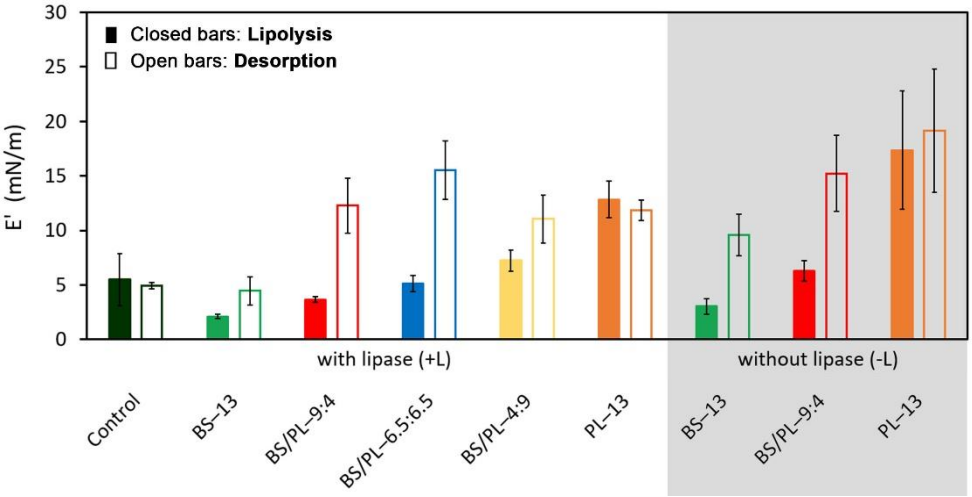


Fig. 6. Interfacial study: comparison of the lipolysis and desorption steps. Influence of BSs, PLs, and their respective ratios (as outlined in Table 1) on the IFT values acquired at the conclusion of the lipolysis step (closed bars) and the desorption step (open bars) (mean ± SD, n = 3 for each condition) in the experiments depicted in Fig. 5. Diamonds represent the relative difference in IFT between the desorption and lipolysis steps (Δ_{IFT}) for each set of experimental conditions (the line serves as a visual reference). Control shows data for the experiments conducted in the absence of biosurfactants. Bars displaying different letters indicate significant difference (* P<0.05).



1178

1179 Fig. 7. Dilatational rheology. Influence of BSs, PLs and their respective ratios (as outlined in Table 1)
1180 on the dilatational elasticity (storage modulus, E') measured at the conclusion of the lipolysis step
1181 (closed bars) and the desorption step (open bars) (mean ± SD, n = 3 for each condition) in the
1182 experiments depicted in Figs. 4 and 5. Control shows data for the experiments carried out in the
1183 absence of biosurfactants. Oscillations were conducted at 5% area deformation and a frequency of 0.1
1184 Hz.

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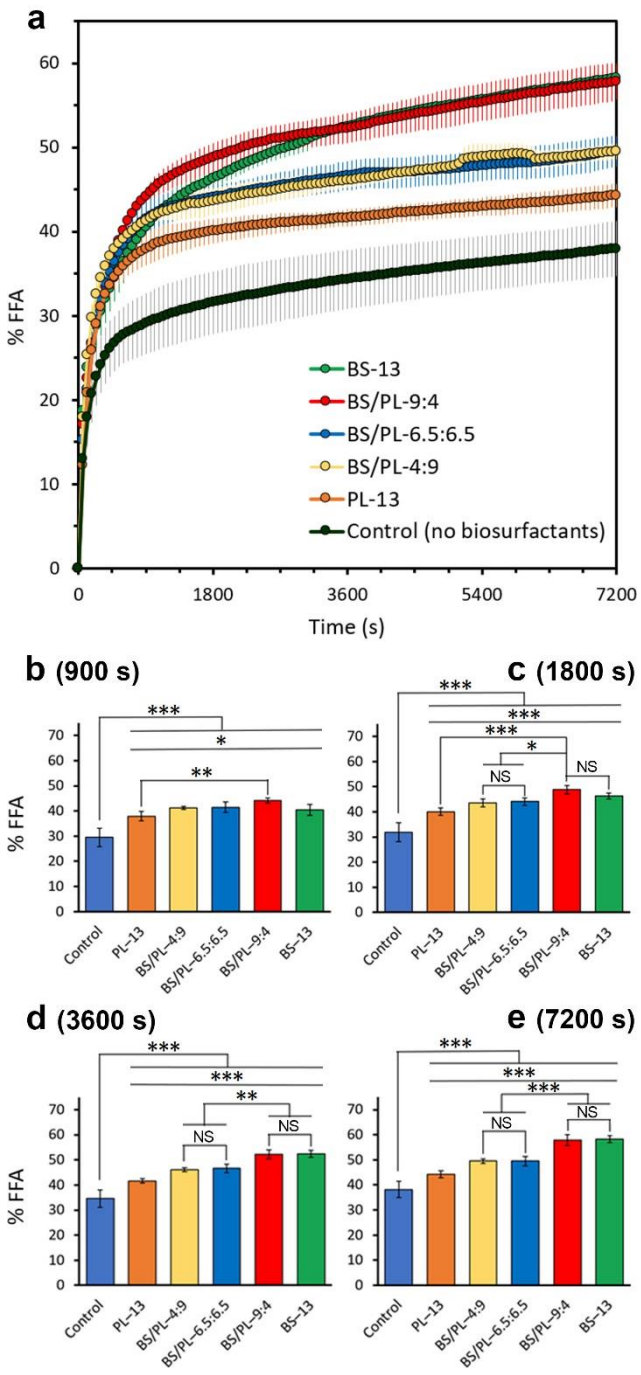
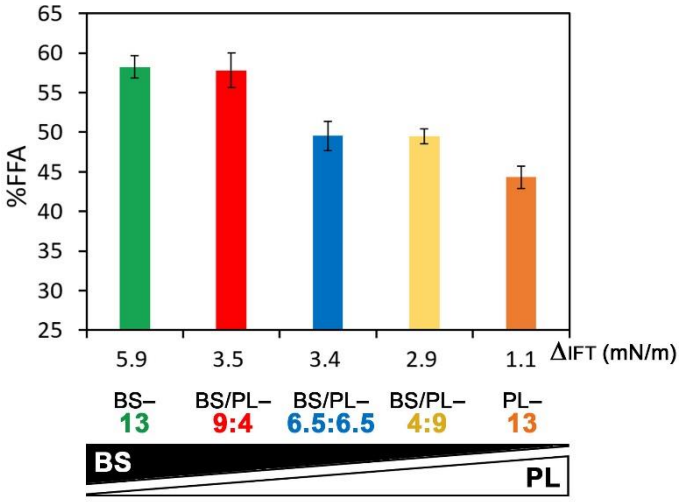


Fig. 8. Lipolysis in emulsion. (a) Progression of free fatty acid release (%FFA) from sunflower oil emulsions during *in vitro* small intestinal digestion conducted at different BS/PL molar ratios (refer to Table 1). (b–e) Statistical comparison of the lipolysis progress after 900, 1800, 3600 and 7200 s. Control lipolysis experiments without biosurfactants were also performed. All experiments were conducted at 37.0 ± 0.1 °C (mean ± SD, n = 3 for each condition). * P<0.05, ** P<0.01, *** P<0.001, NS (not significant, P>0.05).



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1195 Fig. 9. Combining results of the interfacial and emulsion studies. Correlation between the quantities of
1196 free fatty acids (%FFA) liberated from sunflower oil emulsions (after 7200 s of *in vitro* lipolysis; refer to
1197 Fig. 8e) and the diminishing Δ_{IFT} values derived from interfacial lipolysis/desorption experiments
1198 conducted for BS/PL mixtures with varying BS and PL molar ratios (from 13:0 to 0:13; refer to Fig. 6).
1199 Means ± SD, n = 3 for each condition.
1200

Tables

Table 1. Experimental conditions. (i) Molar proportions of the two types of biliary surfactants (BS/PL) and their concentrations under experimental conditions. (ii) Activities of intestinal lipolytic enzymes (U per mL of reaction mixture) in the interfacial and the emulsion *in vitro* lipolysis experiments.

BS/PL	**BS conc. (mM)**	**PL conc. (mM)**	**Lipase[a] or pancreatin[b] (lipolytic activity)**	**Colipase[a]**	**PLA_2[a]**
BS–13	1.3[a], 13[b]	0	2000 U/mL	-	-
BS–13	1.3[a], 13[b]	0	-	-	-
BS/PL–9:4	0.9[a], 9[b]	0.4[a], 4[b]	2000 U/mL	2:1 (w/w) lipase:colipase	-
BS/PL–9:4	0.9[a], 9[b]	0.4[a], 4[b]	2000 U/mL	-	170 U/mL
BS/PL–9:4	0.9[a], 9[b]	0.4[a], 4[b]	2000 U/mL	-	-
BS/PL–9:4	0.9[a], 9[b]	0.4[a], 4[b]	-	-	-
BS/PL–6.5:6.5	0.65[a], 6.5[b]	0.65[a], 6.5[b]	2000 U/mL	-	-
BS/PL–4:9	0.4[a], 4[b]	0.9[a], 9[b]	2000 U/mL	-	-
PL–13	0	1.3[a], 13[b]	2000 U/mL	-	-
PL–13	0	1.3[a], 13[b]	-	-	-
No biosurfactants	0	0	2000 U/mL	-	-

[a]used only in the interfacial lipolysis experiments (Section 3.2.), [b]used only in the emulsion lipolysis experiments (Section 3.3.).

The bile salt/phospholipid ratio determines the extent of *in vitro* intestinal lipolysis of triglycerides: Interfacial and emulsion studies

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S1. Materials and methods

S1.1. Literature search of PL and BS physiological concentrations

Table S1.1. Scientific articles reporting on the physiological concentrations of biliary surfactants (bile salts and phospholipids) in the hepatic bile, the gallbladder bile and in the small intestinal lumen of the adult human

Bile/Intestinal lumen	Literature references
Hepatic bile	1-3
Gallbladder bile	2-14
Small Intestinal Lumen, Fasted State	15-28
Small Intestinal Lumen, Fed State	20, 23, 25, 29-39

S2. Results

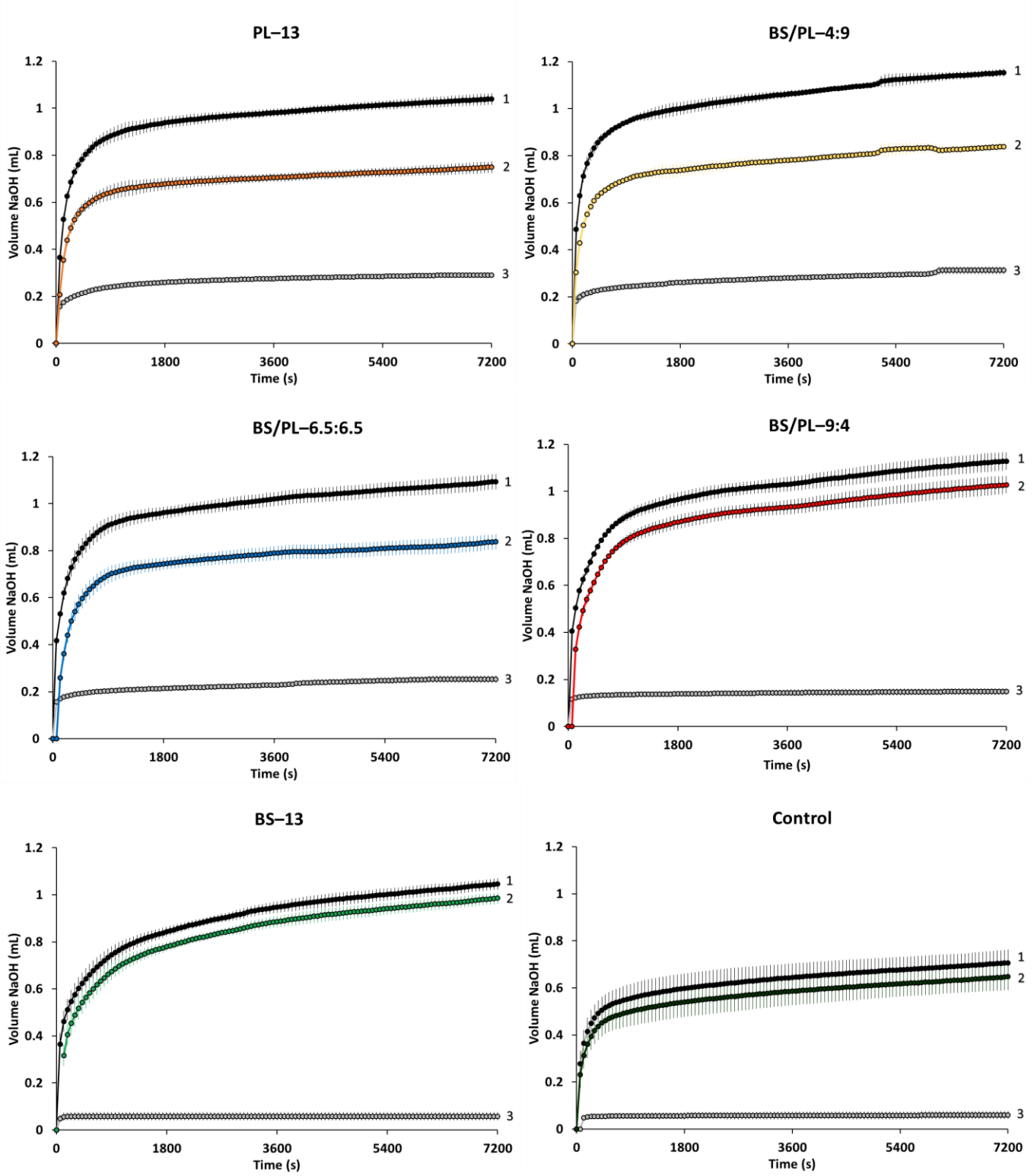

Fig. S2.1. The raw data obtained from pH-stat measurements during emulsion digestion under various BS/PL ratios; the volumes of NaOH titrant required to maintain a pH of 7.0 throughout the *in vitro* lipolysis experiments. The annotated numbers represent:
1 – Initial readings of TG emulsion digestion with pancreatin, conducted in the presence of PLs, BSs, BS/PL mixtures, or without any biosurfactants;
3 – Control measurements involving pancreatin but excluding the emulsion, where SIF was used in place of TG emulsion. These controls were also performed in the presence of PLs, BSs, BS/PL mixtures, or without any biosurfactants, similar to the initial measurements (1);
2 – The actual amounts of NaOH titrant used, calculated as the difference (**3 minus 1**), for neutralising the FFAs exclusively released from the TG emulsion during lipolysis. These data were used for calculating the results shown in Fig. 8.
The data are presented as the mean ± SD (n = 3) under each experimental condition.

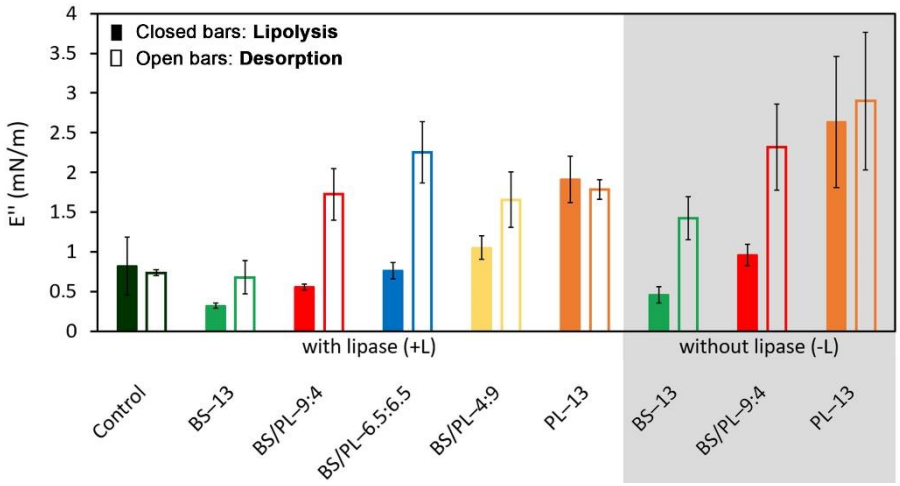

Fig. S2.2. Influence of BSs, PLs and their respective ratios (as outlined in Table 1) on the dilatational viscosity (loss modulus, E") measured at the conclusion of the lipolysis step (closed bars) and the desorption step (open bars) (mean ± SD, n = 3 for each condition) in the experiments depicted in Figs. 4 and 5. Control shows data for the experiments carried out in the absence of biosurfactants. Oscillations were conducted at 5% area deformation and a frequency of 0.1 Hz.

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