Załącznik nr 1/2 do Zarządzenia
Rektora PG nr 30 /2022 z kwietnia 2022 r.

GDAŃSK UNIVERSITY OF TECHNOLOGY

The author of the doctoral dissertation: Natalia Łozińska

Scientific discipline: Chemical sciences

DOCTORAL DISSERTATION

Title of doctoral dissertation: Effect of bile salts and their conjugation on the process of lipolysis

Title of doctoral dissertation (in Polish): Wpływ soli żółciowych i ich konjugacji na mechanism lipolizy

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Supervisor   Signature
dr hab. Christian Jungnickel

Gdańsk, 2024

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DESCRIPTION OF DOCTORAL DISSERTATION

The Author of the doctoral dissertation: Natalia Łozińska

Title of doctoral dissertation: Effect of bile salts and their conjugation on the process of lipolysis

Title of doctoral dissertation in Polish: Wpływ soli żółciowych i ich conjugacji na process lipolizy

Language of doctoral dissertation: English

Supervisor: Christian Jungnickel

Date of doctoral defense:

Keywords of doctoral dissertation in Polish: sole żółciowe, trawienie in-vitro, lipoliza, napięcie powierzchniowe, emulsja

Keywords of doctoral dissertation in English: bile salts, in-vitro digestion, lipolysis, interfacial tension, emulsion

Abstract of doctoral dissertation in Polish: Głównym celem rozprawy doktorskiej było wskazanie znaczenia koniugacji soli żółciowych na proces lipolizy, określenie czynników wpływających na zmianę składu soli żółciowych oraz ustalenie procesów kontrolujących szybkość lipolizy. Wyniki eksperymentów (modele trawienia in vitro, badania międzyfazowe) i meta-analizy danych literaturowych połączono w celu określenia najbardziej istotnych czynników wpływających na szybkość procesu lipolizy. Otrzymane wyniki wykazały, że kilka czynników, takich jak antybiotyki, stan chorobowy i skład mikroflory jelitowej, może wpływać na proces trawienia lipidów poprzez działanie soli żółciowych. Wykazano, że sprzężone formy soli żółciowych – taurochlorany sodu zwiększają uwalnianie wolnych kwasów tłuszczowych do znacznie wyższego poziomu niż nieskoniugowane formy soli żółciowych – dezoksycholany sodu, obecnych w naszym przewodzie pokarmowym. Taurochloran sodu wykazał większy potencjał adsorbcji na powierzchni kropli lipidu, zwiększając adsorpcję lipazy i sprzyjając procesowi emulgowania. Co więcej, taurochloran sodu potrzebował mniejszej liczby cząsteczek i stężenia środka powierzchniowo czynnego, aby stworzyć agregaty odpowiedzialne za zbieranie produktów lipolizy z interfazy olejowej. Lipoliza napędzana taurochloranem sodu może osiągnąć większe uwalnianie wolnych kwasów tłuszczowych dzięki szybszemu usuwaniu produktów lipolizy w procesie desorpcji, umożliwiając ciągły proces trawienia lipidów. Wykazano, że lipoliza jest kontrolowana przez stężenie skonjugowanych soli żółciowych poprzez modulację pięciu zidentyfikowanych procesów.

Abstract of the doctoral dissertation in English: The main aim of the PhD dissertation was to indicate the importance of conjugation of bile salts (BS) on the level of lipolysis, determine factors influencing the alteration of BS composition, and establish processes controlling the rate of lipolysis. Experimental results (in-vitro digestion models, interfacial studies) and meta-analysis of literature data were combined to determine the most influential factors affecting the rate of lipolysis. The results demonstrate that several factors such as antibiotics, disease state, and gut microbiota composition may affect the lipid digestion process via the action of BS. Conjugated forms of BS – sodium taurocholate (NaTC) were shown to enhance free fatty acids (FFA) release to a significantly higher level than unconjugated forms of BS – sodium deoxycholate (NaDC) present in our gastrointestinal tract. NaTC showed greater potential to adsorb to the lipid droplet enhancing the adsorption of lipase and promoting the emulsification process. Moreover, NaTC required fewer molecules and surfactant concentration to create aggregates responsible for incorporating lipolysis products from the oil interphase. The lipolysis driven by NaTC could achieve greater FFA release due to faster removal of lipolysis products via the desorption process, allowing the continuous process of lipid digestion. The lipolysis was shown to be controlled by the concentration of conjugated BS by modulation of five identified processes.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. hab. Christian Jungnickel, for his support, alike scientific and private, and encouragement to take on new challenges and achieve goals. I am very grateful for all his advice which built my personality, help, and patience. You were a great mentor for me throughout my studies.

I also would like to thank Prof. Julia Maldonado-Valderrama from the University of Granada, Department of Applied Physics for hosting me at her University and providing me the opportunity to conduct scientific research. I am very grateful for her support during my time in Spain.

I would like to thank all the people from the Department of Colloid and Lipid Science for their friendly environment, support, and willingness to share experience. Especially, I would like to thank Małgorzata Borecka who always found time to give advice and explain everything. Thanks to you, working in the department has always been a pleasure.

Chciałaym również podziękować mojej Mamie, której nieocenione wsparcie i wiara we mnie pozwoliły na ukończenie doktoratu.

Also, I want to thank my best friend Marianna Tomaszewska for her support and for constantly providing me with an incentive to act.

Dedications

Rozprawę doktorską dedykuję mojemu Tacie.

Table of Contents

Scientific achievements constituting a Doctoral dissertation
"Effect of bile salts and their conjugation on the process of lipolysis"

L.p	Title of scientific publication	MNiSW	IF
A1	**Łozińska, N.,** & Jungnickel, C. (2021). Importance of Conjugation of the Bile Salt on the Mechanism of Lipolysis. *Molecules*, *26*(19), 5764 DOI: 10.3390/molecules26195764	140	4.6
A2	Krupa, Ł., Staroń, R., Dulko, D., **Łozińska, N.,** Mackie, A. R., Rigby, N. M., ... & Jungnickel, C. (2021). Importance of bile composition for diagnosis of biliary obstructions. Molecules, 26(23), 7279. DOI: 10.3390/molecules26237279	140	4.6
A3	**Łozińska, N.**, Maldonado-Valderrama, J., Del Castillo-Santaella, T., Zhou, Y., Martysiak-Żurowska, D., Lu, Y., & Jungnickel, C. (2024). Bile conjugation and its effect on in vitro lipolysis of emulsions. Food Research International, 114255. DOI: 10.1016/j.foodres.2024.114255	140	8.1
	Summary	420	17.954

Scientific achievements that do not constitute a doctoral dissertation

L.p	Title of scientific publication	MNiSW	IF
A4	**Łozińska, N.**, Głowacz-Różyńska, A., Artichowicz, W., Lu, Y., & Jungnickel, C. (2020). Microencapsulation of fish oil–determination of optimal wall material and encapsulation methodology. *Journal of Food Engineering*, *268*, 109730. DOI: 10.1016/j.jfoodeng.2019.109730	140	5.7
A5	Jungnickel, C., & **Łozińska, N.** (2019). Predicting the Environmental Fate of Ionic Liquids, Article Chapter 51-1 , 1-10. DOI: 10.1007/978-981-10-6739-6_51-1	NA	NA

Abbreviations

BA – bile acids
BS – bile salts
BSH – bile salt hydrolase
CMC – critical micelle concentration
FFA–free fatty acids
MSR – molar solubilisation ratio
NaDC – sodium deoxycholate
NaTC – sodium taurocholate
NaGCDC – sodium glycochenodeoxycholate
NaGDC – sodium glycodeoxycholate
PC – primary conjugated
SU – secondary unconjugated

1. Introduction

The increasing problem with obesity in the last decades (recent statistics have shown that the obesity problem increased by up to 50% in Europe (Stival et al. 2022) and 55% in Poland (Rychlik et al. 2022)) highlight the importance of the digestion process, as the controlling factor of calorie uptake. Essential nutrients can be used for energy, repair of cells, and growth, etc are obtained during the digestion process by breaking down food. Absorption of food and the final stage of digestion takes place in the small intestine. One of the main components of our diet is lipids, which are broken done through the process of lipolysis. This process uses BS as the key factor responsible for emulsification and creating micelles which may transport digestion products to our body.

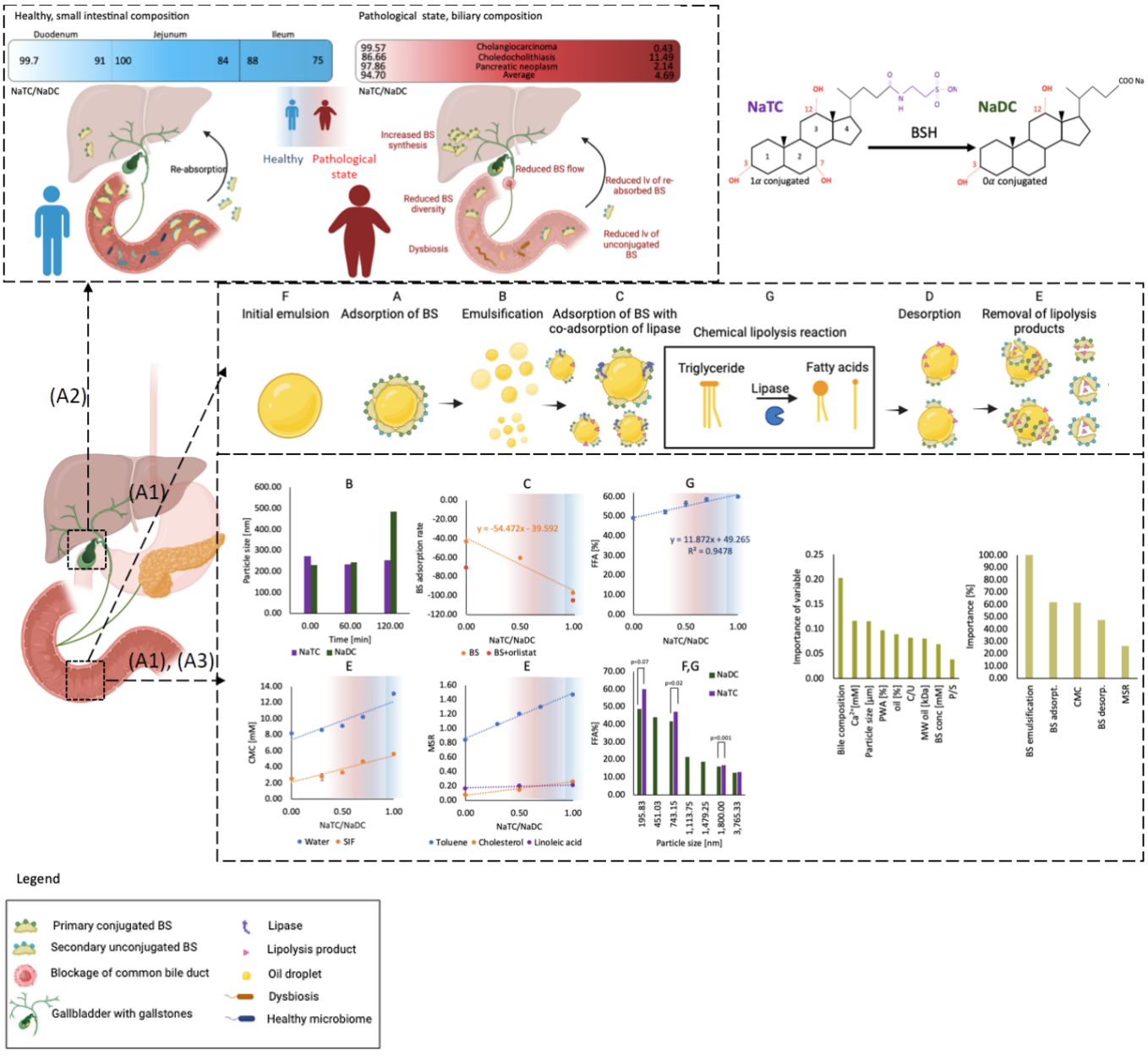


Figure 1 The workflow presents the graphical representation of the results from three scientific articles covering the subject of the dissertation. Publication A1 presents the steps of the lipolysis process: initial emulsion, adsorption of BS and lipase/colipase complex, emulsification process, desorption, and removal of lipolysis products. Each of the steps of lipolysis (A-G) is linked with results from publication A1 and publication A3. The reddish-blue shadow behind the graph shows changes in BS ratio and corresponding alteration of results concerning healthy and pathological states. Publication A2 represents

changes in BS composition in healthy and pathological patients concerning four diseases: cholangiocarcinoma, choledocholithiasis, and pancreatic neoplasm. The composition of BS in pathological patients is disturbed due to the reduction of the flow of BS which results in alteration of gut microflora composition. BS- bile salts, NaTC – sodium taurocholate, NaDC – sodium deoxycholate, CMC – critical micelle concentration, MSR – molar solubilisation ratio, PWA – protein weight average, MW – molecular weight, Ca – calcium, FFA – free fatty acids, C/U – conjugated/unconjugated, P/S – primary/secondary, SIF – simulated intestinal fluid, BSH – bile salt hydrolase.

In our first publication (publication A1) we performed the meta-analysis for three main defined lipolysis parameters: (1) critical micelle concentration (CMC), (2) aggregation number, and (3) molar solubilization ratio (MSR) for four types of BS: Primary conjugated (PC) and unconjugated and secondary conjugated and unconjugated. Our analysis revealed that the type of BS influences the main parameters of the lipolysis process. Further analysis revealed that bacterial transformation in the small intestine results in deconjugation of PC BS into secondary unconjugated (SU) BS. Therefore, representatives of two predominant forms of BS in our gastrointestinal tract were chosen for further analysis.: PC – sodium taurocholate (NaTC) and SU – sodium deoxycholate (NaDC). We used the static pendant droplet technique to screen the difference in interfacial properties of BS. Finally, the in-vitro digestion model was used to check the extent of lipolysis influenced by NaTC and NaDC. Our results indicated that NaTC and NaDC yield significantly different surface activity properties and could influence lipolysis efficiency to a significantly different extent. NaTC was shown to promote the release of free fatty acids (FFA) to a higher extent than NaDC.

Our second publication (publication A2) discussed BS as a disease indicator and also as a factor that is sensitive towards changes in health state. The data collected on the composition of BS during different diseases state: cholangiocarcinoma, choledocholithiasis, pancreatic neoplasm, and stricture have shown significant change concerning the concentration of BS in healthy individuals. The reason for the significant alteration of BS composition was mostly connected with blockage of the flow of BS to the small intestine providing a decreasing concentration of re-absorbed BS and by modulation of molecular receptors increasing BS synthesis. Even though the analysis showed that BS is not specific enough to serve us markers, the results bring attention to the connection between disease development and the possible effect on the lipolysis process due to significant changes in BS concentration. Moreover, the performed analysis revealed that reduced BS concentrations in the small intestine resulted from the development of disease state, and enhanced BS synthesis due to absorption of low concentrations of BS. Normal BS synthesis results in a concentration of BS in the small intestine in the range of 5-10mM (Naso et al. 2019). Increased BS synthesis leads to the formation of excessive concentration of BS, which is correlated with two effects: (1) a greater concentration of conjugated BS in the small intestine may significantly enhance the rate of the lipolysis process which can promote the development of the obesity problem and (2) a formation of excessive concentration of unconjugated BS by intestinal bacteria, which may further disturb BS synthesis and promote development of diseases connected with the toxicity of SU BS.

Finally, our last publication (publication A3) aimed to develop the main outcomes and conclusions from two previous publications: publication A1 and publication A2. First of all the idea of the dominant process during lipolysis was developed including adsorption, co-adsorption of lipase, desorption, formation of micelles, and MSR of lipolysis products. The influence of particle size of initial emulsion in a high range of 200-3800nm was also investigated, to consider the possible variation of delivered food. Results also indicated that the efficiency of previous stages, gastric digestion, also influences the final rate of lipolysis. Moreover, the results showed that with decreasing particle size of the digested emulsion, the efficiency of digestion increases. Duodenum consists of 98% of conjugated BS, it is also where most lipolysis takes place. Conclusions from publication A2 allowed us to consider additional factors influencing lipolysis efficiency, such as the development of disease state, but also the possibility of consuming antibiotics connected with specific diseases and resulting changed BS profile in the small intestine. Therefore, all experiments in publication A3 were performed within the whole range of NaTC/NaDC ratio assuming 0 NaTC/NaDC (100% NaDC) and 1 NaTC/NaDC (100% NaTC). The digestion process was studied by performing experiments covering in vitro digestion models (release of FFA) and surface science (interfacial tension measurements). To determine the most influential parameter controlling the lipolysis process the meta-analysis was performed covering the lipolysis process for single systems (pure BS) and complex systems (BS of various animals). All of the previously collected data, as well as analysis of meta-analysis results, allowed us to consider emulsification as the predominant step. BS by modulating five separate processes have been shown to influence the lipolysis process. Results revealed the possibility of controlling the rate of lipolysis by modulating lipolysis processes.

Modulating the food digestion process is a worldwide challenge. Digestion of the lipid component may be controlled either by altering the food structure or modulating the digestion process. BS in respect to their form may demonstrate different properties, which will influence the final rate of lipolysis and regulate nutrient absorption. The impact of the BS on the lipolysis efficiency is modulated by five processes. The results of publication A1, publication A2 and publication A3, as shown in Figure 1 indicated BS as the agent's modulation of the lipolysis process.

1.1. Digestion of lipids

The research of publication A1 and publication A2 includes the digestion of lipid droplets and the complexity of their evolution during the lipid digestion process. Unravelling the mechanism of lipid digestion gives the potential to modulate the lipolysis process. The research has been focused on lipid digestion, as lipids are common diet components. Humans need to eat as our bodies require sources of energy and building blocks that we cannot provide for ourselves. Digestion is a complex and long process, which starts when we consume food and is responsible for processing material to the form that is useful for our organism. Lipids are an important part of our diet as they are responsible for delivering energy to our body and enable absorption of vitamins A, D, E, and K. They are also widely available and are ingredients of many food products. However, our body cannot just absorb lipids in the form as is delivering to our body. Instead, it needs to be broken down into preliminary parts which we can absorb.The research covered in this work covers small intestinal digestion of lipids, however, the potential impact of digestion in the mouth and stomach were also considered (particle size reduction), as they influence the final efficiency of digestion

1.2. Digestion in the mouth

Consumed food, starts to be digested in the mouth when saliva is released, which is a neutral fluid composed of a mixture of proteins and minerals (Bansil and Turner 2006). The emulsion is destabilized due to the flocculation and coalescence process.(Vingerhoeds et al. 2005; Silletti et al. 2007a, b). During the limited time that food bolus spends in the mouth, the exposure to mastication and temperature results in phase inversion of the emulsion.

1.3. Gastric digestion of lipids

Before small intestinal digestion, food enters the stomach, where it is exposed to the acidic environment created by gastric juices, which results in a decrease in the colloidal stability of the emulsion due to the electrostatic screening of protein. Digestion of lipids in the stomach is driven by gastric lipase which is characterized by high-range activity (pH 3-7) in comparison to intestinal lipase, with an optimal working pH of 6.5 (Hamosh 1990; Carriere et al. 1993; Porter et al. 2007). The optimal activity of gastric lipase was estimated to be around pH 5.4.(Carriere et al. 1993). Therefore digestion of the lipids in the stomach occurs mostly during the first hour, later on, the consumed product may disturb pH level and reduce lipase activity. One hour may not yield enough time to digest lipids, resulting in only partially digested products. The gastric lipase hydrolyses dietary lipids into fatty acids and diglycerides. Gastric hydrolysis yields 5-30% of the total lipid digestion(Armand et al. 1999). Gastric digestion helps to emulsify lipid droplets and, therefore, may enhance the efficiency of further intestinal digestion by increasing the surface area of the lipid droplet. However, gastric hydrolysis yields 5-30% of the total lipid digestion (Golding and Wooster 2010). In the case of emulsions that were tested during my research gastric hydrolysis would not have a significant effect, as the emulsions could be fully digested during the intestinal process. Moreover, partially digested emulsions from the mouth and gastric phase could hide the full action of BS and, therefore were not considered during research.

1.4. Small intestinal digestion of lipids

When food is moved from the stomach to the intestine, pH rapidly increases due to the secretion of alkaline bile juice. Intestinal digestion of lipids is driven by pancreatic lipase(Carriere et al. 1993). The lipolysis process promotes breaking down lipids into FFA and glycerol with the assistance of BS – the key factors responsible for the digestion and absorption of lipids.

The lipolysis process starts when BS is transported to the small intestine. The first role of BS is to improve the adsorption of lipase by increasing the accessibility of the oil droplet. BS acts as an emulsifying agent and increases the surface area of the lipid droplet (Macierzanka et al. 2014). BS are also responsible for displacing the lipolysis material from the oil interface, therefore promoting the adsorption of the lipase(Torcello-Gómez et

al. 2011). BS also play an important role in the transportation of FFA generated during the lipid digestion process to enterocytes where they are absorbed (Maldonado-Valderrama et al. 2011).

BS are multi-tasking biosurfactants, necessary during the digestion process of lipids. Due to its complex and rate-limiting functions during the digestion process, BS were the main interest of our research. Although it was observed that lipolysis is controlled by different parameters acting simultaneously, still there insufficient data to indicate a distinct rate-limiting factor. More detailed research on the relation between lipolysis rate and type of BS should be investigated to understand their contrasting role in this process. The behaviour of the BS is associated with their molecular structure, therefore, understanding the source of their action would give perspective to control the lipolysis process.

1.5. Nature of BS

In the third century, Hippocrates created a concept of the human body being composed of four "humours", consisting of two biles (Guzior and Quinn 2021). The four humours were referred to as *blood*, *phlegm*, *yellow bile*, and *black bile* (Thompson and Turner 1913; Goodacre and Naylor 2020). According to Hippocrates' idea, the body is healthy when humours are balanced and a disease state develops when any of the "humour" is in excess or deficiency (Goodacre and Naylor 2020). Greek physician, Galen, developed the Hippocrates idea by describing personalities with unbalanced "humours". A person with an excessive concentration of *yellow bile* was named choleric and one with an excessive concentration of *black bile* was named melancholic (Goodacre and Naylor 2020). Still, since then we have been trying to understand and we are developing ideas of how the BS ratio influences our health state. The role and importance of BS was more widely understood by discovering the structure of BS.

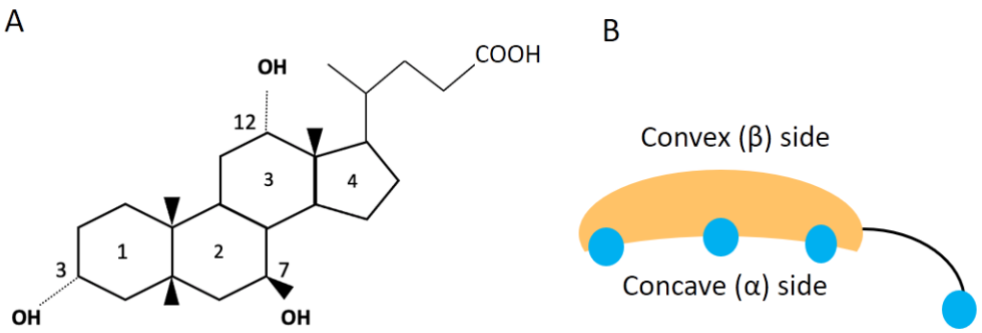


Figure 2 A Structure of BA. Cholic acid is synthesized from cholesterol in the liver and is known as primary unconjugated BS. BS consists of 4 rings. B Planar polarity of BS. The hydrophobic part is located on the convex side (yellow part) consisting of methyl groups and the hydrophilic part is located on the concave side (blue part) consisting of hydroxyl groups. This yields the unique structure of BS, different from standard surfactants.

Over the years the knowledge about BS expanded and the concept of "humour" evolved, and an understanding of the structure and real impact of BS on the human body emerged. The discovery of the chemical structure of BS in 1932 was the breakthrough moment, which enabled further research development (Hofmann and Hagey 2014). BS are surface active, steroid and ionic compounds with an amphiphilic nature (Moghimipour et al. 2015). As shown in Figure 2 A they consist of a steroid skeleton, composed of four rings, three six-carbon rings (1-3) and five carbon rings (4). Differently from traditional surfactants, mostly consist of a polar head and non-polar tail (Holm et al. 2013), BS possessed planar polarity, as shown in Figure 2 B (Warren et al. 2006). The hydrophilic part of BS with hydroxyl groups is located on the concave (α) side and the hydrophobic part with methyl groups is located on the convex (β) side. Different types of BS differ by the number of hydroxyl groups and functional groups, which were shown in publication A1 and publication A3 to have a direct influence on the lipolysis efficiency process. Therefore their concentration and type are of high importance.

1.6. Enterohepatic circulation and alteration of BS structure

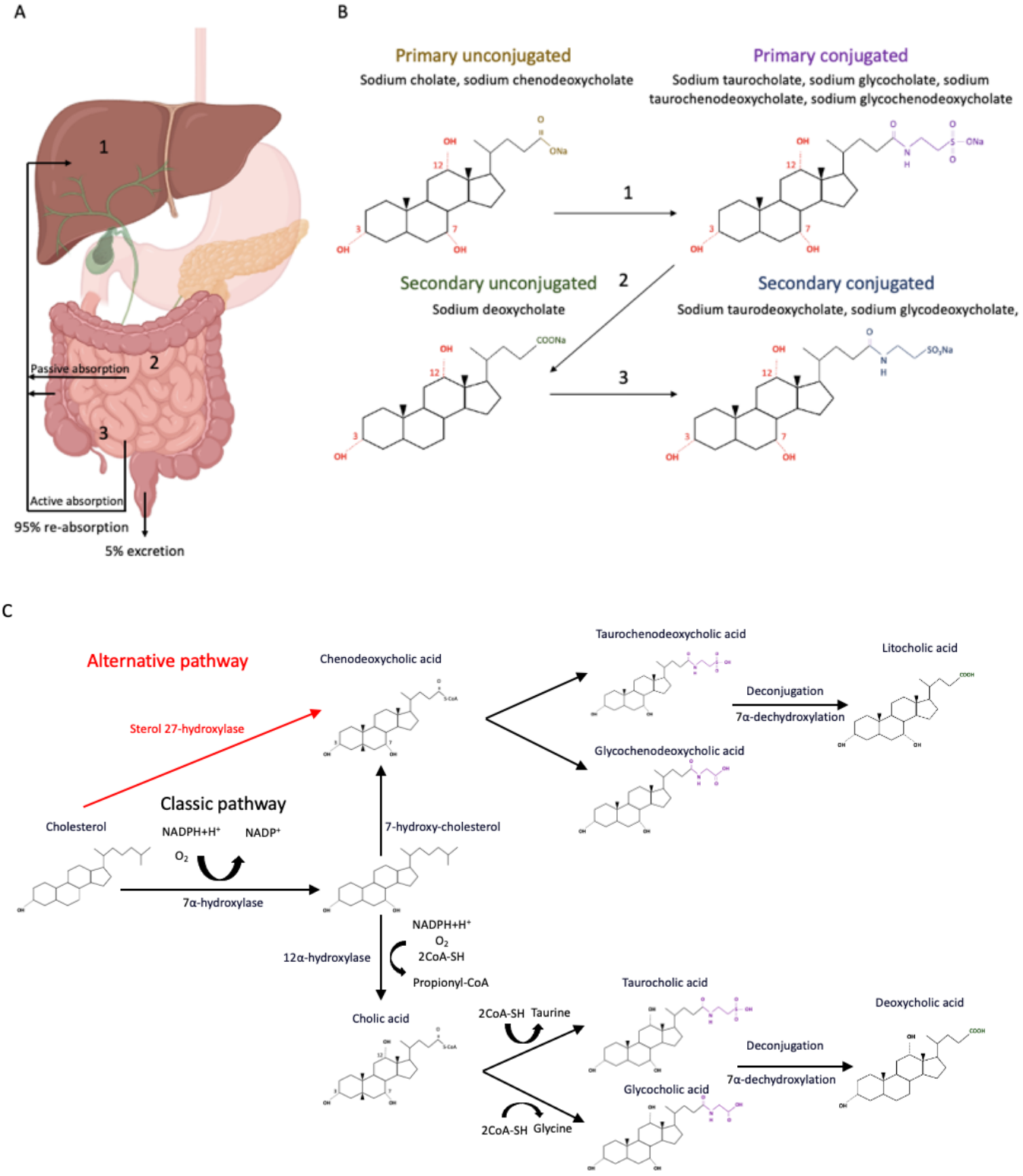


Figure 3 A. Enterohepatic circulation of BS in our gastrointestinal tract (Ridlon et al. 2006). *B. Structures of exemplary four predominant forms of BS in our organism. The number above the arrows corresponds to the transformation processes of BS taking place in marked locations. C. Schematic representation of bile acid synthesis from cholesterol as shown by Moghimipour et al.* (Moghimipour et al. 2015). *Classic pathway represents the formation of cholic acid and chenodeoxycholic*

acid from 7-hydroxy-cholesterol and alternative pathways, knows also as acidic pathways, represent the formation of chenodeoxycholic acid from cholesterol.

The disease state results in changes in BS composition, which in publication A2 was concluded to be an effect of blockage of BS flow. The BS enterohepatic recirculation mechanism controls the synthesis of BS. Increased or reduced concentration of BS synthesis was linked to alteration of lipolysis efficiency. Therefore the proper BS flow and re-absorption are crucial for our organism.
Bile acid (BA) are synthesized from cholesterol in the liver by two possible pathways. Neutral pathways, also known as classical pathways are responsible for synthesizing almost 90% of BA from the liver, while alternative pathways synthesize about 10% of BA. BA either cholic acid or chenodeoxycholic acid are formed from cholesterol, as shown in Figure 3C (Moghimipour et al. 2015). The conjugation of primary BA with glycine or taurine is catalyzed by BA CoA: amino acid N acyltransferase , created respectively by taurocholic acid, glycocholic acid, taurochenodeoxycholic acid and glycochenodeoxycholic acid (Ridlon et al. 2006; Chiang and Ferrell 2018), as shown in Figure 3A number 1. Healthy human bile consists of 75% of glycol-conjugated BS and 25% of tauro-conjugated BS. In the small intestine, BA is transformed into BS due to deprotonation. PC BS is further transformed in the small intestine by bacterial action to create secondary forms, as shown in Figure 3B. The enterohepatic recirculation process controls the flow of BS through our body. Disturbance of the flow of BS, as it was shown in publication A2, leads to a disorder of BS synthesis which is correlated with changed concentration of BS in the small intestine, alteration of the efficiency of the lipolysis process, and development of disease state.

1.7. Effect of changing microbiota on BSH

Intestinal microbiota plays a crucial role in our organism by ensuring a healthy balance (Shreiner et al. 2015). One of its main roles is the deconjugation process of BS. The deconjugation process is known as the removal of the amino acid side chain and as a result, the secondary BS are created (Begley et al. 2005). The formation of secondary BS is catalyzed by bile salt hydrolase (BSH). Most of the bacteria with the ability to promote the deconjugation process are gram-positive bacteria: *Lactobacillus, Enterococcus, Bifidobacterium, Clostridium,* and *Bacteroides spp* (Urdaneta and Casadesús 2017) with the exceptions of two strains of gram-negative Bacteroides (Begley et al. 2006).
There is a strong relationship between BS and gut microbiota composition, as one of the roles of BS is to control gut microbiota composition and the intestinal microflora influence the BS pool size (Sayin et al. 2013). For this reason, the alteration of BS-microflora homeostasis may result in the alteration of BS composition and the development of dysbiosis. Overgrowth of bacterial species not possessing BSH over another one may lead to a change in the ratio of conjugated/unconjugated BS. Therefore, modification of gut microbiota species may influence the concentration of BS. One of the most common reasons for overgrowth the of bacterial species without BSH over another one is antibiotics.

1.8. Antibiotics and gut microbiota

Consumption of antibiotics has become a global trend (Klein et al. 2018). In 2000-2015 using of antibiotics increased up to 65% globally (Nandi et al. 2023). Poland is a country with one of the highest rates of antibiotic consumption (Wojkowska-Mach et al. 2018). Antibiotic utilization was shown to reduce microbial diversity (Ianiro et al. 2020). Results presented by Palleja et al. (Palleja et al. 2018). have shown that consumption of antibiotics by adults results in an increased concentration of *Enterobacteriaceae* and a reduced concentration of *Bifidobacterium* Therefore, it is important to note that antibiotics by themselves do not necessarily decrease the overall number of bacteria but they lead to alteration of their diversity (Duvallet et al. 2017). Bacteria which are sensitive to antibiotics may be eliminated and antibiotic-resistant bacteria can multiply and replace them.
Moreover, BSH activity can be reduced by the administration of antibiotics(Wang et al. 2012). Smith et al. (Smith et al. 2014) have shown the impact of different antibiotic classes on the potential to inhibit BSH. Recent studies have revealed that the concentration of *Lactobacillus*, the main bacteria strain possessing BSH in our small intestine was reduced in the presence of antibiotics (Dumonceaux et al. 2006; Guban et al. 2006). Khodakivskyi et al. (Khodakivskyi et al. 2021) examined the alteration of BSH based on bioluminescence image due to deconjugation of BS. The result revealed the potential of antibiotics to reduce BSH of about 30% of gut microbiota. Furthermore, antibiotics, by changing gut microflora composition, were linked to the development of obesity (Vallianou et al. 2021). Antibiotics are also used as therapeutic agents. Children with malnutrition are treated with *amoxicillin* which results in weight gain (Francis et al. 2023). Studies performed on mice concerning transferring microbiota from obese adult twins to germ-free mice resulted in weight gain of mice (Lange et al.

2016). Antibiotics are external factors, which may influence the composition of the gut microflora and consequently, indirectly influence the BS composition, leading to disturbance of the enterohepatic recirculation system.

1.9. BS and obesity

The research covers the topic of the digestion process of food. However the diet is a key parameter as it may lead to the development of disease, therefore we should control the process of food modulation in our organism. Obesity appears to be a worldwide problem that negatively influences our body development. The diet was considered as a direct factor influencing weight profile. High-fat diet enhances obesity development, while a diet rich in vegetables results in a healthy homeostasis (Sakamaki et al. 2005). Diet was also proven to impact the gut microbiota diversity and complexity, by changing intestinal environmental conditions and influencing the BS composition (Scott et al. 2013).

Except for diet, obesity may be also promoted by changes in microbiota composition, induced for example by taking antibiotics or the development of disease (Li et al. 2021). Antibiotics were considered to reduce the composition of gut microflora and decrease the activity of BSH. (Guban et al. 2006). This reduces the concentration of deconjugated BS and increases the C/U ratio.

Results presented in publication A1 have shown that conjugated BS enhance the lipolysis process to a higher extent than unconjugated BS. The formation of high concentrations of conjugated BS over unconjugated ones may result in the unbalanced process of lipid digestion. Conjugated BS, as it was shown in publication A3, has a faster adsorption rate on the oil droplet than unconjugated BS results in greater surface area and ensures a more effective lipolysis process. Moreover, conjugated BS may more effectively remove lipolysis products through the desorption process than unconjugated BS, which gives more space for another BS to adsorb to the oil droplet and continue the digestion of lipids. Excessive lipolysis rate promoted by conjugated BS may result in the development of obesity. Exorbitant conjugation levels may be considered as a factor contributing to the obesity problem due to the enhanced lipolysis process. Moreover, excessive concentration of conjugated BS may disrupt BS synthesis resulting in reduced BS concentration. Unbalanced BS concentration and disturbed BS synthesis, as shown in publication A2, results in the development of diseases, such as gallstone formation or choledocholithiasis.

Several factors induce changes in BS concentration, by affecting intestinal microbiota, which are responsible for lipolysis efficiency. Therefore, it can be concluded that BS play an important role as a factor controlling the development of obesity by modulating the calorie uptake.

1.10. Role of BS in the lipolysis process

Digestion of lipids takes place in the stomach by hydrolysis of lipids driven by gastric lipase, however, this process was estimated to occur to a limited extent (10-30%). Predominantly (70-90%) lipolysis process takes place in the small intestine due to the hydrolysis of lipids by lipase (Maldonado-Valderrama et al. 2011). Therefore, the research covers the lipid digestion process in the small intestine.

Lipolysis is a well-known and broadly studied process. Its efficiency is mostly measured in the form of FFA releases. It has been previously shown that different compositions of emulsion influence the final FFA release (Wilde et al. 2019). Different forms of BS have also been shown to promote lipolysis efficiency to various extents (Pabois et al. 2020). Previously it was concluded that lipolysis is controlled by three main parameters: 1. Adsorption of BS/lipase to the oil interphase, 2. Emulsification of lipid droplets and 3. Desorption and solubilization of lipolysis products by BS micelles (Golding and Wooster 2010; Bellesi and Pilosof 2021; Łozińska and Jungnickel 2021). Taking into consideration the influence of BS on each aspect of lipolysis, it can be assumed that these parameters are too general. Therefore, based on previous assumptions, there were proposed six separate factors, including five unique parameters (as shown in publication A1 and publication A3) of which lipolysis consists. First (1), BS **adsorb** on the oil interface, promoting the emulsification process, removing surface materials such as proteins or emulsifiers and facilitating adsorption of the pancreatic lipase/colipase. Higher adsorption of the BS on the oil surface may suggest that they can facilitate the lipolysis process by enhancing pancreatic lipase/colipase to adsorb. Moreover, the higher the ability of the BS to break down the fat droplet (**surface tension of oil droplet**) into smaller droplets, the higher the surface area would be available for lipase/colipase to adsorb (2). Next, lipase/colipase promotes the hydrolysis of triglycerides into FFA and monoglycerides, which stay at the interface of the oil droplet, inhibiting further digestion by blocking the contact of lipase/colipase with the oil interface (3). BS create small aggregates called mixed micelles, which act as

vehicles for lipolysis products. BS can incorporate those products, remove them from the interphase and further transport them (4). After solubilizing lipolysis products into their structures BS desorb from the soil surface (5). **CMC** displays the minimum concentration of the substance to create those aggregates. Therefore, a lower CMC of the BS would indicate a lower concentration necessary for starting agglomeration which may be beneficial for lipid digestion. A smaller **aggregation number** of the BS would mean that a lower number of surfactants is required for micelle creation. Thus, within the same concentration of the BS, assuming that conjugated forms of the BS reveal smaller CMC and aggregation numbers, a greater number of the micelle might be created in comparison to unconjugated BS. Moreover, the ability of the BS to incorporate lipolysis products into mixed micelles is evaluated by the **MSR** of the compound. The higher the MSR, the greater amount of the substance would be incorporated, therefore more lipolysis products might be removed from the oil surface, providing more space for lipase/colipase to adsorb. The final rate of the lipolysis will be measured by the FFA released. The greater the number of the FFA released, the higher the extent of the lipolysis may be achieved.

1.11. Adsorption of BS

The ability of the lipase to adsorb to the lipid droplet is assisted by BSs, and therefore the BS adsorption kinetics (Pilosof 2017). First, the ability of the BS to adsorb at the oil/water interface would indicate their potential to remove the surface materials and facilitate lipase adsorption. It is a rate-limiting step influencing the lipolysis efficiency. The number and position of the hydroxyl group were shown to affect the adsorption profile of BS, as shown by Castillo-Santaella et al. (del Castillo-Santaella and Maldonado-Valderrama 2023). The results pointed out that NaTC would yield the highest surface tension at air/water interphase, concerning other investigated BS: NaGCDC and NaGDC, due to its highly hydrophilic nature. The study conducted by Parker et al. (Parker et al. 2014) allowed us to distinguish two groups characterized by different adsorption behaviour. The first group demonstrates reversible adsorption behaviour (NaGDC, NaTDC), while the second group displays a significant degree of irreversibility (NaTC, NaGC, NaGCDC). The study demonstrated that the adsorption behaviour follows the micellization properties. Faster desorption was represented by BS which had low CMC and large aggregation numbers and high CMC and low aggregation numbers promoted irreversibility adsorption.

Secondly, the interaction between the BS-lipase complex may give the information about potential of the BS to change lipase structure. To better understand the influence of the type of BS on the lipolysis process, the conformational structural changes of lipase introduced by BS were investigated, which may further reflect the potential of lipase adsorption. The molecular dynamic simulation performed by Haque et al. (Haque and Prakash Prabhu 2018) revealed the alterations in the interfacial activity of pancreatic lipase. The binding of NaTC to porcine pancreatic lipase resulted in changing the structure of the lipase. Moreover, this interaction prevents the loss of helical structure. The binding of NaTC prevents against conformation and induces an open-conformation (Haque and Prabhu 2016). Open conformation helps lipase to stay active without the co-lipase. Thus, the interaction between BS and lipase complex may induce conformational changes in the lipase, influence the lipase activity and stimulate the lipid digestion process.

Stronger adhesion of the BS may facilitate adsorption of the lipase/co-lipase to the surface of the lipid, which may promote lipolysis. However, reduction of the residence time at the interface can decrease the adhesion of the lipase/colipase but at the same time can facilitate displacement of lipolysis products from the surface. Therefore, the examination of BS behaviour at the interface is a key aspect considering their role in the lipolysis process.

1.12. Emulsification of fat droplet

Lipolysis efficiency was strongly correlated with particle size of the emulsion. Greater particle size was observed to reduce FFA release, which is correlated with a smaller surface area available for BS/lipase complex for the adsorption (Wilde et al. 2019). The smaller particle size of the initial emulsion was shown to promote higher lipolysis efficiency than the emulsion with a bigger particle size (Sarkar et al. 2016). The composition of digested emulsion may also influence the final FFA release due to the interaction of components of emulsions with BS. Wilde et al. (Wilde et al. 2019) examined the effect of phytosterol, a known component to reduce blood cholesterol levels (Dumolt and Rideout 2017) on the lipid digestion process. The results show that phytosterol accumulates at the surface of the oil, reducing space for BS to adsorb at the interphase, therefore reducing FFA release. Recently, plant-based diets gained popularity due to health and environmental concerns and animal welfare (Alcorta et al. 2021). Dietary fibres, the components of plant-based diets, were shown to trap BS in an aggregated structure during the digestion process and reduce the FFA release (Bellesi et al. 2018). The properties of emulsions, both composition and size of emulsion influence the lipid digestion process.

1.13. Micellization of BS

BS forms small aggregates in the aqueous solutions when their concentration exceeds CMC. The formation of micelles allows BS to complete their roles during the lipolysis process. It ensures the solubilization of lipolysis products into their structures and removal them from the oil interphase, which gives a greater surface for the BS/lipase complex to adsorb (Holm et al. 2013). Moreover, micelles play an important role as transport vehicles. Thanks to them lipolysis products can be delivered to our organism, which would be impossible due to their hydrophobic nature.

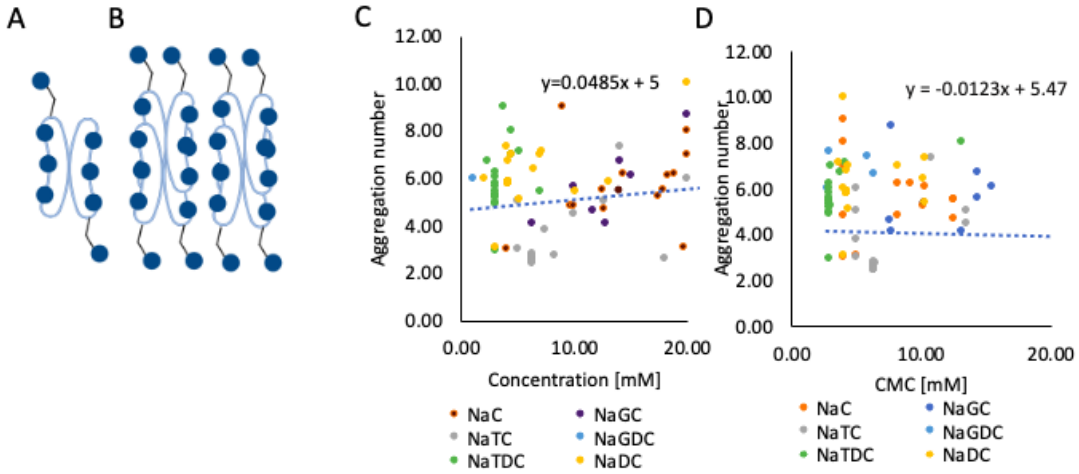


Figure 4 Arrangement of BS micelles into A. Primary structures, resulting from hydrogen interactions. Aggregation number was estimated to vary between 2-10. B. Secondary structures resulting from hydrogen bonding. The aggregation number was estimated to vary between 10-100. The scheme was prepared according to Moghimpour et al. (Moghimipour et al. 2015). *C. Aggregation number in respect to the concentration of different BS. Aggregation number increases with increasing concentration of BS. PC BS (NaTC) had the lowest aggregation number. D. Aggregation number with respect to CMC of BS. The increasing CMC did not result in a decreasing aggregation number. The results of CMC and aggregation number were taken from the meta-analysis. The data of aggregation numbers from C. and D. were previously published by Łozińska et. al.* (Łozińska and Jungnickel 2021). *NaC – sodium cholate, NaTC – sodium taurocholate, NaTDC – sodium taurodeoxycholate, NaGC – sodium glycocholate, NaGDC – sodium glycodeoxycholate, NaDC – sodium deoxycholate, CMC – critical micelle concentration.*

Over the years various techniques have been used to determine CMC (surface tension, dye solubilization, light-scattering, fluorescent, conductivity, and potentiometry). Each of the techniques is characterized by different selectivity and sensitivity. Moreover, CMC depends on BS type. Maestre et al. (Maestre et al. 2014) indicated that the higher number of hydroxyl groups and more hydrophilic character will contribute to higher CMC concentration due to greater water solubility of the molecule. Roda et al (Roda et al. 1983) observed that the CMC values of BS increase with an increasing number of hydroxyl groups. Trihydroxy BS have less hydrophobic character than dihydroxy BS which results in a lower CMC value of dihydroxy BS (Mukherjee et al. 2016). I Partay et al. (Pártay et al. 2007) indicated that the CMC of SC dihydroxy BS – NaDC was smaller than that of PU trihydroxy BS- NaC.

CMC of BS is one of the key parameters controlling the rate of lipolysis. The formation of micelles by BS is preceded by the creation of small aggregates such as dimers and trimers. (Duane and Gilboe 1995) Micelles are not only responsible for solubilizing lipolysis products, therefore allowing them to be removed from the oil interphase during the lipid digestion process but also ensure the safe transport of digested components through our body. Adsorption of BS was mentioned to be the most influential factor in controlling the process of lipid digestion (Macierzanka et al. 2019). However, the attention was also directed to the presence of unadsorbed BS, which inside the mixed micelles could effectively influence the lipolysis processes by solubilizing and removing products from the oil interface (Sarkar et al. 2016).

The formation of micelles of BS has multiple roles during the lipolysis process. It serves as aggregates in which lipolysis products can be incorporated and removed from oil interphase by the desorption process, which influences lipolysis efficiency and it also ensures the safe transport of necessary for our body components. Therefore, the tendency of BS to form micelles was studied by meta-analysis, for all types of BS, and experimental approach, for specific BS, in publication A1 and publication A3.

1.14. Desorption of BS

The ability to desorb from the lipid surface plays an essential role in the lipolysis process (Maldonado-Valderrama et al. 2014). Adsorption-desorption process is a rate-limiting step controlling the rate of the lipolysis. Increasing the surface tension of the surface layer indicates the depletion of the BS and it is desorption from the surface. The study performed by Maldonado-Valderrama et al. (Maldonado-Valderrama et al. 2014) showed different desorption properties of two conjugated forms of BS. While NaGDC fully desorbs from the surface within the whole concentration range, the NaTC tends to form irreversibly adsorbed structures at the interface. Desorption of NaTDC at lower concentrations was linked to reduced lipolysis efficiency (Pabois et al. 2020).
Desorption of BS from the oil interphase is a key parameter ensuring the removal of lipolysis products from the interphase and providing free space for BS/lipase to adsorb and continue the lipid digestion process. Finally, the desorption of BS ensures the incorporation of lipolysis products and delivers them to our bodies.

1.15. The solubilisation function of BS

Lipolysis products are solubilized by BS into created aggregates. BS forms vehicles that facilitate the transportation of lipolysis products. (Pigliacelli et al. 2023). Hofmann and Borgstrom (Hofmann and Borgstrom 1962) performed an ultracentrifugation experiment on human lipid digestion products, where they demonstrated that it consists of an oily phase and a solubilized BS mixed micellar phase. The studies comparing the effect of the ratio of BS and surfactant concentration (cationic, anionic, nonionic) on lipid digestion were performed, showing that the nature of surfactant plays an important role in the lipolysis process (Vinarov et al. 2012).
Formation of the micelles and solubilization of lipolysis end products is known as the process that completes the digestion of lipids. Created micelles transport lipid-digested products by absorption through enterocytes (Leal-Calderon and Cansell 2012). This requires micelles to diffuse through the protective layer of intestinal mucus. The small intestinal mucus is known as a complex colloidal system that protects the intestinal epithelium from exposure to luminal contents by creating a protective layer for the entire intestinal epithelium (Macierzanka et al. 2019). The mucus layer is a natural filter, that prevents epithelium against pathogenic microorganisms and ensures absorption of nutrients, so they can reach enterocytes (Cone 2009). Intestinal mucus is composed of a range of organic compounds, among which are two major ones that create a coherent network: gel-forming biopolymers, MUC2 mucin glycoprotein, and extracellular DNA (Hansson 2012; Macierzanka et al. 2014). The intestinal epithelium is composed of goblet cells that produce and secrete mucin (Zhang and Wu 2020). Viscoelasticity and strength of the gel depend on many factors such as concentration of mucin, DNA, size of pores, and level of entanglement (Macierzanka et al. 2019). Peristaltic movements cause a decreasing thickness of the mucus layer by inducing shear force. Penetration through a thinned mucus layer is possible only by diffusion (Cone 2009). Because lipids are insoluble in water they require transportation by BS in micelles.

1.16. Intestinal absorption

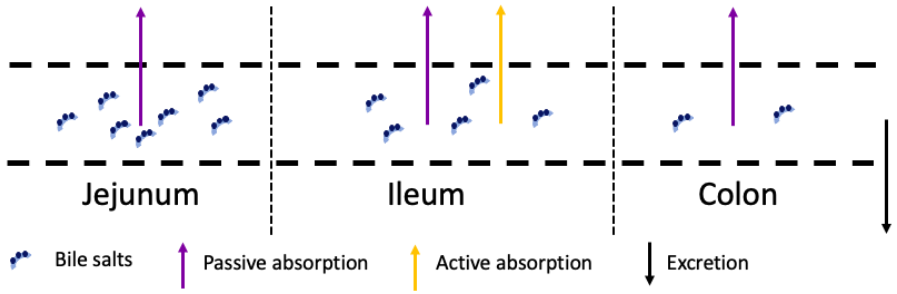


Figure 5 Intestinal absorption of BS. The jejunum and colon allow only for passive absorption, whereas the ileum transport BS through passive and active transportation mechanisms.

At the end of the enterohepatic recirculation mechanism, 5 % of BS is excreted from our body and 95% of BS is transported back to the liver through intestinal absorption. The intestinal transport mechanism of conjugated and unconjugated BS is a crucial process, ensuring the enterohepatic recirculation flow. The BS present in the small intestine can be reuptake by both active transport and passive ionic and nonionic transport mechanisms. Based on the BS features they may be transported in three main ways: by passive absorption in the jejunum, active and passive mode in the ileum, and passive mechanism in the colon, as shown in Figure 5. The active transport plays an essential role in the reabsorption of BS from the ileum. Tyor et al. (Tyor et al. 1971) concluded

that the majority of the BS present in the small intestine is transported back to the liver In the ileum. They also pointed out that limited absorption of BS may appear in other parts of the intestinal tract. The ionized and conjugated form of the BS reduces its absorption ability to the ileum transport mechanism and potential absorption by brush border membrane limits the action of a transporter (Dawson and Karpen 2015). However passive transport system was proved to exhibit good absorption ability for unconjugated or secondary conjugated BS. Passive absorption is based on the concentration gradient rule. Unconjugated BS can be absorbed at any level in the small intestine by passive absorption.
Aldini et al. compared the transport concentration of BS in the jejunum and ileum (Roda et al. 1983; Aldini et al. 1996). The mechanism of absorption for conjugated and unconjugated BS was examined. They noticed that unconjugated BS undergo passive transport in both the jejunum and ileum, the taurocholate was more likely to be absorbed in the ileum by an active transport system, while glycol-conjugated BS possessed an ability to undergo jejunum and ileum passive absorption as well as the ileum active absorption. Glycine-conjugated BS were examined to have a more hydrophobic nature than taurine-conjugated BS (Podda et al. 1990). Moreover, Merkus et al. pointed out that the conjugation of BS reduces the hydrophobicity of the created BS (Merkus et al. 1996). This process ensures maintaining the ionized form of BS, which prevents undergoing the absorption process before the fat absorption (Heaton 1969), which also indicates that the diffusion rate of BS in the small intestine depends on its form. Tyor et al. (Tyor et al. 1971) pointed out that the absorption concentration of the BS depends on the presence of specific microorganisms with the capability to deconjugate BS present in the small intestine. An important aspect is connected with the fact that conjugated BS possess a stronger ability to be absorbed, thanks to their enhanced water solubility, than their deconjugated forms, which are more likely to undergo an excretion process (McHugh et al. 2004).

So far there has been no clear investigation of how individual forms of BS may impact each of the mentioned parameters about lipolysis efficiency. There were performed individual studies of the physiochemical functions of BS, including CMC, aggregation number, solubilization, adsorption and desorption properties (Heuman 1989; Nagadome et al. 2001; Maestre et al. 2014; Maldonado-Valderrama et al. 2014; Mukherjee et al. 2016), as well as physiological functions such as FFA release (Bellesi and Pilosof 2021; Łozińska and Jungnickel 2021). However, based on the existing research, it is impossible to determine the rate-limiting step during the lipolysis process. BS performs its action simultaneously, therefore, investigation of all parameters concerning the lipolysis process may be sufficient for fully understanding the impact of BS on the digestion process. Imbalance in the BS concentration, caused by various diseases, non-healthy diet, environmental stress, etc, may inhibit the lipid digestion process and promote weight imbalance, as well as further development of diseases. Therefore, the investigation of the influence of BS in the lipolysis process was further developed in the publication A1 and may give a perspective to modulate the lipolysis process in a controlled way.

1.17. Physiological function of BS

In addition to their role in the lipolysis process BS are multifunctional biosurfactants. They act as anti-microbial agents in the small intestine, towards gram-positive bacteria (Hagey et al. 2010). The anti-microbial activity was related to oxidative DNA damage, disrupting cell membranes and cellular homeostasis (Moghimipour et al. 2015). BS act as signalling molecules, they regulate activation of G-protein coupled receptor and FXR.and they are responsible for stimulating lipid, glucose and energy metabolism (Da Silva et al. 2013). BS also regulate the secretion of lipoproteins from hepatocytes (Torchia et al. 2001), and colonic mucosal growth and stimulates the proliferation of colonic epithelium (Strauch et al. 2003). BS are responsible for the stimulation of intestinal immunity and regulating immune cells in the mucosa (Keating and Keely 2009; Soroka and Boyer 2014). BS also are responsible for removing toxins and excessive concentrations of cholesterol, preventing the formation of gallstones (Krupa et al. 2021).

1.18. Methods of measuring the lipolysis process

The increasing interest and awareness of the importance of digestion in the human body contributed to the search for a way to measure the effectiveness of the digestion process. To represent physiological conditions during digestion, both in-vitro and in-vivo techniques have been developed. In-vitro static models are commonly used as they may reflect the biochemistry of specific regions of the gastrointestinal tract, are easy to use and cheap. Single compartment pH stat model requires cheap and easily accessible equipment but it does not consider processes in the stomach, for example, gastric emptying, gastric and intestinal phases have to be performed separately and transferring the sample requires pre-conditioning (Lee et al. 2018). To overcome the

limitations two-compartment model can be used as they simulate both the gastric and the intestinal phase and connection by peristaltic pump ensures transportation of gastric medium to the intestinal vessel (Huang et al. 2021). In-vitro static techniques are well-standardized models and allow capturing the individual key parameters but they can not reflect the complexity of the intestinal microbiota, don't mimic the peristaltic movement and do not reflect the shape of the specific organ of the body (Huang et al. 2021). In-vitro dynamic models were developed to overcome multiple limitations of static techniques and ensure the reflection of digestion kinetics of the gastrointestinal tract and reproduction of the gastrointestinal environment. However dynamic models are very complicated, time-consuming, and not standardized and they do not focus on a simple parameter, but rather on the complexity of biochemical changes in the gastrointestinal tract therefore their accessibility is much lower than static models (Mulet-Cabero et al. 2020). Another alternative in-vitro method is a pendant drop surface film balance implemented with multi-subphase exchange(Maldonado-Valderrama et al. 2014). This method uses a single droplet immersed in the oil phase to simulate in-vitro digestion of emulsion. The droplet solution is exchanged with simulated digestive media that mimics the lipolysis process.
Due to the complexity of the process, reflection of lipolysis with a validation level comparable to our digestion system is a huge challenge. Recently, due to increasing interest and awareness of the importance of the digestion process, several in-vivo noninvasive and invasive methods have been developed. Magnetic resonance imaging is a non-invasive technique used to visualise changes in meal composition in the gastrointestinal tract, mainly in the stomach (Mariani et al. 2004). Another technique that allows following the gastric emptying process and uptake of nutrients is stable isotope breath testing with MS. This technique allows following the meal by using specific compounds that depend on the tested material (Golding and Wooster 2010). In-vivo invasive techniques may give a greater range of information. These studies are mainly performed with clinical assessment, for example, blood tests (Degen et al. 2007). The main aim of developing the in-vitro techniques is to ensure the appropriate validation level, reflecting in-vivo actions. In our research, we were using non-invasive techniques, which effectively reflect the complexity of the lipolysis process.

1.18.1. Static in-vitro digestion model

Digestion models are used to measure the efficiency of the lipolysis process, based on the formation of the final product however, they cannot measure the efficiency of each step of the lipolysis. The main advantage of this model is that they are cheap, non-complicated to use have good reproducibility, easy to use. The most popular and widely used model is the Brodkorb model (Brodkorb et al. 2019) based on a systematized in-vitro digestion model. Unfortunately, static models do not reflect the multi-complexity of biochemical reactions, absorption, secretion, peristaltic movements and emptying. We used the Brodhorb model, as its standardized method was created due to over three years of cooperation between the INFOGEST group, allowing results to be easily comparable between each other, to compare the efficiency of the lipolysis process in the presence of two different forms of BS: NaTC and NaDC.

1.18.2. OCTOPUS technique

We used the pendant drop technique which allows us to measure digestion efficiency in a single droplet. The work of OCTOPUS is based on a subphase device that allows to replacement of digestive media. Experiments performed on in-vitro static digestion models allowed us to compare the efficiency of lipid digestion in the presence of different forms of BS by measuring the final FFA release as an indicator of the efficiency of the lipolysis process. These experiments proved that the form of BS modulates lipid digestion and showed that NaTC increases the final FFA release, leaving us with the question – why does NaTC promotes higher FFA release? Lipolysis is a process consisting of cumulative effects. The pendant drop technique was used to determine the continuous evolution (changes in interfacial tension and dilatational modulus) of the interface during the lipolysis process, considering the influence of NaTC and NaDC. BS during the lipolysis process has to adsorb on the surface of the oil to allow lipase and co-lipase to perform the emulsification process and has to also desorb from the oil surface, ensuring removal of lipolysis products from the interface. The desorption process is also important because if the accumulated lipolysis products would not be removed from the oil interface, further digestion would be blocked due to a lack of place for the adsorption of the BS-lipase complex. Therefore, interfacial properties would strongly affect the extent and rate of lipolysis and were further examined in our research.

2. Purpose and scope of the work

The PhD thesis aimed to determine the effect of two predominant forms of BS: NaTC and NaDC on the process of lipolysis by identifying their influence on individual processes of mechanism of lipolysis. Lipolysis mainly takes place in the duodenum, which consists of PC forms of BS, and SU, ones due to the deconjugation process (Corstens et al. 2017).

The scope of the doctoral dissertation includes (1) the identification of predominant forms of BS influencing the lipid digestion process, (2) the investigation the potential of which different BS influence the specific parameter (CMC, N_a, and MSR), (3) determine factors disturbing BS synthesis and its effect on BS composition, (4) establish influence of choledocholithiasis, cholangiocarcinoma and pancreatic neoplasm towards changes in BS composition and its potential to disturb lipid digestion process, (5) identification the processes of lipolysis mechanism, (6) determination the influence of predominant forms of BS in our small intestine: NaTC and NaDC on each process of lipolysis, (7) assessment the impact of BS action on the lipolysis efficiency, (8) identification the rate-limiting process of lipolysis.

The results of the PhD work allowed us to reveal the potential to control the lipolysis mechanism via the action of BS. The influence of BS on each lipolysis process was experimentally measured and the final rate of lipolysis was asses.

3. Discussion of scientific literature results

This part of the work presents the results of the research included in the doctoral dissertation in the form of a series of three original scientific publications on the presented research issues, published in two journals from the JCR list with a total IF = 17.954. A short description of the works is presented below.

3.1. Publication 1 -A1

Łozińska N, Jungnickel C. Importance of Conjugation of the Bile Salt on the Mechanism of Lipolysis. *Molecules*. 2021; 26(19):5764. DOI: 10.3390/molecules26195764.

3.1.1. Objective of research

Publication A1 was focused on meta-analysis and statistical analysis of three parameters covering aggregation properties of BS: (1) micellization properties (CMC, β parameter), (2) aggregation number and (3) MSR.
The second aim of the publication A1 was to determine the influence of two predominantly present forms of BS in our small intestine: PC NaTC and SU NaDC, on digestion efficiency. The goal was to use a standardized, easy method which results will be, in the future, comparable with other experiments. Therefore, the static in-vitro digestion model, according to the Brodkorb protocol, was used to determine the progress of digestion by measuring FFA release.

3.1.2. Reason for undertaking the research problem

The main research problem that encouraged undertaking work on publication A1 was that there was a poor understanding of the relation between BS, aggregation properties (CMC, aggregation number, MSR) and lipolysis efficiency.

The type and concentration of BS determine its effect of action on lipolysis parameters. Alterations of those parameters may impact the role of BS in the lipid digestion process and modulate the final rate of lipolysis. Moreover, the nature of BS influences the processes modulating lipolysis efficiency. Increasing hydrophilicity of BS was correlated with the reduction of CMC, which is contradictory to linear surfactants. The factor responsible for this difference was no of BS-water hydrogen bonds. Therefore, an investigation effect of the simultaneous action of those parameters on the lipolysis process had to be performed.
The meta-analysis of experimental data has revealed that there is a limited number of data points from experiments performed under physiological conditions.
Lipolysis experiments are often performed under various, changeable conditions (type of oil, conc of BS, particle size of emulsion etc.) which limits comparability between each other. Moreover, it could be observed that there is a limited number of studies on the influence of the BS ratio on lipolysis efficiency.

Meta-analysis of CMC of BS showed a high variation of data for a single BS. The results strongly depend on the technique used to determine CMC. Some of the techniques with high sensitivity, allowed to detect the primary CMC and techniques with low sensitivity were only able to detect secondary micelles. The review of the literature data (Roda et al. 1983; Astrup 2001) revealed that dihydroxy BS may form both primary and secondary micelle, while trihydroxy BS mostly forms primary micelles. Dihydroxy BS forms primary micelles at a concentration of n 10-50mM and secondary micelles at a concentration above 100mM (Mishra et al. 2019). The formation of secondary micelles for trihydroxy BS was reported to be above the 300mM (Pártay et al. 2007). For this reason, only experiments that were carried out within the intestinal composition of BS, 10mM (Łozińska et al. 2024) were taken into consideration. This approach allowed to solve the problem with the high variation of CMC data, especially for dihydroxy BS such as NaDC. Moreover, the divergence of the CMC data was also connected with the year of the performed experiments, CMC increased with time, probably due to the increased purity of BS.

Analysis of β of different BS: BS systems revealed two occurring effects within the systems: antagonistic (with high CMC, positive β) and synergistic (with low CMC, negative β). In our research, we assumed the individual effect of BS on lipolysis efficiency taking into consideration only the CMC of the single BS. However, during the digestion process are present different types of BS and we decided to expand our research to two questions: (1) why does the antagonistic effect occur and (2) what is the reason for the synergistic effect? Moreover, the research revealed that the system composed of the PC and the SU BS balances the impact.

The increasing hydrophilicity of BS resulted in lower CMC, which was contradictory to the behaviour of linear surfactants. A similar situation appeared in the case of the BS: BS systems, where both the antagonistic and the synergistic effects could be observed, while systems created by linear surfactants mainly result in synergistic effects. The contradictory behaviour of BS towards linear surfactants resulted from their planar polarity.

To assess the level of difference of influence on the lipolysis process by two BS: NaTC and NaDC, stable and uniform emulsion had to be designed. When an emulsion with high particle size was created the emulsion met the conditions of establishment but the difference of FFA between NaTC and NaDC was very low (2%). When the emulsion with the smaller particle size was tried to be created the final emulsion was uniform with low PDI index. Moreover, the long work of the sonicator with high frequency resulted in the destabilization of the emulsion due to the high temperature of the end of the sonicator. Lower frequencies of work of the sonicator were not efficient enough to create an emulsion with a smaller particle size. Changes introduced at the formation of pre-emulsion – replacing vortexing with homogenization - allowed to decrease in the particle size of the emulsion and obtained a uniform and stable emulsion. Formed emulsion allowed to obtain a difference in FFA release between NaTC and NaDC of 15%.

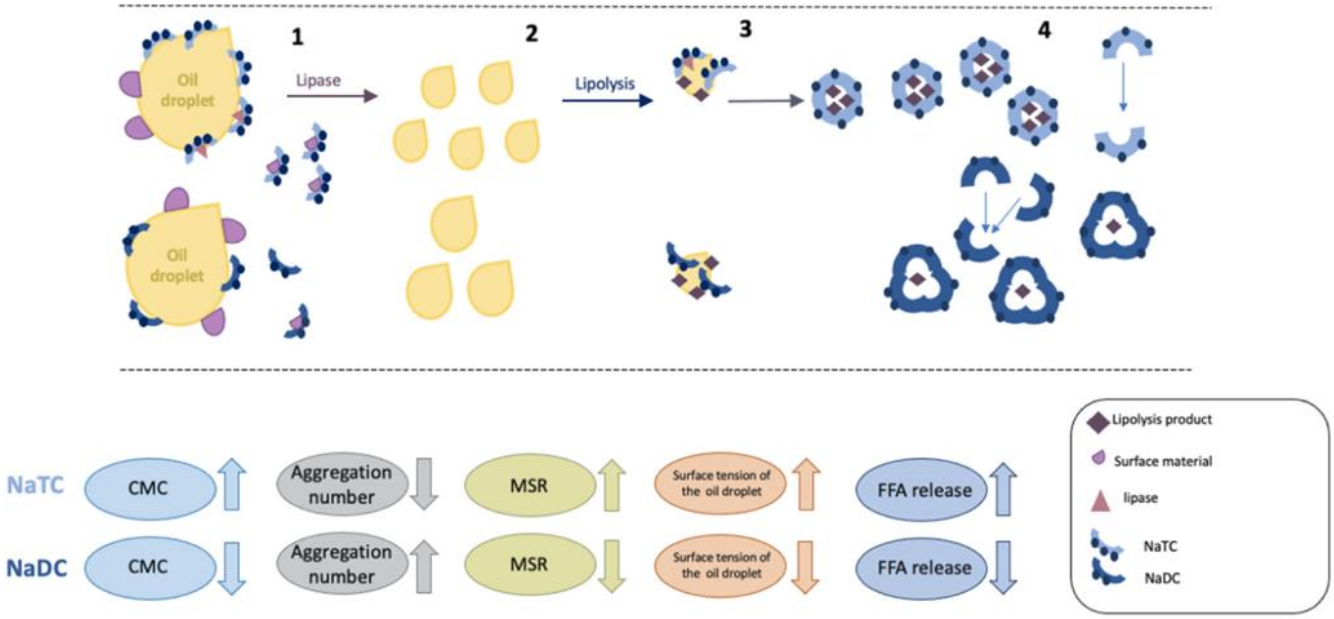
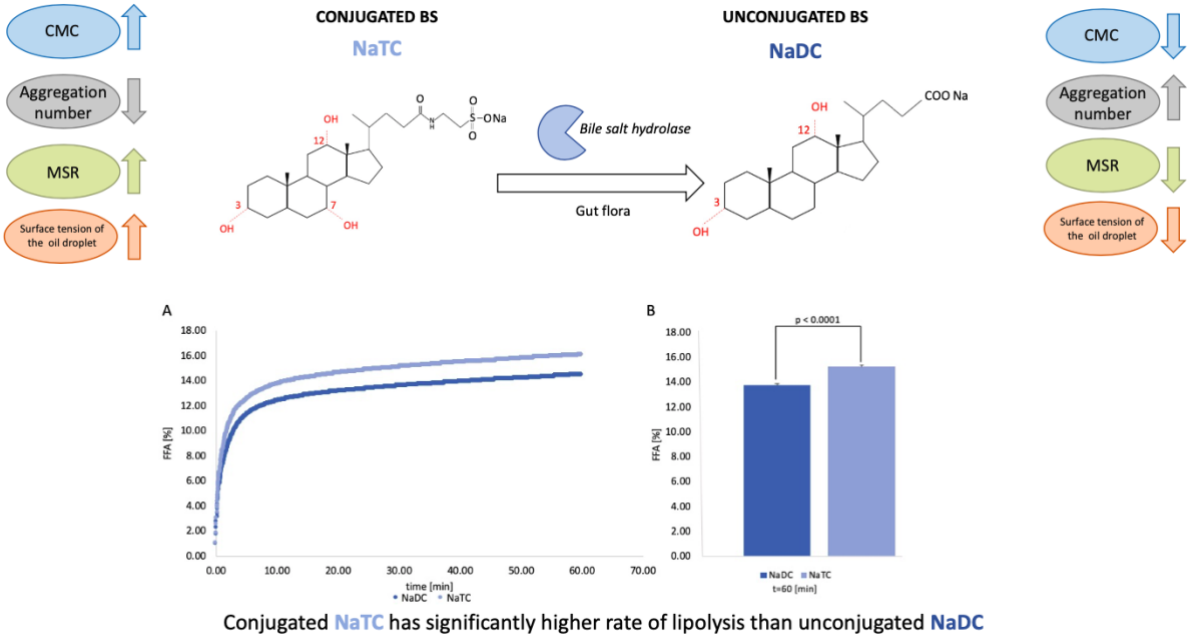


Figure 6 Schematic representation of the role of BS during the lipolysis process. The role of BS can be reflected by its influence on different parameters. The impact of each of the processes influences the final rate of lipolysis. CMC – critical micelle concentration, MSR – molar solubilisation ratio, FFA – free fatty acids, NaTC – sodium taurocholate, NaDC – sodium deoxycholate.

3.1.3. Main outcomes and conclusions

- The deconjugation process affects the physiochemical properties of BS in GIT
- NaDC have lower CMC than NaTC
- NaDC showed greater emulsification properties of oil droplets than NaTC
- The higher hydrophilic character of NaTC allows them to desorb easier from the surface of the emulsion
- LogK_{OW} showed a negative contribution towards MSR
- NaTC enhances FFA release to a higher extent than NaDC.
- The interface activity of NaDC is higher than NaTC, indicating that lipolysis is dominated by other factors

3.1.4. Graphical abstract of publication A1



Conjugated NaTC has significantly higher rate of lipolysis than unconjugated NaDC

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Importance of Conjugation of the Bile Salt on the Mechanism of Lipolysis

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Abstract: We aim to advance the discussion on the significance of the conjugation of bile salts (BS) in our organism. We hypothesize that conjugation influences the rate of lipolysis. Since the rate of lipolysis is a compound parameter, we compare the effect of conjugation on four surface parameters, which contribute to the rate. Since deconjugation is due to gut microbiota, we hypothesize that microbiota may affect the rate of lipolysis. A meta-analysis of literature data of critical micelle concentration, β, aggregation number, and molar solubilization ratio has been performed for the first time. In addition, critical micelle concentration (CMC), interfacial tension, and lipolysis rate measurements were performed. It was found that the unconjugated BS in mixed micelles increases the antagonism between the BS, therefore, increasing the CMC. This correlated with the effect of unconjugated BS on the solubilization capacity of mixed micelles. The collected literature information indicates that the role of the BS and its conjugation in our organism is a key factor influencing the functioning of our organism, where too high levels of unconjugated BS may lead to malabsorption of fat-soluble nutrients. The experimental lipolysis results irrevocably showed that conjugation is a significant factor influencing the rate.

Keywords: bile salts; lipolysis; CMC; aggregation number; MSR

Citation: Łozińska, N.; Jungnickel, C. Importance of Conjugation of the Bile Salt on the Mechanism of Lipolysis. *Molecules* **2021**, *26*, 5764. https://doi.org/10.3390/molecules26195764

Academic Editor: Adele Papetti

Received: 3 August 2021
Accepted: 13 September 2021
Published: 23 September 2021

1. Introduction

Bile salts (BS) are planar surfactants, which have methyl groups on the convex side and hydroxyl groups on the concave α-side [1]. These rigid amphiphiles [2] lack the typical flexibility of linear surfactants, which results in occasional flipping of the molecules so that the hydrophilic parts may remain inside the core, while hydrophobic parts may remain in water [3]. Self-assembly of BS is driven both by induced dipole interaction and hydrogen bonding between the BS molecules.

BS plays a crucial role in the digestion and absorption of nutrients, extraction of waste products from our body and are known to function as steroid hormones regulating nutrient metabolism [4]. The compounds originate as bile acids (BA) that are synthesized from cholesterol in the liver and stored in our gallbladder [5]. BA are conjugated in hepatocytes with either a molecule of glycine or taurine at the C-24 carboxyl group by amino acid N acyltransferase [4,6]. Conjugation is possible due to the conversion of the bile acids to their coenzyme (CoA) thioester [7]. Conjugation reduces the pK_a of the formed BS and increases their hydrophilicity. The pK_a value was measured to be 6 for the unconjugated BA, 4.5 for glycine conjugated BA, and 1.5 for taurine conjugated BA [8]. The conjugated form of the BA present in our gastrointestinal tract is therefore called bile salts (BS), as they are commonly deprotonated [9].

BS plays many important roles in our organism. One of these is their ability to create mixed micelles, which act as vehicles for a variety of molecules, including cholesterol in the liver, thereby becoming the major path of removal of cholesterol from our body [10]. In the small intestine, bile salt is responsible for decreasing the surface interfacial tension of lipid

droplets, which promotes the emulsification of oil droplets [11]. Moreover, BS contributes to the adsorption/desorption process of lipase [12]. BS are responsible for the removal of the lipolysis products from the interphase, solubilizing them in the mixed micelles, which allows for transport from intestinal lumen across into the intestinal mucosa to the gastrointestinal epithelium (enterocytes) [13–15]. They are removed from the bloodstream by the active transporters on the sinusoidal membrane of hepatocytes and are secreted back into the bile [16]. After entering the bloodstream, BS are transported to the liver.

In addition, BS regulates the composition of gut microbiota [17] with their known antimicrobial activity. Part of the BS undergo deconjugation and create secondary BA: deoxycholic acid and lithocholic acid, due to bacterial action [4]. They also act as a signaling molecule by modulating the BS receptors FXR and TGR5 [16]. In the ileum, BS are absorbed and transported back by the portal vein to the liver. In the small intestine, the flow and reabsorption of the BS and secondary BA through our body is known as the enterohepatic recirculation process [18]. Emulsification, formation of mixed micelles, regulating gut microbiota, and binding to the Farnesoid X receptor (FXR) are influenced by the level of conjugated BS [19]. FXR signaling reduces the expression of cholesterol 7α-hydrolase (CYP7a1), a rate-limiting enzyme in bile acid synthesis, and as a consequence, primary BA synthesis is reduced when its level is already high [20]. Unconjugated BS will have a higher affinity to the FXR than conjugated [21], and this will consequently inhibit new BS synthesis [22].

However, the concentration and type of the BS present in our gastrointestinal tract depend on many different factors, acting simultaneously. The most significant factor controlling the level of conjugation of BS is the intestinal flora [23]. These bacteria, mostly Gram-positive (such as *Lactobacillus*, *Enteroccocus*, *Bifidobacterium*, *Clostridium*), as well as some Gram-negative (*Bacteroides* spp.) possess bile salt hydrolase (BSH) [20], which catalyze the deconjugation of the BS. There are five known transformation mechanisms of conjugated BS by intestinal bacteria: dehydroxylation, dehydration, and epimerization, and most reported recently, the amide conjugation of the cholate backbone with phenylalanine, tyrosine, and leucine [24] and deconjugation of the amino acids glycine or taurine [24,25]. Deconjugation is investigated here since it is the most well-studied transformation and is a prerequisite for further transformation with CYP7a1 [26,27].

It has been suggested that the disruption of the composition of the intestinal microflora will result in a change in the BSH activity [28]. This, in turn, leads to a number of diseases. Weight gain may result from the change in lipolysis, which is enhanced by the dysregulation of BA homeostasis, and consequent reabsorption of BS [29]. Higher levels of BS in the colon will lead to the development of colon cancer [30–32]. Cholelithiasis may form with higher levels of unconjugated bile by reducing the removal of cholesterol from the liver [33], resulting in gallstones, among others [16].

The aim of this paper, therefore, is to analyze the effect that changing conjugation has on the lipolysis rate, which is commonly expressed graphically. Therefore, we investigate four interface parameters, which directly influence the lipolysis rate. These parameters are CMC, MSR, BS interaction, and aggregate number. The analysis will lead to a better understanding of why changes in the level of conjugation of bile salts have such profound effects on our bodies. In addition, we will show experimentally how the level of conjugation influences the rate of lipolysis in in-vitro digestion experiments.

2. Results

Conjugation changes of bile salts in the small intestine, as shown in Table 1. As can be seen, the range of the average ratio of conjugated to unconjugated is 94:5% in the duodenum, 93:7% in the jejunum, and 82:18% in the ileum. This change in the ratio is due to the higher presence of BSH, which is found in Gram-positive bacteria that commonly reside in the ileum [34].

Table 1. BS composition in the small intestine. Composition of the BS in the small intestine in respect to the concentration of their conjugated and unconjugated forms. The BS appears in its conjugated form in the duodenum and jejunum; the deconjugation process is mostly observed in the ileum.

Duodenum			Jejunum			Ileum		
Conjugated [%]	Unconjugated [%]	Ref.	Conjugated [%]	Unconjugated [%]	Ref.	Conjugated [%]	Unconjugated [%]	Ref.
99.70	0.30	[35]	100.00	0.00	[35]	88.00	11.79	[36]
94.20	5.00	[35]	96.50	3.50	[35]	75.00	25.00	[37]
91.00	9.00	[37]	84.00	15.50	[37]			

2.1. Micellization

Micellization of BS is a representative and often measured parameter, which provides information on which concentration of BS micelle will form, where a lower CMC would indicate a lower concentration of BS is required to form micelles.

Not much CMC data exists in literature dating back to 1962. Performing a meta-analysis on CMC data is hindered by the large variety of methods that are used to determine the CMC (such as potentiometric, calorimetric, or conductometric) and measurements being performed in various conditions. For this analysis, we assumed that each method was equally valid, and we looked only at sodium cholate and sodium chenodeoxycholate and their transformation products, measured at 298.25 K. Even though the temperature dependence of the CMC is weak [3], including another variable (such as temperature) into a meta-analysis will reduce the strength of conclusions that can be drawn.

Figure 1A shows the results of the CMCs determined by other groups. It can be seen that primary unconjugated sodium cholate and sodium chenodeoxycholate generally have the highest CMC, whereas the conjugated form, with the addition of a taurine or glycine reduces the CMC. Interestingly, the addition of more hydrophilic groups to the molecule reduces the CMC (as shown in Figure 1B). This behavior is contradictory to the usual linear surfactants, where a higher hydrophobicity results in a lower CMC [38]. This evident decrease in the CMC with the conjugated BS is due to the stabilization of the micelle due to the hydrogens bonds on the amino residue [39]. The hydrogen bonds between these groups result in added induced dipole interactions of the hydrophobic sections. This has been shown by molecular dynamics simulations [40,41]. Conjugated BS offers additional sites for H-bond formation, especially between the peptide amino group and the steroid hydroxyl groups. Hydrogen bonds were found to be missing in unconjugated primary BS. In addition, the flipped molecules might serve to further stabilize the aggregate by offering more sites for hydrogen bond formation [3]. Comparing primary to secondary BS, dihydroxy secondary unconjugated BS create micelles in a smaller concentration than trihydroxy primary unconjugated BS [42], which follows the usually expected influence of hydrophobicity. After primary micelles are formed, the micelles may further aggregate to form secondary micelles, which are held together by hydrogen bonds [43,44].

The primary unconjugated CMCs had the largest standard deviation ($\sigma = 2.33$) because it is the most frequently measured ($N = 28$) and thus was determined with the largest variety of methods (conductivity, fluorescence, light-scattering, potentiometry, and surface tension), whereas secondary unconjugated BS had the lowest standard deviation ($\sigma = 0.68$) since it was determined with a lower variety of methods (tensiometry, conductivity, light-scattering, and conductometry).

In addition, the CMC was shown to change over the years of publication, as shown in Figure S2, where it is evident that the CMC of the measured BS actually increases with time, specifically for the BS sodium deoxycholate increased from ~3.92 mM in the 1960s to 1970s to an average of ~4.16 mM from 2010 to 2020. The only reasonable justification is the increased purity of the tested BSs, which removed a synergistic contaminant.

These results clearly indicate that the level of conjugation is a significant factor influencing the properties of the BS (as shown in Figure 2). However, it should be noted that BS

do not exist as pure compounds in the human body, but as a mixture of primary/secondary and conjugated/unconjugated. Therefore, the interactions of BS in these mixtures still need to be understood.

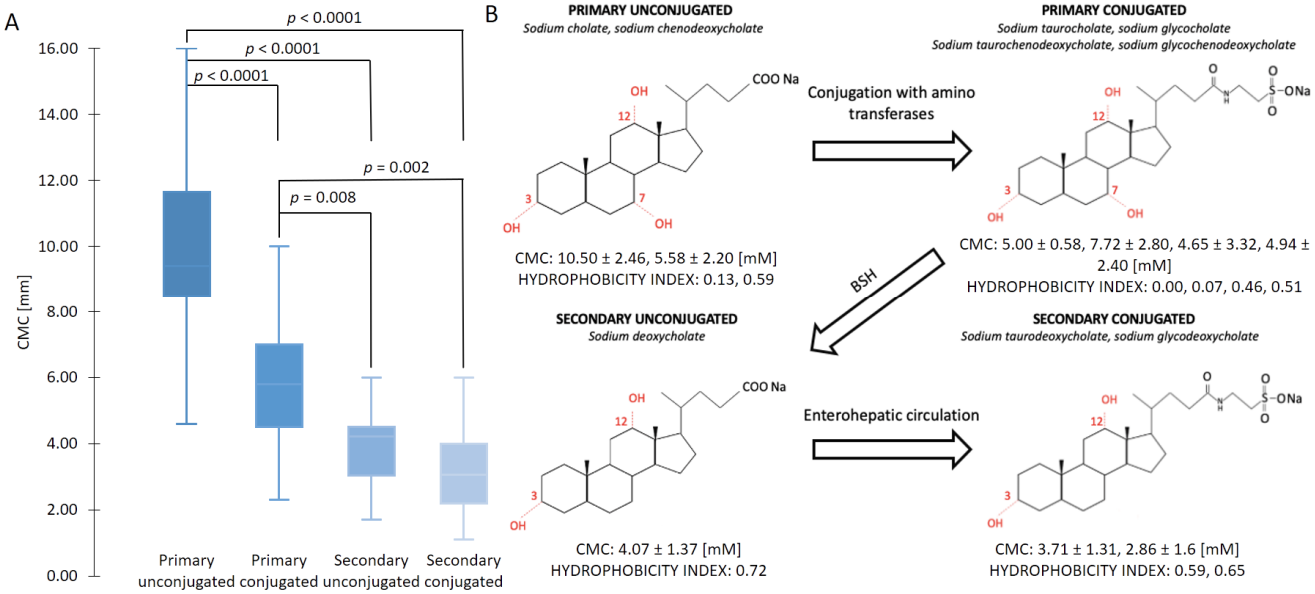

Figure 1. (**A**) Comparison of CMCs of conjugated/unconjugated, primary/secondary BS at the temperature of 298.15 K. The conjugated forms of the BS generally display a lower CMC than their respective unconjugated forms. (**B**) Structural changes are shown together with the average CMCs values of conjugated and unconjugated BS at 298.15 K as well as their hydrophobicity index. The hydrophobicity index values are taken from Heuman et al. [45]. The conjugated form of the BS is characterized by a smaller average CMC than their unconjugated form with a lower hydrophobicity index. The *p*-value of primary conjugated and secondary unconjugated was calculated to be 0.008.

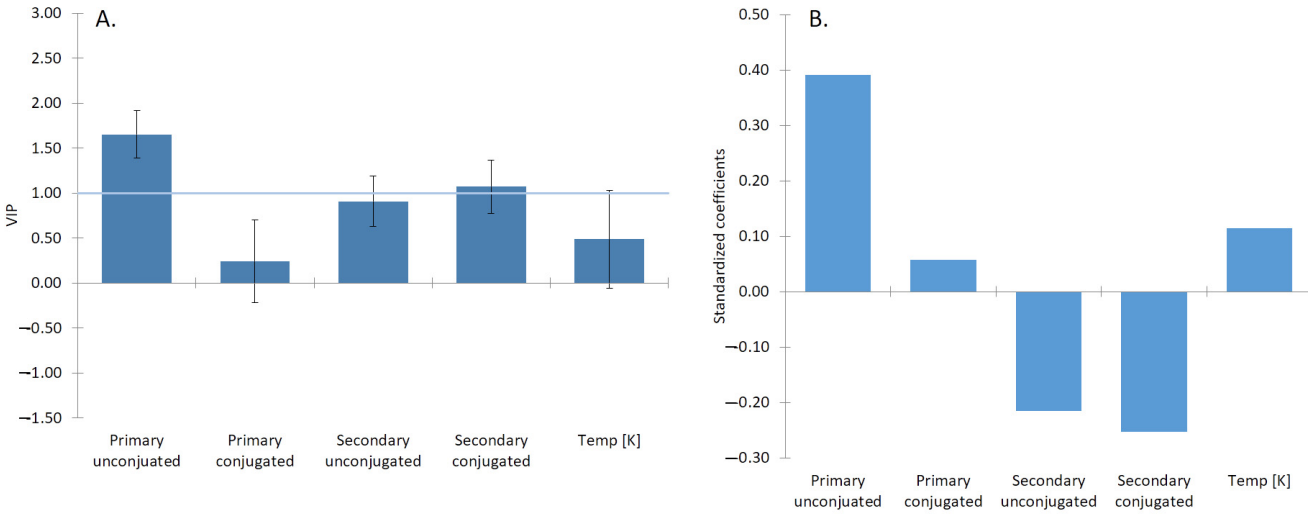

Figure 2. (**A**) The most influential parameters (with VIP larger than 1) affecting the CMC are the type of the BS, specifically the primary unconjugated and the secondary conjugated BS. (**B**) The primary unconjugated BS: cholic and chenodeoxycholic acids showed a positive impact on the creation of the micelles. The location of the OH group at positions 3α and 7α of NaCDC and 3α, 7α, and 12α of NaC promote micellar growth. The same orientation of the OH groups enhances the micelle formation. The location of the OH group and its position can be recognized as the most influential factor promoting micelle formation [3].

2.2. Analysis of β Parameter

The interactions of surfactants are characterized by β, as described by Rosen [46]. The beta values were taken from three publications, looking at the synergism or antagonism of mixed micelles of BS systems. All BS were categorized into primary conjugated/unconjugated, and secondary conjugated/unconjugated. The effect of conjugation is shown in Figure 2. However, to determine the contribution of each of the factors (conjugation/deconjugation) to the β, the data were analyzed by partial least squares (PLS) regression, which allowed us to extract the variable importance, as shown in Figure 3. The various categories were included as "one hot encoded" variables. Temperature was included as the degree of counter-ion binding changes with temperature [47].

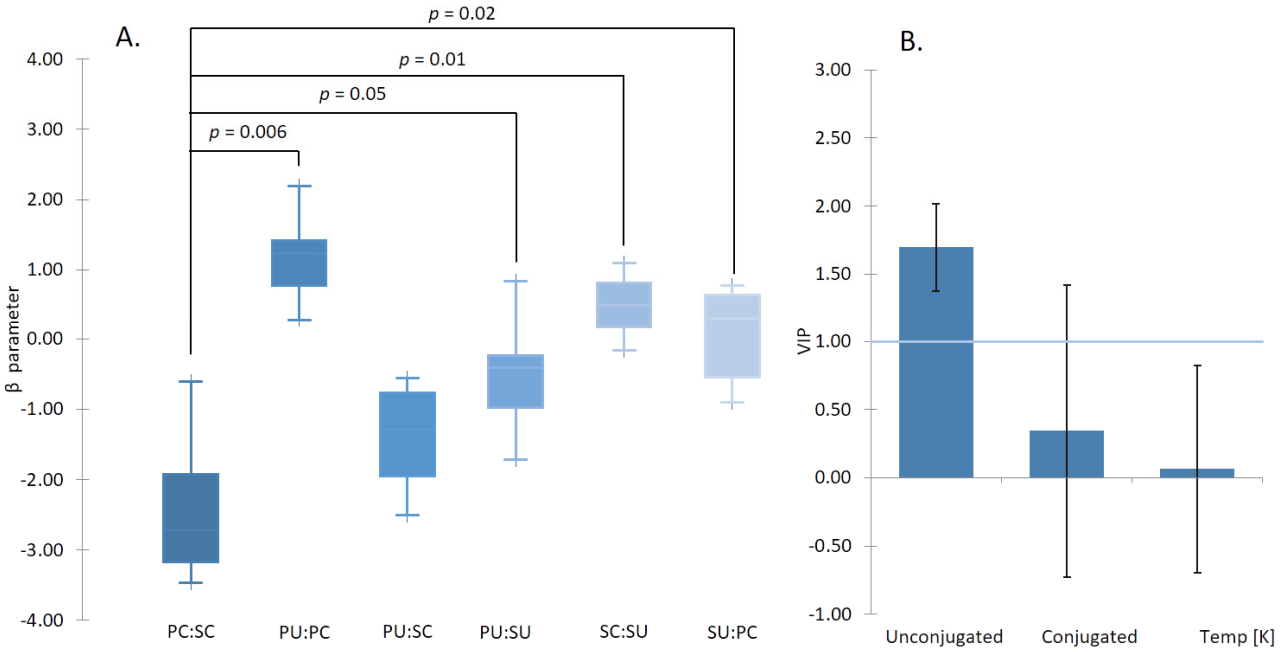

Figure 3. (**A**) The plot of β parameter for different BS:BS mixed systems. A positive β indicates the antagonistic effect and thus a higher CMC, and a negative β indicates a synergistic effect, and thus a lower CMC. The system composed of two conjugated forms of the BS showed the most synergism. The graph was created using six different BS systems. The system composed only of conjugated forms of the BS (PC:SC) showed to be statistically different from almost all other investigated systems, composed of at least one unconjugated form of the BS. (**B**) The most significant factor affecting the BS:BS mixed system was the secondary conjugated form of the BS. The synergistic effect was enhanced in the systems composed of the secondary conjugated aggregates. The *p*-value of PC:SC and SU:PC were calculated to be 0.02.

It can be seen that conjugated secondary BS has the strongest contribution to the β value of mixed BS micelles. The effect is negative, which means a synergistic effect reduces the CMC. Both the primary conjugated, as well as primary unconjugated, have small antagonistic effects. This synergism is due to the lack of the additional hydroxyl group, making the molecule more hydrophobic and enhancing its insertion into the micelle, while the conjugated chain allows for more hydrogen bonds with other molecules, thereby stabilizing the molecule inside the micelle. This can be seen when comparing the number of hydrogen bonds with water, where both GDCH and TDCH have on average 14 hydrogen bonds, compared to 16.5 for conjugated primary BS. The number of hydrogen bonds between conjugated primary and secondary BS is the same.

It clearly shows that conjugation, especially in conjunction with secondary conjugated BS is an important synergistic factor enhancing the micellization of the BS.

When comparing the β values of mixtures of traditional linear surfactants and bile salts, mixtures of linear surfactants generally have synergistic effects due to better packing of a variety of tails lengths into the core of the micelle [7]. This synergistic effect is

evident also in the BS mixtures of the same type (conjugated/conjugated and unconjugated/unconjugated), as it can be observed in Figure 3A, and Table 2. However, interestingly mixtures of conjugated/unconjugated (e.g., PC:SU or PU:PC) exhibit an antagonistic effect. This is the result of the columbic repulsion between the negative charge of the carboxyl group of the unconjugated BS with the slightly electro-negative ester of the amino acid. The action of BSH and formation of unconjugated BS, therefore, reduces the ability of the bile to form micelles.

Table 2. The binary mixtures of the cationic/cationic, anionic/anionic and BS/BS surfactants at 298.15 K. The common linear surfactants showed a synergistic effect, while BS yielded both synergistic and antagonistic effect. Exemplary data for BS is given, where a complete BS mixture data set is given in Table S3.

Type of Surfactant	Composition	CMC [mM]	β	References
NaC/NaTC	0.2	6.1	1.33	[48]
(PU:PC)	0.4 0.6 0.8	8.1 9.18 9.93	2.19 1.39 1.48	[48]
NaC/NaDC	0.2	3.6	–0.40	[48]
(PU:SU)	0.4 0.6 0.8	4.15 4.8 5.41	−0.31 −0.41 −0.84	[48]
C_{12}TAB/C_{10}TAB	0.3	25.00	−1.4	[49]
C_{14}TAB/C_{10}TAB	0.3	8.00	−4.7	[49]
C_{14}TAB/C_{12}TAB	0.3	6.00	−1.4	[49]
C_{16}TAB/C_{10}TAB	0.3	3.00	−7.7	[49]
C_{16}TAB/C_{12}TAB	0.3	3.00	−5.1	[49]
C_{16}TAB/C_{14}TAB	0.3	2.00	−1.5	[49]
C_{16}Br/C_{16}BzCl	0.10 0.25 0.5 0.75 0.90	13.20 9.33 10.70 22.90 24.10	−4.24 −4.83 −2.95 −1.27 −1.79	[50]
SOS/SAE2S			−4.98	[51]

2.3. Aggregation Number

Aggregation number indicates the number of the molecules present in the individual micelle created by the surfactant. To analyze the effect of conjugation on the aggregate number, we have collated both experimental (11 papers with 64 datapoints) and molecular dynamics calculations (4 papers with 14 datapoints). The aggregation number of the individual BS can be observed on the Figure 4A,B; raw data is provided in Table S2. The aggregate number is a crucial parameter considering the amount of surfactant incorporated in the aggregate. Factor influencing aggregation number at 303 K are shown in Figure 5.

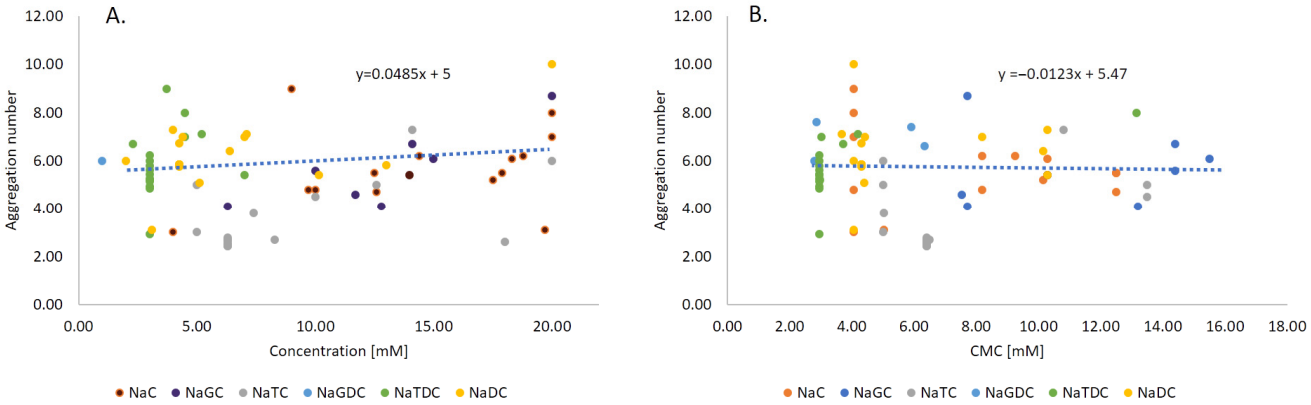

Figure 4. (**A**) Increasing concentration of the BS increases the aggregation number. The lowest aggregation number was seen in primary conjugated BS. (**B**) The data of aggregation number were collected via meta-analysis, and for CMC, we used the averaged CMC from our meta-analysis at temperature range 283.15–323.15 K. The results showed that the aggregation number does not decrease with increasing CMC, which is in contradiction to Madenci et al. [3]. The lack of CMC dependence of the aggregation number is due to the H-bond interaction between the BS molecules and the formation of secondary micelles. The aggregation number of classical surfactants, however, depends on CMC, where the aggregate number increases with increasing CMC [52].

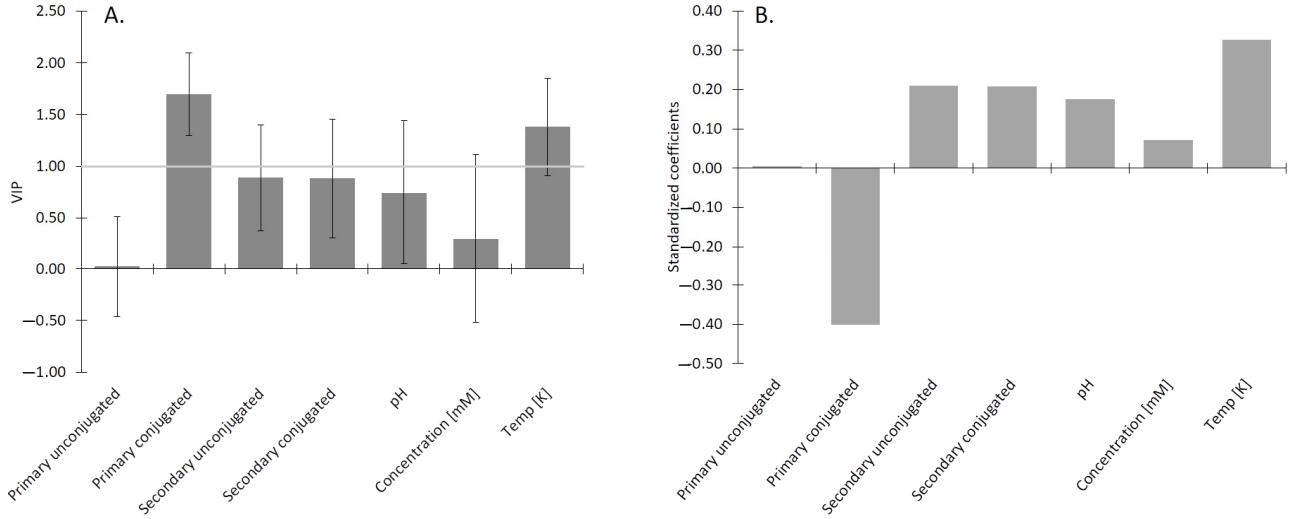

Figure 5. (**A**) The most influential factors influencing the aggregation number are types of the BS, and the temperature. CMC of the BS tends to decrease with increasing temperature up to 303 K, beyond which the CMC starts to increase, leading to an increasing aggregation number [47]. (**B**) Conjugated forms of the BS have a tendency to have lower CMC than their unconjugated forms, therefore, the aggregate number for the conjugated BS should be smaller than for unconjugated ones. The primary conjugated BS showed a high negative correlation towards aggregation number. Conjugated forms of the BS are stabilized not only by the hydrophobic interaction but also by the hydrogen bonding, which means that they require fewer molecules than their unconjugated forms. The positive relation of the concentration [mM] of BS towards aggregation number yields the relation that with increasing BS concentration, the number of the incorporated molecules will increase [42].

Bile salts may form both primary and secondary micelles, as originally stated by Small and Kawamura [43,44]. Primary aggregates which are spherical or slightly oblate in shape, are created by the hydrophobic interaction [53,54], and those aggregates may interact with hydrogen bonds when linked together by the outwardly directed hydrophilic part of the ion constituents, result in the formation of various, complex shapes of secondary micelles such as flattened or rod-like objects known as secondary aggregates [40]. The creation process of the primary and secondary micelles was confirmed by the molecular dynamics

simulations [40,42,55,56]. The mechanism of the formation of the micelles was noticed to be different for deoxycholate (di-hydroxy BS) and cholate (tri-hydroxy BS) [40] specifically a micelle created by the cholate remains a dimer even at 30 mM, which are linked by H-bonds, while the deoxycholate creates primary micelles by hydrophobic interaction and the secondary micelles by hydrogen bonding [40]. Cholate, due to the presence of the three hydroxyl groups, possessed a more hydrophobic character than the deoxycholate, which is mainly characterized by hydrophilic edge [40]. It should be noted that in the human body, the concentration of the BS in the gallbladder varies between 10–50 mM [57], where BS are not favorable to form the secondary micelles.

The results from the PLS regression, shown in Figure 6, represent the contribution of different parameters to the aggregation number. The VIP indicated that the type of the BS was the most meaningful parameter affecting the aggregation number. For the conditions of the experiments, the temperature had a higher significance than pH since temperature predominately affects the formation of large aggregates ($N_a \geq 10$). The increasing temperature balances two opposite effects: the repulsion between anionic polar heads and hydrophobic interaction, ensuring the stability of the small aggregates (primary micelles $N_a < 10$). It has previously been shown that increasing temperature decreases the size of secondary BS micelles [58], which is due to the structure of the secondary aggregates, where hydroxyl groups are hidden inside the micelle and the anionic amino acid residue predominates on the outside of the micelle. pH was shown to have the positive contribution towards the aggregation number since acidification of sodium glycodeoxycholic acid promotes the formation of the helical aggregates [58,59]. Additionally, increasing pH may result in dehydration of nonionic moiety and the formation of hydrogen bonding between nonionic polar parts, allowing larger micelles to form [60].

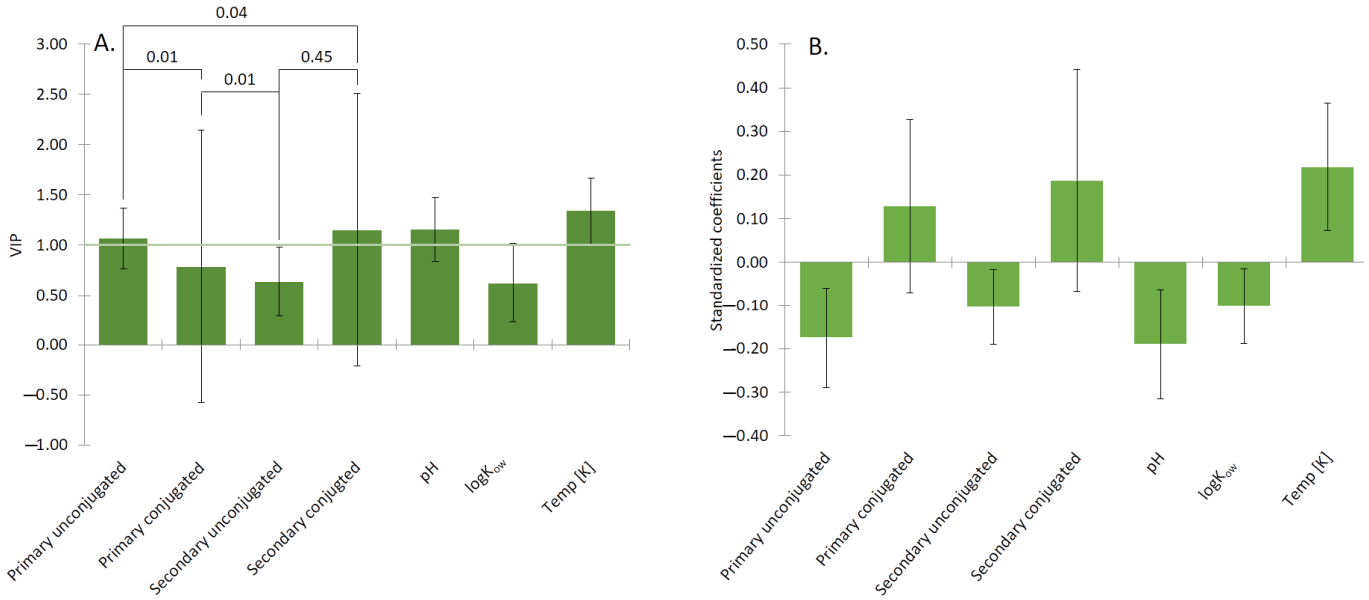

Figure 6. (**A**) The VIP analysis showed that the most significant factors influencing the MSR are the types of the BS, either primary or secondary, and conjugated or unconjugated. (**B**) The standardized coefficient of MSR for different forms of the BS. Elements with the longest bar have the greatest impact on MSR. Their contribution is positive if they are above the line and negative if they are below the line. Statistical significance was determined by using a *t*-test. The MSR of conjugated forms (PC, SC) of the BS have shown to be statistically significant to the MSR of unconjugated BS (PU and SU).

2.4. Molar Solubilization Ratio

The molar solubilization ratio is known as the ratio of the molecules solubilized inside the aggregate. For bile salts, the MSR has a crucial meaning, as it is the size of the MSR that will dictate the efficiency of removal of the lipolysis products from the lipid droplet.

We may observe in Figure 6B that the $\log K_{ow}$ has a negative contribution to the MSR, which is the result of the correlation between the $\log K_{ow}$ of the solubilizate and its molecular volume (Pearson correlation for the 21 solubilizates analyzed here was 0.990, $p < 0.0001$). Therefore, more hydrophobic solubilizates in our analysis resulted in a lower MSR due to their larger volume. The meta-analysis of six papers for the first time highlights the effect of conjugation on the MSR. Both primary and secondary unconjugated BS have a negative contribution to the MSR. That is, the higher is the level of the unconjugated forms of the BS the lower will be MSR for a given substance. In addition, the MSR is also influenced by the locus of solubilization within the micelle. Steroid compounds were found to be more effectively incorporated into the NaDC than NaC due to the less hydrophilic character of the NaC molecule [61]. Aromatic compounds not only have the ability to solubilize inside the hydrophobic interior, but also occupy external positions on BS micelles, as determined by Kolehmainen et al. [62]. Unconjugated BS was more favorable to incorporate fatty acids into their structure than their glycine conjugates [63]. Therefore, vitamins undergo lipolysis before they are absorbed by the BS micelles.

2.5. Measurements of Interfacial Tension at Oil/BS Interface

The ability of the two different BS NaTC and NaDC to decrease the surface tension of the oil droplets was investigated to determine their role in the lipolysis process. Surface tension reduction of BS follows a similar trend as shown by the CMC. A higher CMC of NaTC (Figure 1A) indicates a lower ability of surface tension reduction of the oil droplet at physiological conditions (Figure 7). NaDC showed a greater ability to reduce the oil droplets' surface tension and may therefore better reduce the droplet size during the lipolysis process.

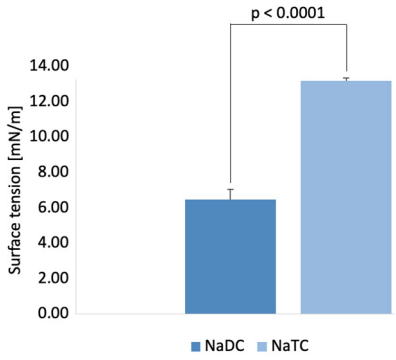

Figure 7. The surface tension of sunflower oil droplets with 10 mM of the two BS, NaDC and NaTC, measured at 310.15 K. Surface tension of PC NaTC showed to be statistically significant lower compared to the SU NaDC. The average surface tension was determined to be 6.45 ± 0.01 for NaDC and 13.17 ± 0.08 for NaTC. The control sunflower oil droplet had a surface tension of 30.64 ± 0.13 mN/m.

2.6. Impact of Conjugation Form of the BS on the Lipid Digestion

To show that the changes in the proposed surface parameters actually affect the rate of lipolysis as hypothesized above, we have conducted experiments to show the significance of the conjugation of the BS. In essence, the rate of lipolysis was determined in in-vitro experiments with both PC and SU bile salts. The results are shown in Figure 8. It is shown that conjugated NaTC shows a faster rate of release of FFA (i.e., lipolysis) as compared to the same concentration of the unconjugated counterpart NaDC.

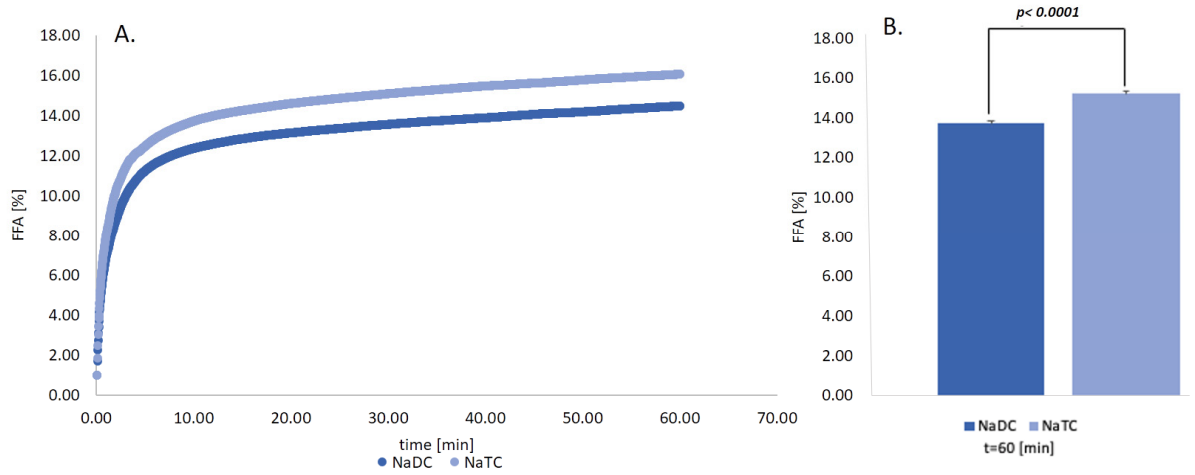

Figure 8. (**A**) FFA released over time from WPI-stabilized emulsion in respect to two different forms of BS: NaTC and NaDC at 10mM under physiological conditions at 310.15 K. Experimental results of CMC of NaTC and NaDC at 310.15 K with errors are also shown. The CMC of NaTC and NaDC have been shown to be statistically significant, $p < 0.0001$, and are comparable to previously determined values (5–20% of FFA after 60 min for NaTC and NaTDC at 10 mM and 50 mM, methylcellulose stabilized emulsion [64], 10–13% of FFA after 60 min for mix BS at 9.7 mM, WPI-stabilized emulsion [65], 6.5–15% of FFA after 60 min. for mix BS at 10 mM [66]. (**B**) Statistical significance was calculated by using the t-sample t-test. The magnitude of the %FFA is in line with results from other researchers who also digested sunflower oil/WPI, or other protein emulsions, with similar emulsion sizes [64,65,67]. $p < 0.0001$ indicates that the values are statistically significant.

Previously it was pointed out that the interfacial process of lipolysis involves three key steps [68]. However, we are showing that the release of the FFA from the emulsion (as shown schematically in Figure 9) is linked to five factors. First, the ability of the BS to further break down the emulsified lipid droplets, which promotes a larger surface area onto which the enzyme can adsorb [9]. Second, the adsorption kinetics of the BS onto the emulsion. Third, assisting the lipase/co-lipase complex to attach to the emulsion surface. Fourth, is the removal of lipolysis products from the oil/water interface, and finally, the desorption kinetics from the emulsion [65,69].

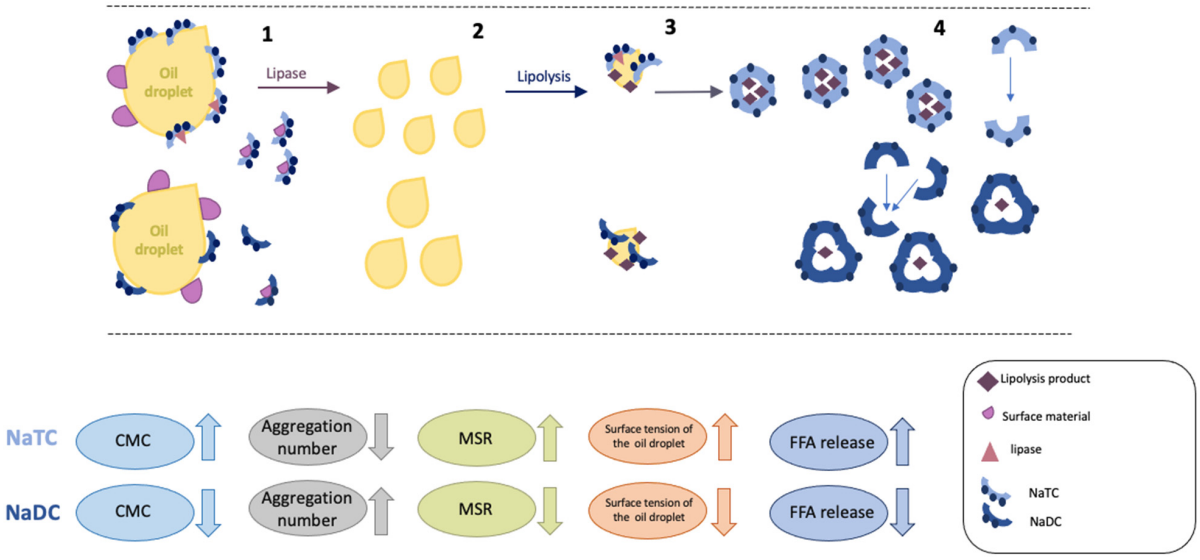

Figure 9. The role of BS during the lipolysis process. 1. Bile salt adsorbs at the oil droplet, leading to the removal of surface materials (proteins, emulsifiers) and promote adsorption of the lipase. 2. BSs break down larger lipid droplets into smaller ones ensuring efficient lipolysis 3. BS will assist lipase and co-lipase in the sorption onto the emulsion. 4. BS incorporates lipolysis products into mixed micelles, ensuring the transport of valuable components into the body.

In our case, it could be observed that the conjugated form of the BS, NaTC, favored the release of FFA to a higher extend than SU form of the BS. From the five factors mentioned above, we hypothesize from Figure 6 that the enhanced MSR of the primary conjugated aids the removal of the lipolysis products from the emulsion, with a small number of BS molecules in the micelle (Figure 5), in addition, the added BS–water hydrogens bonds (as shown in Table 3) allow the primary conjugated BS to desorb easier from the surface of the emulsion. Thus, the lower CMC of NaDC (with less BS–water hydrogens bonds) (Figure 1) would yield a lower release of FFA (Figure 5) as in comparison to NaTC [70]. Interestingly, as shown in Figure 7, the interface activity of the unconjugated BS is higher than the conjugated, which therefore indicates that lipolysis is dominated by other factors. BS have previously been shown to have different ability to adsorb at the oil–water interface [64,71], and thus to reduce the interfacial tension of the droplet. Micellar state affects the BS adsorption [72]. NaDC showed a better ability to reduce interfacial tension on the adsorbed droplet (Figure 7), thus promoting a greater surface area for lipase and co-lipase adsorption. However, accumulated products on the emulsion interface and the lower ability of SU BS to remove surface materials may interrupt adsorption of the lipase and co-lipase and, therefore, can result in lower FFA release for SU BS. In the future, more detailed adsorption/desorption studies of BS behavior at oil interface should be performed to better understand their role in the lipolysis process, as well as to determine which of the five factors dominate the lipolysis process.

Table 3. The number of BS-BS hydrogen bonds and number of BS–water hydrogen bonds of four groups of BS [41]. Secondary unconjugated BS could be characterized by the smallest number of BS-BS HBs and number of BS–water HBs, while the primary conjugated has the highest number of BS–water HBs.

Molecule	No. of BS–BS HBs	No. BS–Water HBs
Primary unconjugated	0.08 ± 0.13	15.00 ± 1.41
Primary conjugated	0.305 ± 0.35	16.50 ± 1.41
Secondary unconjugated	0.06 ± 0.11	13.00 ± 1.41
Secondary conjugated	0.305 ± 0.30	14.00 ± 1.41

In summary, the significantly lower release of FFA obtained by NaDC indicates that deconjugation of the BS affects lipid metabolism. It, therefore, follows that excessive microbiota with BSH may impact on the efficiency of lipid digestion. Moreover, the reduction of digestion performance by NaDC suggests that the bacterial action and composition of gut microflora in our organism have an important impact on our health and are therefore indirect factors regulating the lipolysis process.

3. Materials and Methods

3.1. Meta-Analysis

To analyze the importance of BS conjugation, a meta-analysis was performed on experimental data that analyzed the critical micelle concentration (CMC), molar solubilization ratio (MSR), and aggregate numbers of bile salts. This was collected from scientific articles ranging from 1962 to 2019, where Google Scholar was used [73] with the following keywords for CMC: "bile salts, critical micelle concentration, mixed micelle"; for aggregation number: "bile salts, aggregation number"; for MSR: "bile salts, molar solubilization ratio". This has resulted in 205 unique datapoints of CMC of pure compounds from 27 publications, in 33 datapoints of CMC of mixed systems from 3 publications, 166 datapoints of aggregation number from 9 publications, and 53 datapoints of MSR from 5 publications.

All units were standardized. Additional parameters were noted and included; for CMC temperature and method of determination, for aggregate number temperature, the concentration of BS, CMC, pH and salt concentration, and for MSR, the solubilizate, and temperature, and the salt concentration were noted. The $\log K_{ow}$ and molecular volume (nm^3) of the solubilizate were determined using Molinspiration Cheminformatics. Direct

comparisons were only made for systems of the same temperature unless stated otherwise. β and MSR, if not presented, were calculated using the CMC values provided by each author.

3.2. Critical Micelle Concentration Determination

The CMC of NaDC (from Sigma Aldrich, St. Louis, MO, USA, 97.0%) and NaTC (from Sigma Aldrich; 97.0%) at physiological temperature (310.15 K) were assessed by using conductivity measurements using an auto titrator (Cerko Lab System CLS/M/07/06, Gdynia, Poland) equipped with a microconductivity electrode (Eurosensor, EPST-2ZAM, Gliwice, Poland). The temperature was maintained using a thermostatic water bath (PolyScience 9106, Niles, IL, USA). The breakpoint determination in the conductivity curves was done using the Phillips method as previously described by Łuczak et al. [74]. The data are given in the Supplementary Materials, Table S1.

3.3. Emulsion

Oil in water (O/W) emulsion (oil to water 20:75% *w/w*) and whey protein isolate (WPI) concentration of 0.5% (*w/w*) was prepared by dissolving WPI in saline buffer (150 mM NaCl and 0.02% *w/w* NaN_3). The mixture was stirred with a magnetic stirrer until dissolution. Sunflower oil, which was previously treated with florisil (Taufkirchen, Sigma, F9127), was used as the oil phase [75]. The mixture of sunflower oil and protein dispersion was further vortexed for 3 min to obtain a coarse emulsion. The pre-emulsion was sonicated with an ultrasound generator (Sonics VCX 500, Sonics & Materials Inc., Newtown, CT, USA) with a 0.13 cm diameter titanium probe with an amplitude of 80%, pulse duration of 5 s on/10 s off for 3 min. Lipolysis results were carried out on split samples, one-half for each bile salt.

3.4. Droplet Size

A zetasizer (Zetasizer Nano, Malvern Instruments Ltd., Malvern, UK) was used to determine mean droplet diameter by using dynamic light scattering. Water was used as a dispersant (refractive index of 1.330). The absorbance value of the oil droplets was 0.001 (refractive index of 1.467) [76]. The results of particle size were recorded as the Z-average mean diameter, which is calculated from the particle size distribution [77]. The 2 µL emulsions were diluted in 7 mL of the saline buffer to avoid back-scattering. Each sample was measured in quadruplicate. Exemplary particle size distribution is provided in the Figure S1

3.5. Interfacial Tension Measurements

Drop shape analysis was done by measuring interfacial tension using a drop shape analyzer (Krüss Drop shape analyzer DSA 10, Hamburg, Germany). The measurements were performed as described previously by Szumała et al. [75] with some modifications. Specifically, the measuring cell was filled with 10 mM BS. Subsequently, the oil drop was formed and BS adsorbed on the oil/water interface and interfacial tension was measured. Each drop was allowed to equilibrate with the BS for 10 min before the surface tension was recorded. All measurements were made at 310.15 K, with five repetitions.

3.6. In Vitro Duodenal Digestion

In-vitro lipolysis [78] was used to simulate the environmental condition of the small intestine (duodenum). 0.8 mL of the simulated intestinal fluid and 0.375 mL of the emulsion were added to the vessel. After gently mixing with a magnetic stirrer (1500 rpm), 0.3 mL of 10 mM BS (NaTC or NaDC) and 3 µL of 0.3 M $CaCl_2$ were pipetted, and the pH was set to 7.0 using 0.1 M HCl. Finally, with the addition of 1.0 mL of freshly prepared pancreatin (80 U/mg of oil) the titration was started.

The reaction vessel was continuously stirred and thermostatically controlled to maintain 310.15 K. All lipolysis experiments were carried out in triplicate.

The extent of the lipolysis was measured by continuous titration with an autotitrator (Cerko Lab System CLS/M/07/06, Gdynia, Poland) of free fatty acids (FFA) with 0.1 M NaOH.

3.7. Statistical Analysis

PLS was applied to determine the most significant influence of the descriptor on the dependent variable. The PLS can be used for qualitative as well as for quantitative data, therefore, the PLS analysis was done according to Łozińska et al. [79], and *p*-values were determined by (one-tailed) students t-test. The aim of the analysis of the data was to investigate the potential of which different descriptors influence the specific parameter (CMC, β, N_a, and MSR). Therefore, we were looking to which extent each descriptor impact on the parameter. The complete data is given in the Supplementary Materials, as Table S1 for the CMC data, Table S2 for β values, Table S3 for aggregation numbers, and Table S4 for the MSR. Statistical analysis was done using XLSTAT (version 2020.1.3.65326) [80]. The workflow for the analysis is schematically represented in Figure 10. Statistical significance is shown in Figures if *p* was determined to be less than 0.05.

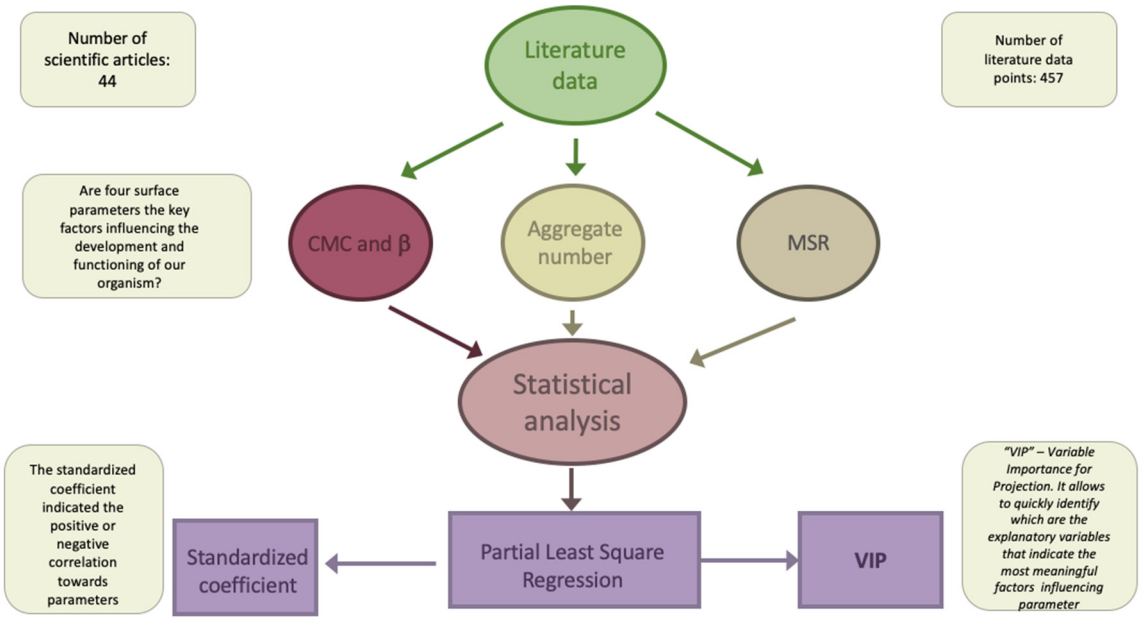

Figure 10. The workflow of the data analysis.

4. Conclusions

To assess the importance of the level of conjugation, this paper represents the meta-analysis of four phenomenological parameters (CMC, β, N_a, and MSR).

The conjugated BS will form micelles with a lower concentration than their unconjugated forms. It was shown by molecular dynamic simulations [42,44,56,57] that the lower CMC of the conjugated BS is a result of the hydrogen bonds on the amino acid residues [39]. Secondary conjugated BS showed the greatest contribution to promote the synergistic effect in combination with other BS. Conjugated BS requires fewer molecules to create aggregates, which means that with the same amount of substance, the conjugated BS will promote the formation of more micelles than their unconjugated forms [3]. Although the deconjugation process promoted by BSH will lead to decreasing the CMC of the existing BS, mixed BS systems composed of unconjugated forms of the BS will be characterized by an antagonistic effect, resulting in a higher CMC of the mixed system [48]. Finally, fewer compounds would be solubilizing inside the micelle of the unconjugated BS, which may

promote the deficiency of the beneficial compounds in our organism, such as vitamins, fats, and sterols [81].

To prove the importance of conjugation, we have measured the in-vitro digestion of an emulsion with both conjugated and unconjugated bile salts, and we show for the first time experimentally that these changes in lipolysis can be modulated by variation of BS conjugation level. That means that an exemplary decrease in BSH activity (by taking antibiotics, for example) may lead to a potential increase in conjugation, and thus an increase in lipolysis and could cause obesity over a longer period [82], which will, in turn, result in bile saturation [83] and can lead to gallstone formation [84]. On the other hand, overactivity of BSH will result in lowering the level of conjugated BS, which binds strongly to the FXR to reduce bile acid synthesis and result in malnutrition.

Supplementary Materials: The following are available online, Table S1: CMC data, Table S2: β values of binary mixtures of bile salts, Table S3: Aggregation numbers of BS, Table S4: MSR, Figure S1: Particle size distribution of O/W emulsion, and Figure S2: CMC of NaDC change with years.

Author Contributions: N.Ł.: conceptualization, data curation, formal analysis, investigation, methodology, software, visualization, writing—original draft. C.J.: conceptualization, data curation, formal analysis, investigation, methodology, project administration, software, supervision, validation, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Provided in the Supplementary Materials.

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Not available.

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3.1.6. Publication A1 Supporting Information

Importance of conjugation of the bile salt on the mechanism of lipolysis
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Table S1 CMC data. NaC – sodium cholate, NaCDC – sodium chenodeoxycholate, NaDC – sodium deoxycholate, NaTC – sodium taurocholate, NaGC – sodium glycocholate, NaTCDC -sodium taurochenodeoxycholate, NaGCDC – sodium glycochenodeoxycholate, NaTDC – sodium taurodeoxycholate, NaGDC – sodium glycodeoxycholate, CMC – critical micelle concentration

Bile salt	CMC	Temperature [K]	Reference
NaC	10.80	283.15	(15)
NaC	12.50	283.15	(15)
NaC	17.90	283.15	(18)
NaC	8.80	283.15	(11)
NaC	8.02	293.15	(22)
NaC	7.78	293.15	(15)
NaC	8.25	293.15	(15)
NaC	8.02	293.15	(7)
NaC	14.40	293.15	(10)
NaC	8.67	298.15	(22)
NaC	9.70	298.15	(18)
NaC	9.00	298.15	(1)
NaC	9.00	298.15	(14)
NaC	10.00	298.15	(4)
NaC	11.30	298.15	(4)
NaC	6.20	298.15	(17)
NaC	12.90	298.15	(3)
NaC	12.78	298.15	(3)
NaC	12.90	298.15	(3)
NaC	12.73	298.15	(3)
NaC	16.00	298.15	(25)
NaC	10.20	298.15	(27)
NaC	13.00	298.15	(21)
NaC	11.10	298.15	(20)
NaC	8.67	298.15	(7)
NaC	14.10	298.15	(10)
NaC	7.98	298.15	(12)
NaC	6.20	298.15	(13)
NaC	11.00	298.15	(16)
NaC	10.20	298.15	(11)
NaC	9.10	298.15	(19)
NaC	8.80	298.15	(19)
NaC	5.18	303.15	(5)
NaC	9.12	303.15	(5)
NaC	7.22	303.15	(5)
NaC	9.06	303.15	(22)
NaC	5.89	303.15	(15)
NaC	7.35	303.15	(15)

NaC	9.06	303.15	(7)
NaC	14.00	303.15	(10)
NaC	6.10	310.15	(15)
NaC	18.80	310.15	(18)
NaC	7.50	310.15	(15)
NaC	9.40	310.15	(11)
NaC	14.30	313.15	(10)
NaC	17.50	323.15	(18)
NaC	19.10	323.15	(11)
NaCDC	3.00	298.15	(17)
NaCDC	9.00	298.15	(21)
NaCDC	4.60	298.15	(16)
NaCDC	5.80	298.15	(19)
NaCDC	5.50	298.15	(19)
NaDC	2.95	283.15	(26)
NaDC	6.30	283.15	(18)
NaDC	4.65	283.15	(15)
NaDC	5.82	283.15	(15)
NaDC	6.60	283.15	(11)
NaDC	3.35	283.15	(26)
NaDC	3.24	283.15	(26)
NaDC	2.30	283.15	(13)
NaDC	3.55	293.15	(26)
NaDC	3.47	293.15	(26)
NaDC	1.50	293.15	(2)
NaDC	2.95	293.15	(22)
NaDC	3.80	293.15	(15)
NaDC	5.36	293.15	(15)
NaDC	2.95	293.15	(7)
NaDC	6.00	293.15	(10)
NaDC	4.10	298.15	(6)
NaDC	2.00	298.15	(24)
NaDC	4.30	298.15	(18)
NaDC	7.94	298.15	(26)
NaDC	1.70	298.15	(26)
NaDC	4.20	298.15	(14)
NaDC	4.50	298.15	(5)
NaDC	2.40	298.15	(13)
NaDC	4.00	298.15	(16)
NaDC	2.40	298.15	(17)
NaDC	6.00	298.15	(25)
NaDC	4.30	298.15	(3)
NaDC	4.25	298.15	(3)

NaDC	4.16	298.15	(3)
NaDC	3.02	298.15	(7)
NaDC	4.30	298.15	(3)
NaDC	4.25	298.15	(3)
NaDC	2.90	298.15	(12)
NaDC	3.02	298.15	(7)
NaDC	3.07	298.15	(20
NaDC	5.56	298.15	(4)
NaDC	5.74	298.15	(4)
NaDC	4.50	298.15	(11)
NaDC	5.40	298.15	(10)
NaDC	4.50	298.15	(27)
NaDC	3.25	298.15	(1)
NaDC	3.97	303.00	(5)
NaDC	2.75	303.00	(5)
NaDC	7.94	303.00	(5)
NaDC	4.80	303.00	(26)
NaDC	4.57	303.00	(26)
NaDC	3.11	303.15	(22)
NaDC	3.02	303.15	(15)
NaDC	4.05	303.15	(15)
NaDC	3.11	303.15	(7)
NaDC	5.10	303.15	(10)
NaDC	8.20	310.15	(11)
NaDC	8.20	310.15	(18)
NaDC	6.10	313.15	(26)
NaDC	3.16	313.15	(15)
NaDC	4.32	313.15	(15)
NaDC	5.90	313.15	(10)
NaDC	10.20	323.15	(11)
NaDC	10.10	323.15	(18)
NaGC	13.60	283.15	(11)
NaGC	12.80	283.15	(18)
NaGC	6.80	298.15	(11)
NaGC	4.20	298.15	(24)
NaGC	6.30	298.15	(18)
NaGC	7.00	298.15	(14)
NaGC	12.00	298.15	(21)
NaGC	10.00	298.15	(16)
NaGC	14.70	310.15	(11)
NaGC	14.10	310.15	(18)
NaGC	16.00	323.15	(11)
NaGC	15.00	323.15	(18)

NaGCDC	6.00	298.15	(21)
NaGCDC	2.30	298.15	(18)
NaGCDC	7.00	298.15	(21)
NaGCDC	7.00	298.15	(21)
NaGCDC	2.40	298.15	(16)
NaGCDC	2.10	310.15	(18)
NaGDC	5.80	283.15	(11)
NaGDC	5.60	283.15	(18)
NaGDC	6.00	298.15	(21)
NaGDC	3.30	298.15	(18)
NaGDC	2.12	298.15	(8)
NaGDC	1.10	298.15	(24)
NaGDC	1.90	298.15	(24)
NaGDC	2.20	298.15	(16)
NaGDC	3.43	298.15	(11)
NaGDC	6.00	310.15	(11)
NaGDC	5.80	310.15	(18)
NaGDC	6.60	323.15	(11)
NaGDC	6.10	323.15	(18)
NaTC	6.79	283.15	(15)
NaTC	7.92	283.15	(15)
NaTC	4.00	283.15	(23)
NaTC	8.30	283.15	(18)
NaTC	3.20	283.15	(2)
NaTC	8.80	283.15	(11)
NaTC	2.80	293.15	(2)
NaTC	4.25	293.15	(7)
NaTC	4.25	293.15	(22)
NaTC	6.68	293.15	(15)
NaTC	7.30	293.15	(15)
NaTC	4.70	298.15	(21)
NaTC	4.50	298.15	(22)
NaTC	4.50	298.15	(7)
NaTC	5.00	298.15	(18)
NaTC	4.70	298.15	(1)
NaTC	6.00	298.15	(14)
NaTC	5.60	298.15	(11)
NaTC	4.75	303.15	(22)
NaTC	4.75	303.15	(7)
NaTC	6.14	303.15	(15)
NaTC	6.81	303.15	(15)
NaTC	3.10	303.15	(2)
NaTC	13.70	310.15	(11)

NaTC	12.60	310.15	(18)
NaTC	6.36	313.15	(15)
NaTC	7.20	313.15	(15)
NaTC	3.00	313.15	(2)
NaTC	3.30	323.15	(2)
NaTC	14.10	323.15	(18)
NaTC	15.00	323.15	(11)
NaTDC	2.01	283.15	(15)
NaTDC	2.21	283.15	(15)
NaTDC	4.50	283.15	(18)
NaTDC	1.80	283.15	(2)
NaTDC	4.56	283.15	(11)
NaTDC	1.88	293.15	(15)
NaTDC	2.62	293.15	(15)
NaTDC	1.50	293.15	(2)
NaTDC	6.00	298.15	(5)
NaTDC	3.05	298.15	(8)
NaTDC	2.87	298.15	(8)
NaTDC	2.30	298.15	(18)
NaTDC	4.07	298.15	(21)
NaTDC	4.00	298.15	(9)
NaTDC	2.30	303.15	(5)
NaTDC	3.98	303.15	(5)
NaTDC	4.07	303.15	(5)
NaTDC	2.43	303.15	(15)
NaTDC	2.90	303.15	(15)
NaTDC	1.80	303.15	(2)
NaTDC	2.88	313.15	(15)
NaTDC	3.50	313.15	(15)
NaTDC	2.10	313.15	(2)
NaTDC	4.53	310.15	(11)
NaTDC	4.50	310.15	(18)
NaTDC	2.10	323.15	(2)
NaTDC	5.30	323.15	(11)
NaTDC	5.20	323.15	(18)
TCDC	2.30	298.15	(18)
TCDC	7.00	298.15	(21)
TCDC	2.10	310.15	(18)

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Table S2 β values of binary mixtures of bile salts. PC – primary conjugated, PU – primary unconjugated, SC – secondary conjugated, SU – secondary unconjugated

System	Temperature [K]	β from literature data	Reference
PU:PC	283.15	0.43	(2)
PU:PC	293.15	0.89	(2)
PU:PC	298.15	1.32	(1)
PU:PC	298.15	2.19	(1)
PU:PC	298.15	1.39	(1)
PU:PC	298.15	1.48	(1)
PU:PC	303.15	1.14	(2)
PU:PC	313.15	0.28	(2)
PU:SC	283.15	-0.84	(2)
PU:SC	293.15	-0.56	(2)
PU:SC	303.15	-2.51	(2)
PU:SC	313.15	-1.76	(2)
PU:SU	283.15	-1.71	(2)
PU:SU	293.15	-1.36	(2)
PU:SU	298.15	-0.41	(1)
PU:SU	298.15	-0.31	(1)
PU:SU	298.15	-0.41	(1)
PU:SU	298.15	-0.85	(1)
PU:SU	303.15	-0.03	(2)
PU:SU	313.15	0.84	(2)
SU:PC	283.15	0.31	(2)
SU:PC	293.15	-0.90	(2)
SU:PC	303.15	0.77	(2)
SU:PC	310.15	-0.54	(3)
SU:PC	313.15	0.64	(2)
PC:SC	283.15	-0.60	(2)
PC:SC	293.15	-3.08	(2)
PC:SC	303.15	-3.46	(2)
PC:SC	313.15	-2.36	(2)
SC:SU	283.15	0.28	(2)
SC:SU	293.15	0.71	(2)
SC:SU	303.15	-0.16	(2)
SC:SU	313.15	1.10	(2)

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Table S3 Aggregation numbers of BS. BS – bile salts, temp – temperature, CMC – critical micelle concentration, NaCl- sodium chloride, NaGC- sodium glycocholate, NaTC – sodium taurocholate, NaC – sodium cholate, NaDC – sodium deoxycholate, NaTDC – sodium taurodeoxycholate, NaGDC – sodium glycodeoxycholate, ref - references

BS	Temp [K]	Concentration [mM]	CMC	NaCl	pH	Aggregate number	Ref
NaGC	283.15	12.80	13.20	0.00	7.00	4.10	(9)
NaGC	291.15	11.70	7.54	0.00	7.00	4.60	(9)
NaGC	298.15	6.30	7.72	0.00	7.00	4.10	(9)
NaGC	298.15	20.00	7.72	150.00	7.50	8.70	(5)
NaGC	310.15	10.00	14.40	120.00	7.00	5.60	(8)
NaGC	310.15	14.10	14.40	0.00	7.00	6.70	(9)
NaGC	323.15	15.00	15.50	0.00	7.00	6.10	(9)
NaTC	273.15	6.30	6.40	0.00	7.00	2.42	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.44	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.46	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.52	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.54	(2)
NaTC	273.15	18.00	6.40	0.00	7.00	2.59	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.65	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.68	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.69	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.69	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.77	(2)
NaTC	283.15	8.30	6.50	0.00	7.00	2.70	(9)
NaTC	291.15	7.40	5.02	0.00	7.00	3.80	(9)
NaTC	298.15	5.00	5.00	0.00	7.00	3.00	(9)
NaTC	298.15	20.00	5.00	150.00	7.50	6.00	(5)
NaTC	298.20	5.00	5.00	0.00	7.00	5.00	(7)
NaTC	310.00	10.00	13.50	120.00	7.00	4.50	(8)
NaTC	310.15	12.60	13.50	0.00	7.00	5.00	(9)
NaTC	323.15	14.10	10.80	0.00	7.00	7.30	(9)
NaC	273.15	19.70	5.02	0.00	7.00	3.09	(2)
NaC	283.15	17.90	12.50	0.00	7.00	5.50	(9)
NaC	283.80	12.60	12.50	0.00	7.50	4.70	(3)
NaC	284.30	12.50	12.50	100.00	7.50	5.50	(3)
NaC	291.15	14.40	9.24	0.00	7.00	6.20	(9)

NaC	298.15	4.00	4.07	0.00	7.00	3.00	(4)
NaC	298.15	9.70	4.07	0.00	7.00	4.80	(9)
NaC	298.15	20.00	4.07	0.00	7.00	7.00	(6)
NaC	298.15	20.00	4.07	0.00	7.00	8.00	(4)
NaC	298.15	9.00	4.07	0.00	7.00	9.00	(1)
NaC	310.00	10.00	8.20	120.00	7.00	4.80	(8)
NaC	310.15	18.80	8.20	0.00	7.00	6.20	(9)
NaC	323.15	17.50	10.15	0.00	7.00	5.20	(9)
NaC	327.90	14.00	10.30	100.00	7.50	5.40	(3)
NaC	328.10	18.30	10.30	0.00	7.50	6.10	(3)
NaGDC	291.15	4.40	2.80	0.00	7.00	6.00	(9)
NaGDC	298.15	3.30	2.86	0.00	7.00	7.60	(9)
NaGDC	310.15	5.80	5.90	0.00	7.00	7.40	(9)
NaGDC	323.15	6.10	6.35	0.00	7.00	6.60	(9)
NaTDC	273.15	3.00	2.95	0.00	7.00	2.92	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	4.87	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	4.96	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	5.15	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	5.28	(2)
NaTDC	273.15	7.00	2.95	0.00	7.00	5.42	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	5.44	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	5.61	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	5.79	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	6.01	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	6.22	(2)
NaTDC	283.15	4.50	3.02	0.00	7.00	7.00	(9)
NaTDC	291.15	3.00	2.97	0.00	7.00	5.20	(9)
NaTDC	298.15	2.30	3.72	0.00	7.00	6.70	(9)
NaTDC	298.20	3.72	372.00	0.00	7.00	9.00	(7)
NaTDC	310.15	4.50	13.15	0.00	7.00	8.00	(9)
NaTDC	323.15	5.20	4.20	0.00	7.00	7.10	(9)
NaDC	273.15	4.24	4.30	0.00	7.00	5.78	(2)
NaDC	273.15	4.24	4.30	0.00	7.00	5.79	(2)
NaDC	273.15	4.24	4.30	0.00	7.00	5.80	(2)
NaDC	273.15	4.24	4.30	0.00	7.00	5.80	(2)
NaDC	273.15	13.00	4.30	0.00	7.00	5.82	(2)
NaDC	273.15	4.24	4.30	0.00	7.00	5.84	(2)
NaDC	273.15	4.24	4.30	0.00	7.00	5.85	(2)
NaDC	273.15	4.24	4.30	0.00	7.00	5.85	(2)
NaDC	273.15	4.24	4.30	0.00	7.00	5.86	(2)

NaDC	273.15	4.24	4.30	0.00	7.00	5.86	(2)
NaDC	273.15	4.24	4.30	0.00	7.00	6.74	(2)
NaDC	283.15	5.10	4.40	0.00	7.00	5.10	(9)
NaDC	285.80	4.40	4.43	0.00	7.50	7.00	(3)
NaDC	291.15	7.10	3.67	0.00	7.00	7.10	(9)
NaDC	298.15	3.10	4.07	0.00	7.00	3.10	(9)
NaDC	298.15	2.00	4.07	0.00	7.00	6.00	(4)
NaDC	298.15	20.00	4.07	0.00	7.00	10.00	(6)
NaDC	310.15	7.00	8.20	0.00	7.00	7.00	(9)
NaDC	323.15	6.40	10.15	0.00	7.00	6.40	(9)
NaDC	327.80	4.00	10.30	100.00	7.50	7.30	(3)
NaDC	328.20	10.15	10.30	0.00	7.50	5.40	(3)
NaGC	283.15	12.80	13.20	0.00	7.00	4.10	(9)
NaGC	291.15	11.70	7.54	0.00	7.00	4.60	(9)
NaGC	298.15	6.30	7.72	0.00	7.00	4.10	(9)
NaGC	298.15	20.00	7.72	150.00	7.50	8.70	(5)
NaGC	310.15	10.00	14.40	120.00	7.00	5.60	(8)
NaGC	310.15	14.10	14.40	0.00	7.00	6.70	(9)
NaGC	323.15	15.00	15.50	0.00	7.00	6.10	(9)
NaTC	273.15	6.30	6.40	0.00	7.00	2.42	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.44	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.46	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.52	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.54	(2)
NaTC	273.15	18.00	6.40	0.00	7.00	2.59	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.65	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.68	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.69	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.69	(2)
NaTC	273.15	6.30	6.40	0.00	7.00	2.77	(2)
NaTC	283.15	8.30	6.50	0.00	7.00	2.70	(9)
NaTC	291.15	7.40	5.02	0.00	7.00	3.80	(9)
NaTC	298.15	5.00	5.00	0.00	7.00	3.00	(9)
NaTC	298.15	20.00	5.00	150.00	7.50	6.00	(5)
NaTC	298.20	5.00	5.00	0.00	7.00	5.00	(7)
NaTC	310.00	10.00	13.50	120.00	7.00	4.50	(8)
NaTC	310.15	12.60	13.50	0.00	7.00	5.00	(9)
NaTC	323.15	14.10	10.80	0.00	7.00	7.30	(9)
NaC	273.15	19.70	5.02	0.00	7.00	3.09	(2)
NaC	283.15	17.90	12.50	0.00	7.00	5.50	(9)
NaC	283.80	12.60	12.50	0.00	7.50	4.70	(3)

NaC	284.30	12.50	12.50	100.00	7.50	5.50	(3)
NaC	291.15	14.40	9.24	0.00	7.00	6.20	(9)
NaC	298.15	4.00	4.07	0.00	7.00	3.00	(4)
NaC	298.15	9.70	4.07	0.00	7.00	4.80	(9)
NaC	298.15	20.00	4.07	0.00	7.00	7.00	(6)
NaC	298.15	20.00	4.07	0.00	7.00	8.00	(4)
NaC	298.15	9.00	4.07	0.00	7.00	9.00	(1)
NaC	310.00	10.00	8.20	120.00	7.00	4.80	(8)
NaC	310.15	18.80	8.20	0.00	7.00	6.20	(9)
NaC	323.15	17.50	10.15	0.00	7.00	5.20	(9)
NaC	327.90	14.00	10.30	100.00	7.50	5.40	(3)
NaC	328.10	18.30	10.30	0.00	7.50	6.10	(3)
NaGDC	291.15	4.40	2.80	0.00	7.00	6.00	(9)
NaGDC	298.15	3.30	2.86	0.00	7.00	7.60	(9)
NaGDC	310.15	5.80	5.90	0.00	7.00	7.40	(9)
NaGDC	323.15	6.10	6.35	0.00	7.00	6.60	(9)
NaTDC	273.15	3.00	2.95	0.00	7.00	2.92	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	4.87	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	4.96	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	5.15	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	5.28	(2)
NaTDC	273.15	7.00	2.95	0.00	7.00	5.42	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	5.44	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	5.61	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	5.79	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	6.01	(2)
NaTDC	273.15	3.00	2.95	0.00	7.00	6.22	(2)
NaTDC	283.15	4.50	3.02	0.00	7.00	7.00	(9)
NaTDC	291.15	3.00	2.97	0.00	7.00	5.20	(9)
NaTDC	298.15	2.30	3.72	0.00	7.00	6.70	(9)
NaTDC	298.20	3.72	3.72	0.00	7.00	9.00	(7)
NaTDC	310.15	4.50	13.15	0.00	7.00	8.00	(9)
NaTDC	323.15	5.20	4.20	0.00	7.00	7.10	(9)
NaDC	273.15	4.24	4.30	0.00	7.00	5.78	(2)
NaDC	273.15	4.24	4.30	0.00	7.00	5.79	(2)
NaDC	273.15	4.24	4.30	0.00	7.00	5.80	(2)
NaDC	273.15	4.24	4.30	0.00	7.00	5.80	(2)
NaDC	273.15	13.00	4.30	0.00	7.00	5.82	(2)
NaDC	273.15	4.24	4.30	0.00	7.00	5.84	(2)
NaDC	273.15	4.24	4.30	0.00	7.00	5.85	(2)
NaDC	273.15	4.24	4.30	0.00	7.00	5.85	(2)

NaDC	273.15	4.24	4.30	0.00	7.00	5.86	(2)
NaDC	273.15	4.24	4.30	0.00	7.00	5.86	(2)
NaDC	273.15	4.24	4.30	0.00	7.00	6.74	(2)
NaDC	283.15	5.10	4.40	0.00	7.00	5.10	(9)
NaDC	285.80	4.40	4.43	0.00	7.50	7.00	(3)
NaDC	291.15	7.10	3.67	0.00	7.00	7.10	(9)
NaDC	298.15	3.10	4.07	0.00	7.00	3.10	(9)
NaDC	298.15	2.00	4.07	0.00	7.00	6.00	(4)
NaDC	298.15	20.00	4.07	0.00	7.00	10.00	(6)
NaDC	310.15	7.00	8.20	0.00	7.00	7.00	(9)
NaDC	323.15	6.40	10.15	0.00	7.00	6.40	(9)
NaDC	327.80	4.00	10.30	100.00	7.50	7.30	(3)
NaDC	328.20	10.15	10.30	0.00	7.50	5.40	(3)

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Table S4 MSR values for BSs with given solubilizates. BS – bile salts, temp- temperature, Na^+ - sodium cation, vit. – vitamin, MSR – molar solubilisation ratio, ref – references, TDC – taurodeoxycholate, TCDC – taurochenodeoxycholate, TC – taurocholate, GDC – glycodeoxycholate, GCDC – glycochenodeoxycholate, GC – glycocholate, C- cholate, DC – deoxycholate.

BS	Solubilizate	$logK_{ow}$	Volume	Temp [K]	pH	Na^+ [M]	MSR	Ref
TDC	Azobenzene	4.13	174.56	310.15	6.30	15.00	0.04	(1)
TCDC	Azobenzene	4.13	174.56	310.15	6.30	15.00	0.03	(1)
TC	Azobenzene	4.13	174.56	310.15	6.30	15.00	0.02	(1)
GDC	Azobenzene	4.13	174.56	310.15	6.30	15.00	0.05	(1)
GCDC	Azobenzene	4.13	174.56	310.15	6.30	15.00	0.04	(1)
GC	Azobenzene	4.13	174.56	310.15	6.30	15.00	0.02	(1)
TDC	Monoolein	6.61	386.27	310.15	6.30	0.15	1.41	(1)
TCDC	Monoolein	6.61	386.27	310.15	6.30	0.15	1.58	(1)
TC	Monoolein	6.61	386.27	310.15	6.30	0.15	0.84	(1)
GDC	Monoolein	6.61	386.27	310.15	6.30	0.15	1.76	(1)
GCDC	Monoolein	6.61	386.27	310.15	6.30	0.15	1.90	(1)
GC	Monoolein	6.61	386.27	310.15	6.30	0.15	1.42	(1)
GDC	vit. K	8.80	483.87	298.15	7.00	0.00	0.03	(4)
GC	vit. K	8.80	483.87	298.15	7.00	0.00	0.02	(4)
GDC	vit. K	8.80	483.87	298.15	7.50	0.00	0.03	(4)
GC	vit. K	8.80	483.87	298.15	7.50	0.00	0.02	(4)
GDC	cholestrol	7.62	423.13	310.15	7.00	0.00	0.46	(5)
TDC	cholestrol	7.62	423.13	310.15	7.00	0.00	0.37	(5)
GC	cholestrol	7.62	423.13	310.15	7.00	0.00	0.36	(5)
GCDC	cholestrol	7.62	423.13	310.15	7.00	0.00	0.29	(5)
TC	cholestrol	7.62	423.13	310.15	7.00	0.00	0.27	(5)
TCDC	cholestrol	7.62	423.13	310.15	7.00	0.00	0.23	(5)
C	Benzene	1.94	84.04	298.00	7.00	0.00	0.90	(2)
C	Fluorobenzene	2.10	88.97	298.00	7.00	0.00	0.45	(2)
C	Hexafluorobenzene	2.63	113.63	298.00	7.00	0.00	0.55	(2)
C	Toluene	2.39	100.60	298.00	7.00	0.00	0.45	(2)
C	p-Fluorotoulene	2.55	105.54	298.00	7.00	0.00	0.45	(2)
C	Styrene	2.79	111.78	298.00	7.00	0.00	0.60	(2)
C	propenylbenzene	3.04	128.02	298.00	7.00	0.00	0.40	(2)
C	Anisole	1.99	109.59	298.00	7.00	0.00	0.50	(2)
C	Fluoroanisole	2.11	114.52	298.00	7.00	0.00	1.00	(2)
C	Acetophenone	1.84	119.59	298.00	7.00	0.00	0.80	(2)
C	Fluoroacetophenone	1.98	124.52	298.00	7.00	0.00	0.50	(2)
C	Nitrobenzene	1.90	107.38	298.00	7.00	0.00	0.25	(2)
C	Mesitylene	3.21	133.73	298.00	7.00	0.00	0.35	(2)
C	Tetraline	3.15	140.41	298.00	7.00	0.00	1.30	(2)
C	Veatrole	1.61	135.13	298.00	7.00	0.00	1.45	(2)
DC	Benzene	1.94	84.04	298.00	7.00	0.00	0.81	(2)
DC	Fluorobenzene	2.10	88.97	298.00	7.00	0.00	0.76	(2)
DC	Fluorotoluene	2.50	105.54	298.00	7.00	0.00	0.72	(2)

DC	Fluoroanisole	2.11	114.52	298.00	7.00	0.00	0.96	(2)
DC	vit. K	8.80	483.87	298.15	7.00	0.00	0.05	(4)
C	vit. K	8.80	483.87	298.15	7.00	0.00	0.03	(4)
DC	vit. K	8.80	483.87	298.15	7.50	0.00	0.05	(4)
C	vit. K	8.80	483.87	298.15	7.50	0.00	0.02	(4)
C	Cholesterol	7.62	423.13	310.15	10.00	0.00	0.04	(3)
C	Stigmasterol	7.87	450.33	310.15	10.00	0.00	0.02	(3)
C	Cholesterol+Stigmasterol	7.70	426.73	310.15	10.00	0.00	0.04	(3)
C	cholestanol	7.80	429.34	310.15	10.00	0.00	0.03	(3)
DC	Cholesterol	7.62	423.13	310.15	10.00	0.00	0.07	(3)
DC	Stigmasterol	7.87	450.33	310.15	10.00	0.00	0.04	(3)
DC	Cholesterol+Stigmasterol	7.70	436.73	310.15	10.00	0.00	0.08	(3)
DC	Cholestanol	7.80	429.34	310.15	10.00	0.00	0.06	(3)

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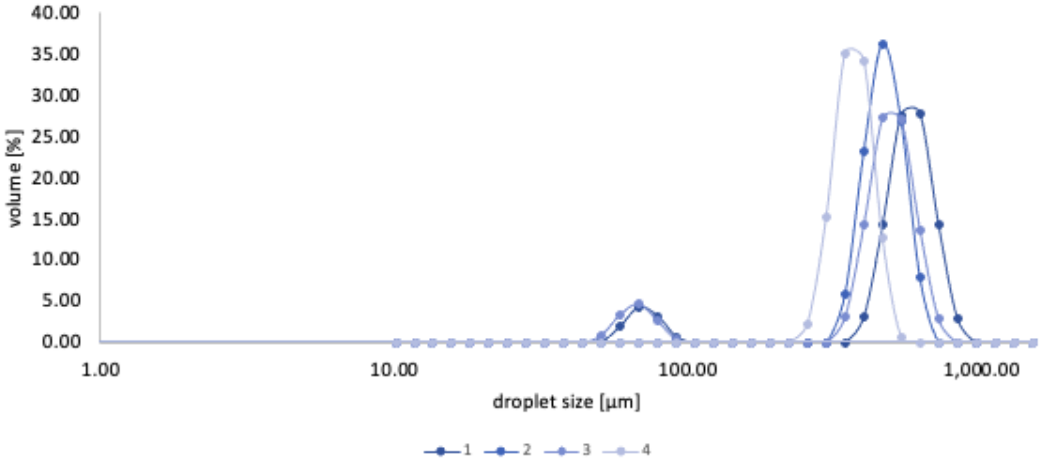

Figure S1. Particle size distribution of O/W emulsion with 0.5% WPI. Numbers: 1,2,3,4 corresponds to each number of run.

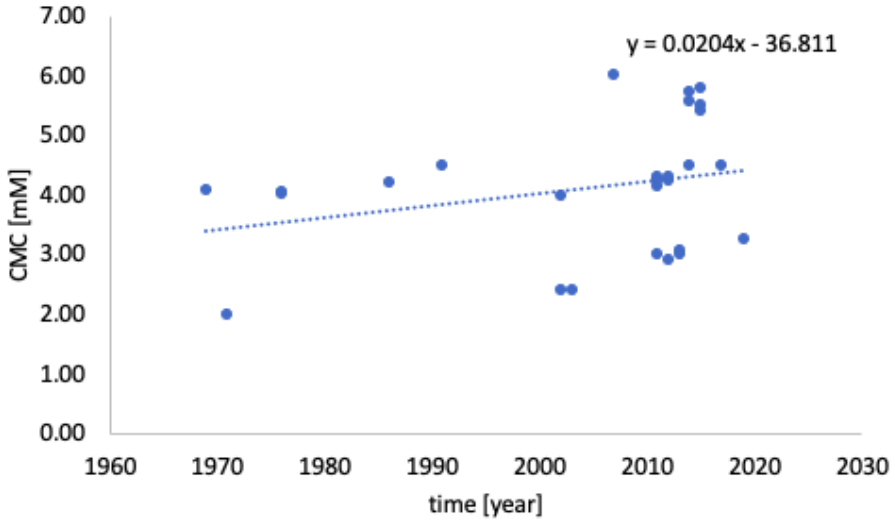

Figure S2. CMC of NaDC at 298.15K increased from 3.92 mM in the 1960-70s to an average of ~4.16 mM in the 2010-2020.

3.2. Publication 2 -A2

Krupa, Łukasz, Robert Staroń, Dorota Dulko, Natalia Łozińska, Alan R. Mackie, Neil M. Rigby, Adam Macierzanka, Aleksandra Markiewicz, and Christian Jungnickel. 2021. "Importance of Bile Composition for Diagnosis of Biliary Obstructions" *Molecules* 26, no. 23: 7279. https://doi.org/10.3390/molecules26237279

3.2.1. Objective of research

The main aim of the research was to determine the importance of BS as the disease indicator. Development of the disease state may change the BS concentration, which may lead to alteration of the BS synthesis, which was hypothesized to result in a reduced health state. Therefore, the main objective was to identify BS as a biomarker for specific diseases. BS undergo specific changes, which allows them to work as disease indicators. The research presented the hypothesized mechanism of changing BS composition concerning the disease state.

3.2.2. Reason for undertaking the research problem

Besides BS's role in the lipid digestion process, they are responsible for stimulating receptors and controlling their synthesis.

The development of gallstones and cholangiocarcinoma may be responsible for the alteration of BS concentration and its synthesis.

There are only a few scientific works that characterized the BS profile in plasma or serum in cholangiocarcinoma and pancreatic neoplasm. Therefore, it was important to analyze those reports and compare them to experimental results for a better understanding of the mechanism under which BS concentration changes.

There is no clear information on how specific diseases alter BS profiles. To reveal new disease indicators, it is valuable to understand the mechanism of changing BS concentration and its ratio

The disease state indirectly induces changes in BS composition, which may consequently influence the rate of the lipolysis process.

Experiments considering different variations of predominant forms of BS in our gastrointestinal tract would be dominant in assessing the efficiency of the lipid digestion process. This would provide us with information on how the disease state and its corresponding BS can influence the lipolysis process.

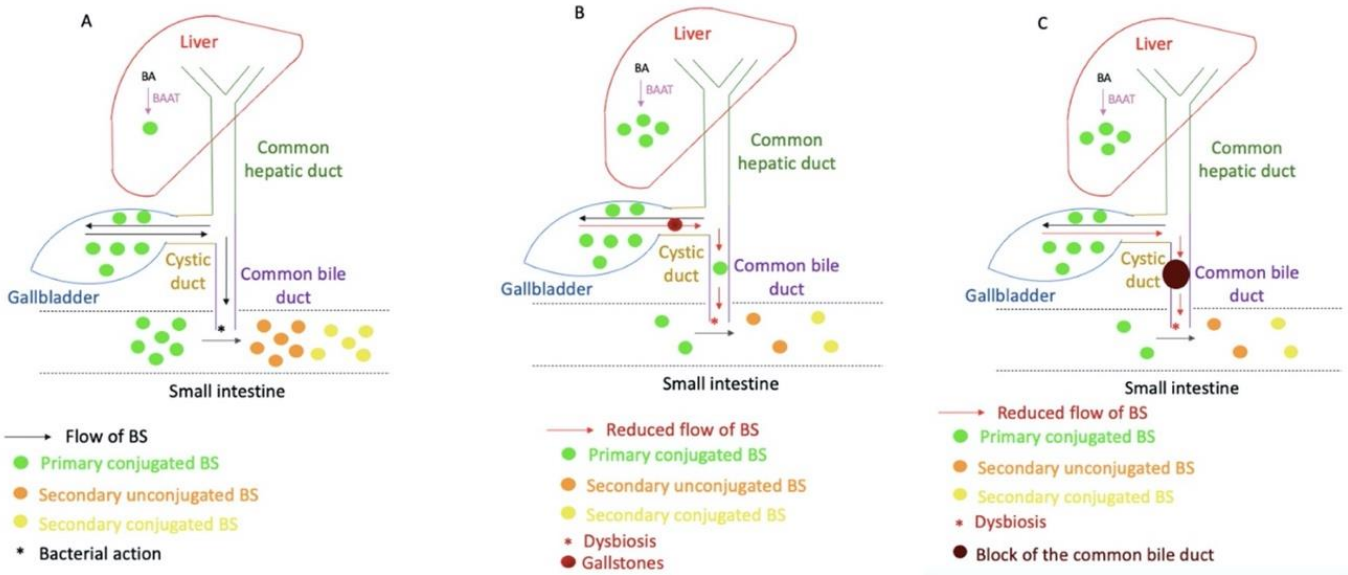

Figure 7 A. The healthy state ensures the normal flow of the BS. B. Formation of gallstones results in reduction of BS flow and dysbiosis which influence the composition of BS in the small intestine. C. Blockage of the common bile duct results in reduced BS flow and promotes dysbiosis in the small intestine. BS – bile salts, BA – bile acids, BAAT - BA CoA: amino acid N-acyltransferase

3.2.3. Main outcomes and conclusions

- BS showed a potential to work as a disease indicator of gallstones cholangiocarcinoma, and choledocholithiasis. However, the changes in BS concentration were not sensitive enough to identify them as disease indicators. We did not have enough data and knowledge to specify if the alteration of BS concentration was only related to the development of disease. A pathogenic state could result in a change of BS concentration but probably BS concentration could be a result of a different factor (for example change in BS synthesis) and therefore result in the development of disease. The process is very complicated and should be investigated in separate research only focused on BS synthesis and concentration.
- Experimental data and literature data collected by meta-analysis of disease state showed to be statistically significant from the reference value of the healthy state, indicating alteration of BS conc with development of disease
- Development of cholangiocarcinoma, choledocholithiasis and pancreatic neoplasm tend to increase the C/U BS ratio from 2.54 in healthy patients to respectively 35.90, 56.03 and 46.45 in experimental data and 135.35, 79.49 and 43.57 of literature data
- The gallstones formed in the gallbladder may reduce the flow of the BS and lead to blockage of the cystic duct and common bile duct leading to dysbiosis in the small intestine and affecting the deconjugation process.

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Importance of Bile Composition for Diagnosis of Biliary Obstructions

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Abstract: Determination of the cause of a biliary obstruction is often inconclusive from serum analysis alone without further clinical tests. To this end, serum markers as well as the composition of bile of 74 patients with biliary obstructions were determined to improve the diagnoses. The samples were collected from the patients during an endoscopic retrograde cholangiopancreatography (ERCP). The concentration of eight bile salts, specifically sodium cholate, sodium glycocholate, sodium taurocholate, sodium glycodeoxycholate, sodium chenodeoxycholate, sodium glycochenodeoxycholate, sodium taurodeoxycholate, and sodium taurochenodeoxycholate as well as bile cholesterol were determined by HPLC-MS. Serum alanine aminotransferase (ALT), aspartate transaminase (AST), and bilirubin were measured before the ERCP. The aim was to determine a diagnostic factor and gain insights into the influence of serum bilirubin as well as bile salts on diseases. Ratios of conjugated/unconjugated, primary/secondary, and taurine/glycine conjugated bile salts were determined to facilitate the comparison to literature data. Receiver operating characteristic (ROC) curves were determined, and the cut-off values were calculated by determining the point closest to (0,1). It was found that serum bilirubin was a good indicator of the type of biliary obstruction; it was able to differentiate between benign obstructions such as choledocholithiasis (at the concentration of >11 µmol/L) and malignant changes such as pancreatic neoplasms or cholangiocarcinoma (at the concentration of >59 µmol/L). In addition, it was shown that conjugated/unconjugated bile salts confirm the presence of an obstruction. With lower levels of conjugated/unconjugated bile salts the possibility for inflammation and, thus, neoplasms increase.

Keywords: bilirubin; conjugated/unconjugated bile salts; biliary obstruction; pancreatic neoplasm; cholangiocarcinoma; choledocholithiasis

Citation: Krupa, Ł.; Staroń, R.; Dulko, D.; Łozińska, N.; Mackie, A.R.; Rigby, N.M.; Macierzanka, A.; Markiewicz, A.; Jungnickel, C. Importance of Bile Composition for Diagnosis of Biliary Obstructions. *Molecules* **2021**, *26*, 7279. https://doi.org/10.3390/molecules26237279

Academic Editor: Farid Chemat

Received: 9 November 2021
Accepted: 27 November 2021
Published: 30 November 2021

1. Introduction

Biliary obstruction refers to a blockage of any duct that carries bile from the liver and from the gallbladder to the small intestine. This can occur at any of the levels of the biliary system. The symptoms of biliary obstruction result from the accumulation of bilirubin in the blood. Biliary obstructions may be caused by benign or malignant diseases of the alimentary tract. The main cause of benign biliary obstruction is choledocholithiasis due to gallstone formations [1]. Other benign causes include strictures post cholecystectomy, inflammatory stricture formation secondary to cholangitis, pancreatitis, and idiopathic

causes. Other rarer causes of nonmalignant obstructions are choledochal cysts, primary sclerosing cholangitis, and Mirrizi syndrome [2]. In addition, liver transplantation may cause biliary track dysfunction in 13–35% of patients [3].

The malignant process also promotes the formation of biliary strictures [4]. Malignant obstructions are most commonly caused by cholangiocarcinoma and pancreatic cancer. Other causes are gallbladder cancer, compression by malignant lymph nodes, and metastasis [5,6]. The differentiation between malignant and benign causes is very important for clinicians as most malignant obstructions, like those caused by cholangiocarcinoma and pancreatic cancer, are unresectable at the time of diagnosis, and treatment options are restricted to palliative management, which typically involves stent insertion as a mainstay of treatment. The survival of patients with malignancies affecting the biliary duct varies between 3–10 months [7]. In cases of pancreatic cancer, the median survival time is 8 months [8]. Inoperable tumors decrease the survival potential further [9].

Types of bile duct strictures are difficult to differentiate by noninvasive methods [10], such as radiological imaging alone. The noninvasive radiological modalities for evaluation of these patients include ultrasonography, contrast-enhanced CT scans, MRI, and magnetic resonance cholangiopancreaticography (MRCP). These noninvasive diagnostic methods may provide information about the level of obstruction, the extent of biliary dilatation, and the presence of a mass or distant metastasis [11,12] and are crucial for further treatment of the patient. On the other hand, endoscopic retrograde cholangiopancreatography (ERCP), percutaneous transhepatic cholangiography (PTC), and endoscopic ultrasound (EUS) are invasive tests, which provide additional imaging information and allow tissue sampling and treatment during the same session. One of the commonly used methods for biliary stricture diagnosis and treatment is ERCP, which provides histopathological tissue diagnosis in 35% of cases and shows a 100% specificity rate for the malignancy diagnosis (pancreatic cancer, biliary cancer, cancer of the ampulla of Vater, metastatic diseases involving bile ducts, and other rare causes) [13]. Techniques providing the tissue for cytological or histological diagnosis include the collection of bile [14], brush cytology [15], or forceps biopsy [16], and direct cholangioscopy.

Despite the advances in imaging modalities and new endoscopic techniques, differentiating between benign and malignant causes of biliary obstructions remains challenging. Endoscopic techniques of tissue acquisition such as biopsies, brushings, and fine needle aspiration (FNA) may provide a definitive tissue diagnosis; however, the combined sensitivity of these techniques is in the region of 60% [17,18]. The sensitivity of endoscopic ultrasound (EUS) and FNA for the diagnosis of a malignant biliary obstruction ranges widely from 43% to 86% [19–21]. The combination of ERCP and EUS may improve the rate of histological confirmation of malignancy. All the endoscopic tests are invasive and associated with risks from complications. The initial diagnosis is based on a review of clinical, biochemical, and radiological features.

Although biliary strictures present a diagnostic challenge and are hard to differentiate, the laboratory parameters may help to indicate the types of strictures [22]. The laboratory values of the liver function tests in the serum and current tumor marker levels lack sufficient specificity to determine the precise cause of a biliary obstruction [23–25]. Serum bilirubin levels are a strong predictor of biliary malignant diseases, with the optimum sensitivity and specificity for malignancy at bilirubin levels of >100 μmol/L [26]. Patients with cholangiocarcinoma had elevated bilirubin levels (60–470 μmol/L) [27]. Raised bilirubin levels were also associated with the development of pancreatic cancer [28] and the increased risk of gallstone formation [29].

Bile salts (BSs) may play an important role in the determination of the cause of stricture formation. BSs are synthesized from cholesterol in the liver and stored in the gallbladder [30]. Cholic acid and chenodeoxycholic acid are two primary bile acids (BAs) synthesized in the human liver. BAs undergo modification by the liver and are conjugated with the glycine or taurine molecule by BA-CoA amino acid N-acyltransferase (BAAT) to form BSs [31]. This process ensures lowering the pK_a value of the formed BSs [32].

Thus, at the physiological pH, the conjugated BSs appear in the ionized form. BSs are transported across the canalicular membrane to the gallbladder, from where they are secreted in the bile to the duodenum after meal intake [33]. In the duodenum and onwards, the formation of deconjugated BSs, deoxycholic acid, and lithocholic acid can occur due to the presence of intestinal bacteria that secrete the bile salt hydrolase (BSH), an enzyme responsible for this conformational change. The secondary BSs may be further conjugated with glycine and taurine molecules to form secondary conjugated BSs. Bile salts are natural ligands for the nuclear BA receptor, Farsenoid X receptor (FXR), and are responsible for the activation of FXR [34]. Activated FXR inhibits the expression of the CYP7A1 enzyme which is responsible for the synthesis of the BSs from cholesterol in the liver [35]. Therefore, BSs are able to control their own synthesis. FXR supresses the CYP7A1 gene expression by induction of the hepatic small heterodimer partner (SHP, NROB2), which inhibits activity of the tissue specific liver receptor homolog 1 (LRH-1), which is responsible for controlling the expression of the CYP7A1 enzyme, and via the induction of the ileal hormone fibroblast growth factor 19 (FGF19) in humans and FGF15 in mice [34,36,37]. Moreover, expression of the hepatic BA transporters is also controlled by FXR. The BA transporters, the Na^+ taurocholate cotransporting polypeptide (NTCP), the bile salt export pump (BSEP), the apical sodium-dependent BA transporter (ASBT), and the organic solute transporter OSTα-OSTβ are responsible for controlling the absorption rate of BSs, the enterohepatic circulation, and the removal of BSs from the body [38]. FXR is also responsible for regulation of BAAT [39].

BSs are transported down from the liver and through the biliary tree to the gallbladder [40]. After the BSs have contributed to food digestion and nutrient absorption, the majority (>90%) are reabsorbed by active transport at the terminal ileum [40] to the liver in a process known as enterohepatic circulation [41]. This signifies that BSs can be obtained from either de novo synthesis or can be recycled from enterohepatic circulation. The transport of the BSs from the blood to the hepatocytes takes place with assistance from sodium-dependent cotransporters [42].

It has been known since 1939 that BSs act as potential carcinogens [43] with a cytotoxic effect on hepatocytes and enterocytes [44] and negatively affect the mucosa of the stomach, intestine, and gallbladder [45]. Specifically, secondary unconjugated BSs, due to their higher hydrophobicity, are more toxic than their primary forms [44]. They promote oxidative/nitrosative stress, cause DNA damage, and promote apoptosis and mutation [46]. Conversely, Dai et al. [47] have indicated that unconjugated BAs promote cell apoptosis and reduce the growth of cholangiocarcinoma cells, whereas conjugated BSs promote cell growth. This would indicate that the exact nature of the role of BAs in cancer formation is as yet poorly understood. However, it is clear that an interaction exists, as it has been shown that the concentration of conjugated BSs in benign biliary diseases has been shown to be statistically lower than in cholangiocarcinoma patients [48]. Zhang et al. [49], as one of the only reports on BAs in serum, have proposed BAs as the biomarkers for cholangiocarcinoma since the ratio of conjugated to unconjugated BAs in cholangiocarcinoma patients was shown to be reduced. Therefore, the imbalance of the BA composition was indicated to play a crucial role in the development of cholangiocarcinoma [50]. Transporters located in the canalicular membrane allow for the secretion of BSs from hepatocytes. Inhibition of the BS secretion results in the pathophysiologic concentration of BSs named cholestasis. How these cause cancer has been postulated through investigation using cultured rat hepatocytes [51]. Jaeschke et al. postulated that elevated BS concentrations cause the translocation of the intracellular Fas ligand (can induce apoptosis and is a tumor necrosis factor) to the cell membrane and trigger cell death [51]. Lower in the biliary tree, BAs have been suggested to be a key factor influencing the development of pancreatic cancer [52–54]. Rees et al. [55] have compared the level of BAs in patients with pancreatic cancer and with benign disease. Increased concentrations of the unconjugated BAs in the malignant group may be explained by the bacteria proliferation in and around the common bile duct.

Therefore, an alteration in the conjugation level and an obstruction-induced reduction of BS concentration in the small intestine may change the BS synthesis via action of FXR and promote excessive BA synthesis. Imbalance in the BA composition can be caused by a number of factors, e.g., 1. altered synthesis due to FXR promotion or inhibition [47,56]; 2. change in the function of BAAT; 3. change in the function of the BA transporters [57]; 4. change in the external concentration of the BS due to a blockage [58–60]; 5. changed function of BSH [58–60].

Therefore, the aim of this study was to analyze the significance of serum bilirubin as an indicator for biliary obstructions as well as to analyze the causal and diagnostic significance of bile salts in biliary obstructions. To achieve this aim, we have collected and analyzed human bile from 150 patients diagnosed with various biliary obstructions occurring at different levels of the biliary tree.

2. Results and Discussion

2.1. Bilirubin as an Indicator of Neoplasm

The importance of bilirubin levels in diseases is shown in Figure 1, which clearly differentiates neoplasms (cholangiocarcinoma and pancreatic cancer) from the nonmalignant diseases (choledocholithiasis and stricture).

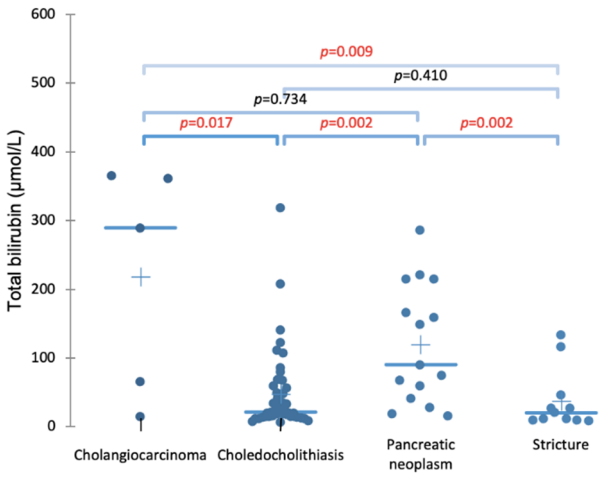

Figure 1. Comparison of the bilirubin levels in the serum of patients with different conditions resulting in biliary blockage. It can clearly be seen that the bilirubin levels in the case of a neoplasm (either cholangiocarcinoma or pancreatic) is significantly higher compared to benign strictures or choledocholithiasis. *p*-values were determined using the Kruskal-Wallis test with the Dunn post hoc nonparametic comparison.

2.2. Importance of BSs as Indicators of Biliary Obstruction

Table 1 shows the average levels of measured compounds with the standard deviation in each of the four classified pathologies. The ratios are calculated as a relative ratio, relative to the number of items. The concentrations of different BSs measured in the bile and blood of 63 patients with pathological biliary obstructions were compared to 11 patients with benign strictures (postinflammatory, postsurgical, iatrogenic, idiopathic), since it is not ethical to extract bile from healthy patients. However, literature values for healthy patients are shown indicatively. Figure 2 shows the cholesterol and liver functions of the recorded patients.

Table 1. Summary of the data from the 74 cases of biliary obstruction. Shown are the average levels of measured compounds with the standard deviation in each of the four classified pathologies. The ratios are calculated as a relative ratio, relative to the number of items. When comparing literature values of serum and bile levels, it was shown that the ratios of conjugated/unconjugated, primary/secondary, and taurine/glycine conjugated BSs did not differ between the serum of healthy patients and the collected bile of patients (with p values of 0.221, 0.053, and 0.355, respectively).

	Cholangiocarcinoma	**Choledocholithiasis**	**Pancreatic Neoplasm**	**Stricture**
Number of patients	5	43	15	11
Age (years)	72.6 ± 13.74	67.14 ± 17.96	71.00 ± 14.40	70.36 ±14.94
BS concentrations:				
Chenodeoxycholic acid (mmol/L)	0.16 ± 0.18	0.31 ± 0.17	0.31 ± 0.17	0.14 ± 0.14
Glycodeoxycholic acid (mmol/L)	5.00 ± 7.03	5.36 ± 8.53	2.02 ± 3.78	7.31 ± 13.23
Glycochenodeoxycholic acid (mmol/L)	11.17 ± 16.02	20.70 ± 17.57	26.81 ± 50.63	25.25 ± 24.28
Glychocholic acid (mmol/L)	6.41 ± 9.98	9.54 ± 6.71	10.46 ± 13.09	15.49 ± 10.91
Taurodeoxycholic acid (mmol/L)	4.11 ± 5.63	2.61 ± 3.72	1.12 ± 1.66	2.18 ± 3.11
Taurochenodeoxycholic acid (mmol/L)	4.80 ± 6.56	7.30 ± 6.77	6.50 ± 5.63	6.20 ± 4.10
Taurocholic acid (mmol/L)	6.25 ± 9.17	9.73 ± 9.36	9.46 ± 8.87	9.27 ± 5.97
Calculated ratios from literature:				
ALT/AST				
Conjugated/Unconjugated BS	112.56–158.14 [61,62]	2.14–156.85 [62–65]	19.2–43.57 [55,62]	
Primary/Secondary BS	14.27–62.69 [61,62,66]	2.91–17.71 [59,62–64,66–71]	2.79–13.17 [55,62]	
Taurine/Glycine conjugated BS	0.66–1.13 [49,61,62]	0.23–0.9 [62–64,69,70]	0.41–1.45 [55,62]	
Healthy patients (literature):	Measured from serum		Measured from bile	
ALT/AST U/L	1.42 ± 0.02 [72]		N/A	
Bilirubin (µmol/L)	12.6 [73]		N/A	
Conjugated/Unconjugated BS	1.28–1.60 [49,74]		2.54 [64]	
Primary/Secondary BS	0.72–2.95 [49,59,66,74,75]		2.47–3.60 [64,69,70]	
Taurine/Glycine conjugated BS	0.27–2.40 [49,52,74,75]		0.26–0.30 [64,69]	

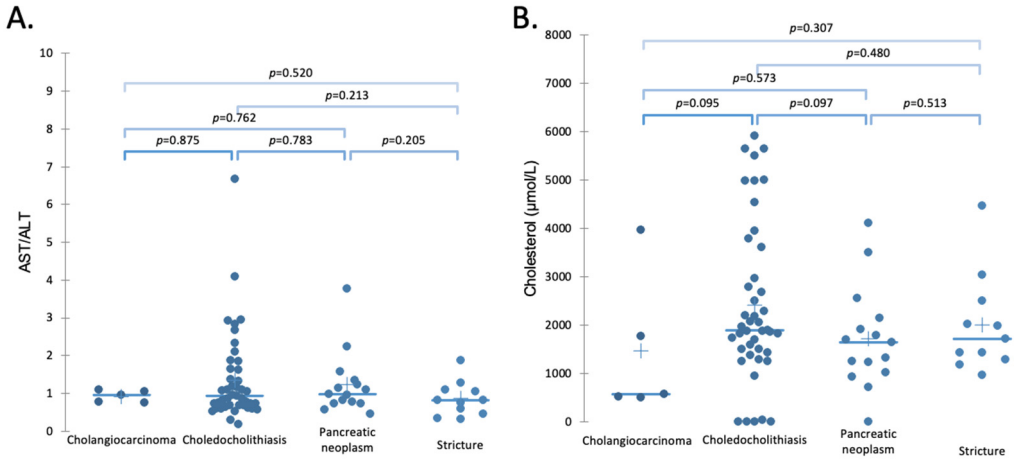

Figure 2. AST/ALT ratio (**A**) and cholesterol levels (**B**) measured in bile collected from patients with different malignancies. (**A**). Traditional liver diagnostic tests. AST/ALT ratio were not able to successfully differentiate the biliary blockages. (**B**). Cholesterol levels of the bile were not significantly different in any of the disease groups; however, the highest cholesterol levels were found for the choledocholithiasis patients.

2.2.1. Choledocholithiasis

Our results have shown that BS concentration may function as the potential indicators of choledocholithiasis (when a gallstone blocks the bile duct). The patients with diagnosed choledocholithiasis have shown elevated primary/secondary (P/S) ratios compared to pancreatic neoplasms and elevated conjugated/unconjugated (C/U) ratios of BSs when compared to benign strictures as shown in Figure 3B,C. Blockage resulting from the gallstone formation inhibits the flow of the BSs from the gallbladder to the small intestine leading to dysbiosis and, therefore, a reduction in BSH [58–60]. Depletion of BSH leads to

suppression of the deconjugation process. Conjugated BSs are better ligands for FXR than their unconjugated forms [56]. Therefore, alterations in the conjugation levels and reduced concentrations in the small intestine due to obstructions may change BS synthesis via action of FXR and promote excessive BA synthesis. Those alterations of the expression of the FXR result in the increased expression of CYP7A1, which promotes the excessive synthesis of the BSs and consequently leads to the changed composition of the BA pool size [76]. Those alterations of BA synthesis result in a predominant concentration of primary BSs in patients with cholestasis [59], as can be observed in Figure 3C. The accumulated primary synthesized BSs may be excessively conjugated by the BA-CoA:amino acid N-acyltransferase (BAAT) enzyme, which is reflected by the elevated C/U ratio observed in Figure 3B. Disorders caused by the reduced flow of BSs and excessive BA synthesis lead to the formation of conjugation forms of BSs, which then may be responsible for the development of cholangiocarcinoma [47]. Interestingly, the cholesterol in the bile was not found to be significantly elevated compared to the other diseases (Figure 2B). The cholesterol level was shown to be correlated with the level of the C/U ratio of BS (Pearson test, $p < 0.001$, r = 0.303). Therefore, excessive levels of the chenodeoxycholic acid (Figure 3A), responsible for the solubilization of cholesterol, may explain the insignificant elevation of cholesterol levels.

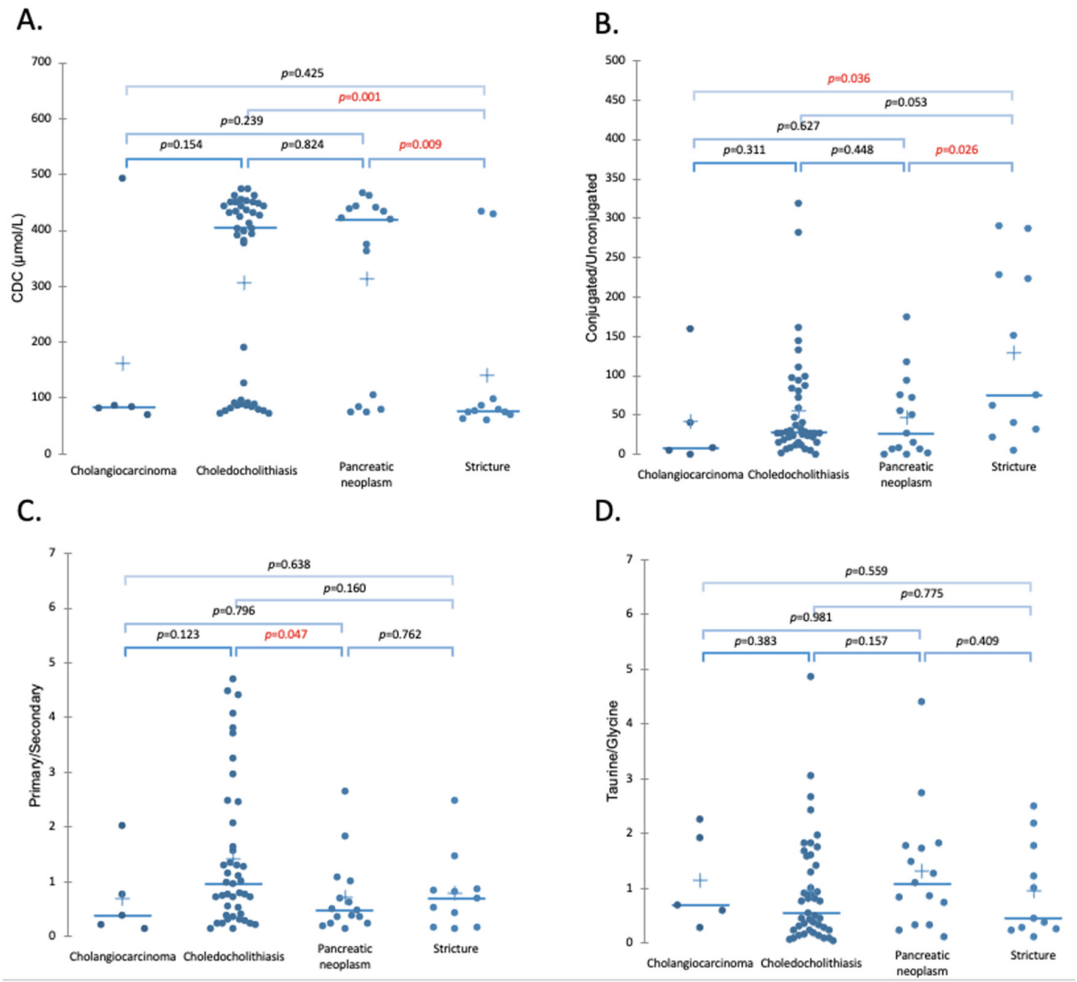

Figure 3. (**A**). Changes of CDC with respect to disease type. The statistical difference between strictures and pancreatic neoplasms as well as choledocholithiasis was indicated. Concentrations of CDC are altered with the development of gallstones and pancreatic cancer. (**B**). Conjugated/unconjugated ratios of BSs in patients with different malignancies. Elevated ratios indicate increasing conjugated forms of the BSs and simultaneous depletion of unconjugated forms. (**C**). Primary/secondary ratios of BSs. The ratios were most influenced by the presence of biliary stones. (**D**). Taurine/glycine ratios do not show any changes with disease types.

2.2.2. Cholangiocarcinoma

Strom et al. [77] noticed the differences in the BA compositions among control groups, subjects with gallstones, and subjects with bile duct cancer. Depletion of deoxycholic and lithocholic acids was noticed for biliary tract cancer [66]. The reduction in the secondary unconjugated BS level was due to the limited flow of the primary BSs to the small intestine. Hence, the decreased level of reabsorbed BSs increase activation of CYP7A1. Increased BS synthesis results in elevated levels of primary over secondary BSs, which can be observed in Figure 3C. Moreover, after reabsorption to the liver, the secondary unconjugated BSs may be conjugated with taurine or glycine, which are catalyzed by BAAT. Reduced flow of the BSs and their excessive accumulation not only change the action of the BS enzymes but also promote the creation of elevated conjugated forms of the BSs, which is reflected in the elevated C/U ratio (Figure 3B). Disorders caused by reduced flow of the BSs and excessive BA synthesis lead to the formation of elevated conjugation forms, which may be responsible for the development of cholangiocarcinoma [47]. Increased ratios of C/U BSs in patients with cholangiocarcinoma were also observed by Zhang et al. [49].

2.2.3. Pancreatic Neoplasms

Increased production of secondary BSs may be correlated with the induction of tumors at the head of the pancreas, which leads to bile duct obstruction as was suggested by Rees et al. [55]. Moreover, elevated secondary BS levels promote the generation of reactive oxygen species and induce DNA damage and cell disruption.

Pancreatic neoplasms have shown different P/S ratios than choledocholithiasis, as can be observed in Figure 3C. Decreased concentrations of secondary BSs can be a result of reduced flow of the primary BSs, caused by the obstruction. Therefore, lower amounts of the primary BSs can be transported to the small intestine and undergo the deconjugation process. The reabsorbed BSs will be further conjugated in the liver to the secondary conjugated (SC) BSs. Moreover, lower concentrations of absorbed BSs may result in the higher activation of the CYP7A1 enzyme and increased BA synthesis as well as the increased production of more primary BSs. Therefore, previously accumulated and newly synthesized BSs may undergo the conjugation process, which may result in excessive conjugation levels, as reflected in Figure 4B by the elevated C/U ratios. The P/S ratios were shown to be statistically different between cholangiocarcinoma and pancreatic neoplasms (Figure 4). The differences could result from the location of the cancer and more efficient blockage of the BS flows.

The schematic representation of possible changes in BS composition due to the development of diseases is shown in Figure 4. Under normal conditions, the BAs are synthesized in the liver and transported to the gallbladder (Figure 4A). They are released to the small intestine during the consumption of a meal, and after fulfilling their roles they are reabsorbed by the liver, and induce the expression of FXR, which inhibits activation of CYP7A1 and regulates BA synthesis. Strictures created during the benign state of disease lead to accumulation of the synthesized BSs and reduce the flow of the BSs to the small intestine (Figure 4B). The gallstones formed in the gallbladder may accumulate and form bigger aggregates in the common bile duct, which lead to decreased BS flow to the small intestine.

As can be seen in Figure 2A, patients with biliary obstructions and completely normal liver function tests (LFTs) are unlikely to have malignant pathologies, where only choledocholithiasis shows elevated AST/ALT ratios, albeit without statistical significance [78].

When analyzing the heatmap (Figure 5), BA ratios were visibly altered for choledocholithiasis compared to the other pathologies.

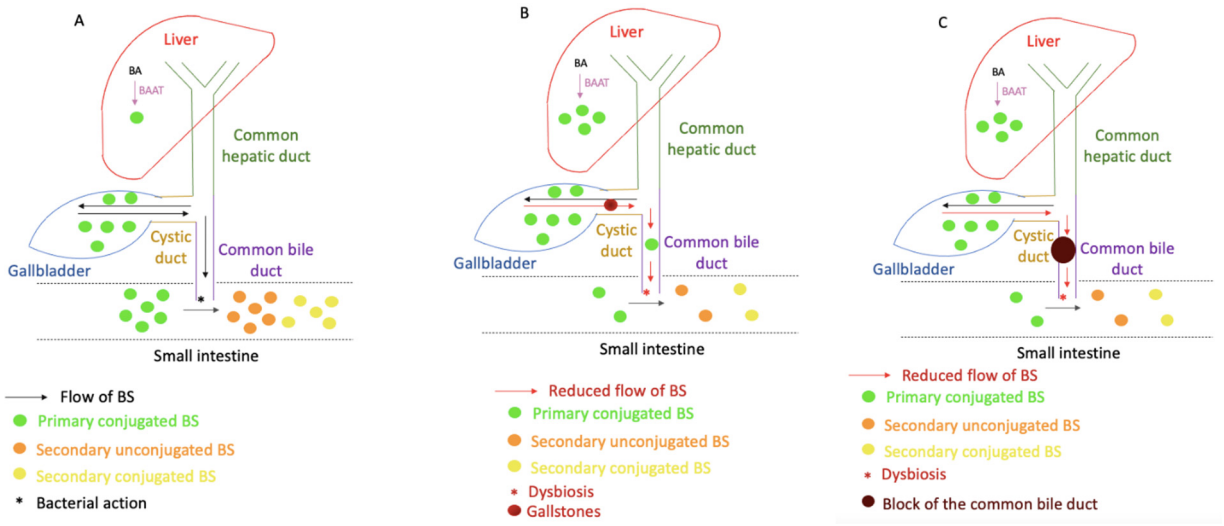

Figure 4. (**A**). The normal flow of the BSs from the liver, through the hepatic duct, cystic duct, gallbladder, and common bile duct to the small intestine. BSs are retransported back to the liver after fulfilling their role. They control their own synthesis by inducing expression of FXR. (**B**). As a result of gallstone formation, the flow of BSs is reduced. (**C**). Blockage of the BS flow caused by the development of cholangiocarcinoma. Decreased levels of reabsorbed BSs reduce the FXR signalling and promote activation of CYP7A1 enzyme leading to increased BA synthesis.

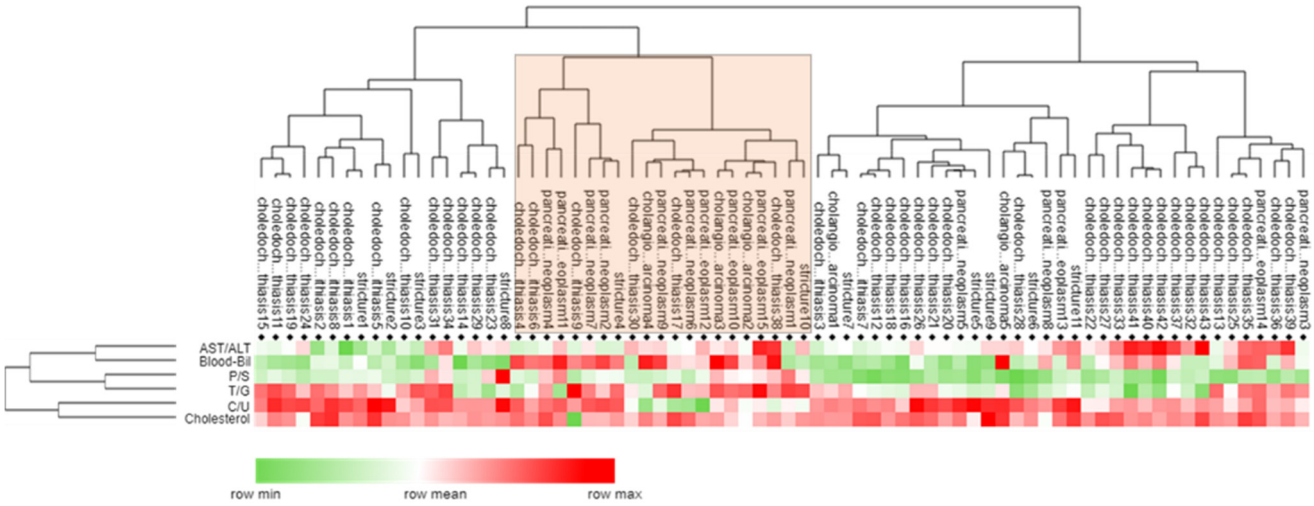

Figure 5. Heatmap and dendrogram of the diagnostic ratios (AST/ALT, conjugated/unconjugated, primary/secondary, taurine/glycine conjugated bile salts), biliary cholesterol, and serum bilirubin in the tested patients with different malignancies. Presence of pancreatic cancer-enriched samples can be identified in the cluster marked with orange (10/15 of pancreatic cancers occur in this cluster).

2.3. Diagnosis of Biliary Obstruction

When looking at the diagnostic significance of individual markers in receiver operating characteristic (ROC) curves (Figure 6), only serum bilirubin shows an appreciable diagnostic relevance, with AUC = 0.793, $p < 0.001$. None of the other values showed the same significance. However, the results indicate that there is a correlative relationship between the BS composition (as represented by the C/U ratio) and the various diseases.

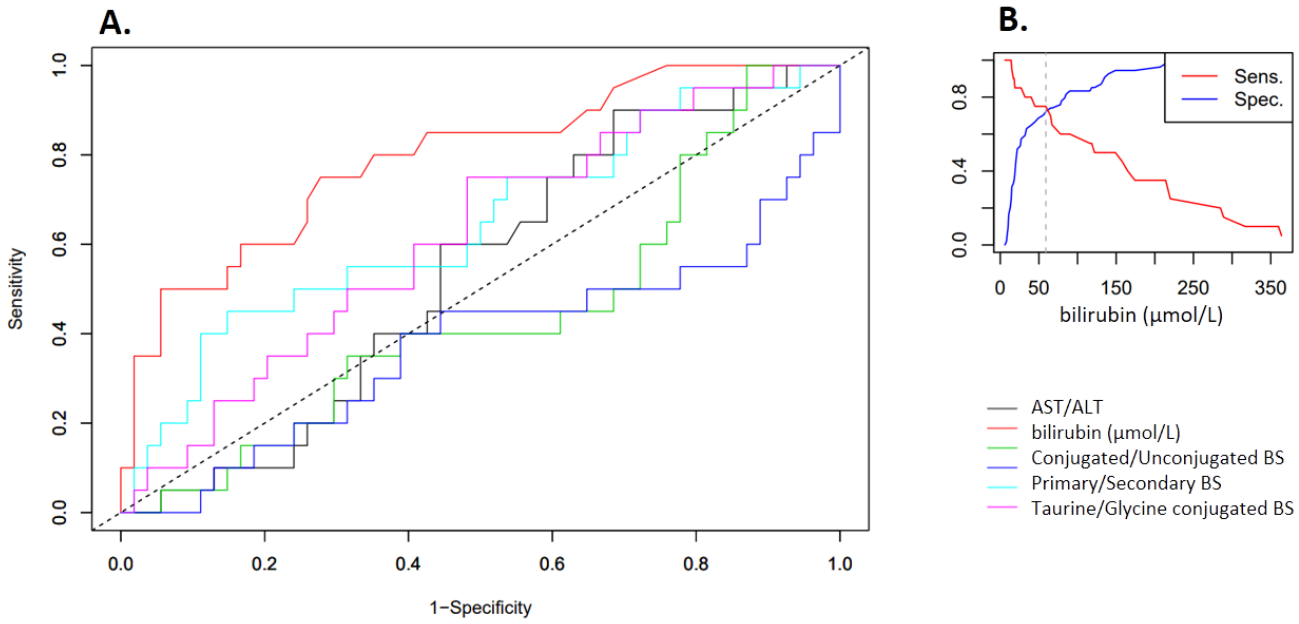

Figure 6. (**A**). ROC curves for the determination of diagnostic values of various markers to discriminate neoplasms (pancreatic cancer or cholangiocarcinoma) vs. nonmalignant changes (stricture and choledocholithiasis). Only bilirubin (AUC 0.793 with a $p < 0.001$). was statistically significant (**B**). Sensitivity and Specificity curve for bilirubin concentration clearly indicates a specificity of close to 77% if serum level is over 55 µmol/L.

Figure 7 clearly indicates the importance of bilirubin as an indicator of malignant biliary obstruction, where the cut-off value for bilirubin concentration serves as an indicator for the type of disease (i.e., above 11 µmol/L choledocholithiasis is likely, and above 59 µmol/L a neoplasm may be suspected). This is in line with the previous model of the less complete closure caused by the cholestasis [79]. The ratio of conjugated to unconjugated BSs was found to be a significant indicator for healthy patients (thus the line is below the diagonal [80]) when compared with cholestasis and neoplasm patients, with a ratio of 80.3 and 48.7, respectively. It indicates that neoplasms are characterized by almost a 50% reduction in the amount of conjugated BSs in the biliary tract (as shown in Figure 3B average C/U changes from ~50 for malignant strictures to ~120 for benign strictures). This could be due to the lower hepatic recycling of the BSs since all the conjugated as well as unconjugated BSs measured derived from a primary synthesis. The only unconjugated BAs included in our test set was a primary bile salt (sodium chenodeoxycholate). Therefore, the stronger the stricture the less bile can recirculate. This can also be seen when grouping bilirubin levels into three categories (as a result of the ROC analysis), low ($\leq$110 µmol/L), medium, and high ($\geq$59 µmol/L), and grouping these (using the Kruskal Wallis test with a Dunn post hoc test) with the three bile salt ratios (C/U, P/S, and T/G). The only correlation was found for C/U at low and medium bilirubin levels (with p values < 0.001, the p values for P/S, T/G and cholesterol where 0.242, 0.192, and 0.647 respectively).

This would indicate that the presence of unconjugated BSs increase the risk of a pathological indication. This is in line with the previous effect of unconjugated BSs on tissue (i.e., cytotoxicity and inflammation). The presence of unconjugated BSs present a higher cellular stress and may result in an inflammatory response. In fact, all biliary diseases are related to rates of inflammation in biliary tissue [81,82]. This indicates that blockage of the biliary tract can lead to self-propagating inflammation, resulting from the increased synthesis of BSs, and a loss of function of BA-CoA:amino acid n-acyltransferase (BAAT). The loss of BAAT is thought to be due to 4-hydroxynonenal (4HNE) in a dose-dependent relationship [83]. 4HNE is a well-studied aldehyde that has been shown to be directly related to oxidative stress [84].

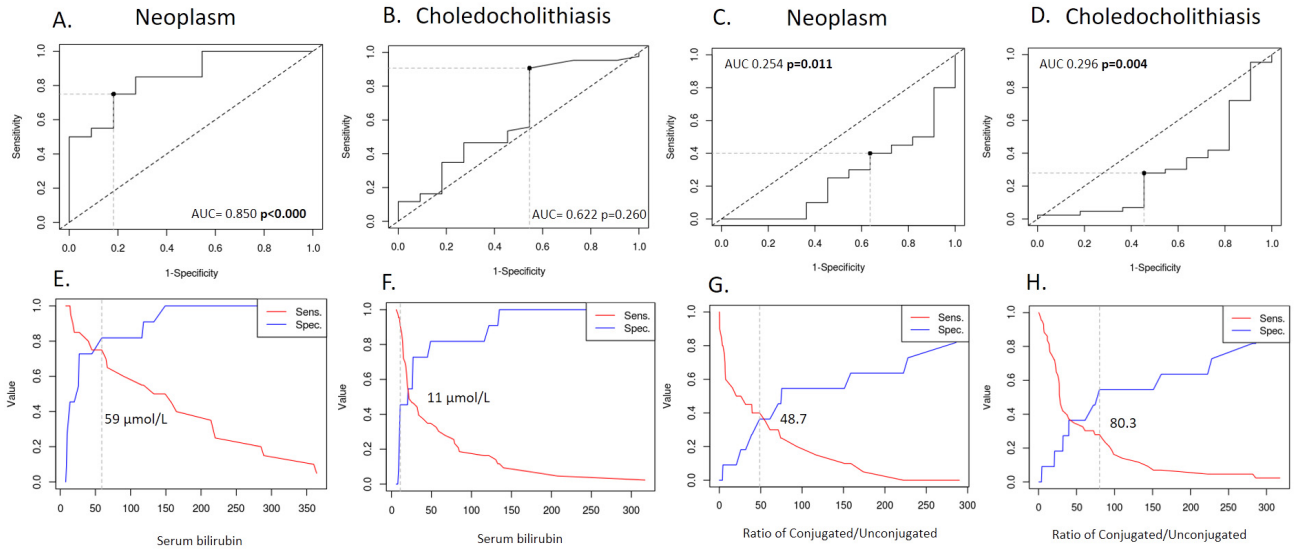

Figure 7. (**A**–**D**) ROC curves comparing neoplasms (both cholangiocarcinoma and pancreatic, (**A**,**C**)) and choledocholithiasis cases (**B**,**D**) to the benign stricture cases using two indicators: serum bilirubin and the ratio of conjugated/unconjugated BSs. In three cases (*p*-value marked in bold), the ROC curve presented a statistically significant indicator. The corresponding cut-off values (**E**–**H**) are given under each ROC curve.

3. Materials and Methods

3.1. Sample Collection

The collection and study of human bile (HB) samples were approved by the ethics committee of the Regional Medical Chamber in Rzeszów, Poland (certificate 15/B/2016). All methods were planned and conducted in accordance with the ethical principles outlined in the Declaration of Helsinki.

Samples of HB were collected at the Department of Gastroenterology and Hepatology (Teaching Hospital No. 1, Rzeszów, Poland) during an endoscopic retrograde cholangiopancreatography (ERCP). ERCP is an endoscopic procedure which involves the assessment and therapy of the bile ducts and/or pancreatic ducts.

For the purpose of our study, we included patients for whom therapeutic procedures on bile ducts were indicated. After insertion of a duodenoscope into the second part of the duodenum, the ampulla of Vater was identified. The ampulla is located at the major duodenal papilla. All patients who undergo the ERCP procedure must have evidence of biliary or pancreatic duct obstruction. This was confirmed by imaging tests such as: transabdominal ultrasound (USS, computed tomography, magnetic resonance imaging (MRI), or endoscopic ultrasound (EUS)). We recruited 150 patients who were qualified for the ERCP procedure due to imaging evidence of biliary obstruction over the period of 4 months. In order to minimize the risk of complications during the procedure, we decided that a bile aspiration attempt could not take longer than 60 s. In the case of difficult procedures such as the need for a contrast injection before cannulation of the bile duct or the need for a precut or prolonged aspiration attempt, the procedure was completed without bile aspiration. Out of 150 patients recruited for the study, bile from 74 subjects was collected.

As a routine part of ERCP, the ampulla is selectively cannulated with a dedicated sterile catheter, which was inserted selectively over the guide-wire into the bile duct. The position of the catheter was confirmed under fluoroscopy (X-ray guidance).

A syringe was attached to one end of the catheter. The assisting endoscopy nurse performed the aspiration of the bile by applying a gentle suction with a syringe. The catheter was moved back and forward from the extrahepatic bile ducts to the intrahepatic bile ducts. Approximately 2–3 mL of fluid was aspirated. Immediately after aspiration, the samples were sealed and instantly immersed in liquid nitrogen for snap freezing. Samples were

stored at −80 °C prior to further examination. The ERCP procedure was completed as planned according to indications.

Serum alanine aminotransferase (ALT), aspartate transaminase (AST), and bilirubin were measured before the ERCP procedure.

3.2. HB Analysis

The BS compositions of all HB samples were analyzed using an Agilent 1260 HPLC system coupled to an AB Sciex 4000 QTrap triple quadrupole MS (Sciex, Cheshire, UK). An aliquot of HB (10 µL) was diluted with 0.9% NaCl (990 µL). Diluted HB (50 µL) was transferred into a HPLC vial and mixed with 50 µL methanol. The MS analysis was carried out according to the method described previously [85]. The following BS reference standards were used: sodium cholate (C; C6445, Sigma-Aldrich, Dorset, UK), sodium glycocholate (GC; G7132, Sigma-Aldrich), sodium taurocholate (TC; 86339, Sigma-Aldrich), sodium glycodeoxycholate (GDC; G9910, Sigma-Aldrich), sodium chenodeoxycholate (CDC; C8621, Sigma-Aldrich), sodium glycochenodeoxycholate (GCDC; G0759, Sigma-Aldrich), sodium taurodeoxycholate (TDC; T0875, Sigma-Aldrich), and sodium taurochenodeoxycholate (TCDC; T6260, Sigma-Aldrich). The workflow is shown in Figure 8.

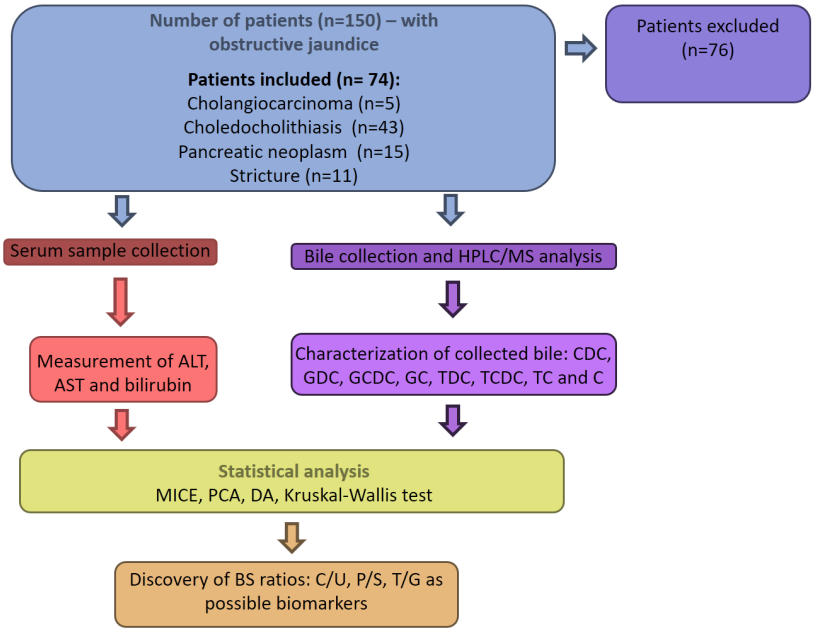

Figure 8. Flowchart from sample collection to analysis and statistical evaluation.

3.3. Statistical Analysis

From the 74 patients, 6 patients had incomplete ALT, AST, and serum bilirubin data. The missing data was imputed using MICE with five imputations. The exact procedure for MICE has been described by Łozińska et al [85].

The Kruskal Wallis test with the Dunn post hoc analysis for comparison of continuous variables in multiple groups was used, using XLSTAT (version 2020.1.3.65326). The heatmap and dendrogram were created using GenePattern Version v3.9.11-rc.5 b234 [86]. Raw data were log2-transformed, Pearson correlation was used as a distance measure in columns and row clustering. Data were row-centered by subtracting the row mean from all the values in each row. A summary of the measured data is provided in Table 1. Receiver operating curves (ROC) [87] were generated using the easyROC (v1.3.1), where the cut-off criteria was determined by minimizing the distance to the corner 0,1. 59 µmol/L. Ratios of conjugated/unconjugated (C/U), primary/secondary (P/S), and taurine/glycine conjugated (T/G) BSs were calculated using the average values of both the denominator and numerator.

4. Conclusions

After analyzing serum and bile markers in 74 patients presenting a biliary or pancreatic obstruction, we show that the level of serum bilirubin can be used as an initial simple, noninvasive screening test for predicting whether the obstruction is likely to be malignant or benign. In addition, we have analyzed the BS composition. Even though BSs play an important role in disease initiation and progression, the changes in composition are not specific enough to serve as markers.

Monitoring changes in the bile composition might allow for possible novel treatment strategies of the disease. For example, patients with cholangiocarcinoma were shown to exhibit significant imbalances in the ratios of conjugated to unconjugated BSs. This might be partially due to the (self-stimulated) excessive BA synthesis, promoted by the reduction of bile flow and the increased activation of the CYP7A1 enzyme, resulting in an elevated level of conjugated BSs. Inhibiting BS synthesis with FXR antagonists such as guggulsterone [47] may present a promising method to reduce inflammation and thereby inhibit the self-propagating disease development.

With the development of diseases, the BSs undergo specific changes. It is important, therefore, to follow the concentration changes for further development of new markers of the diseases. We recognize the limited cohort size of this study; however, the indications that disruption of BS homeostasis leads to the development of cholangiocarcinoma is a clear conclusion from our work. Furthermore, detailed studies, including high throughput metabolomic profiling are therefore required. Even though the lack of healthy controls is a clear issue with the analysis, obtaining aspirated bile from healthy patients raises ethical questions.

Author Contributions: Ł.K.: Investigation, Methodology, Writing—original draft, Writing—review & editing; R.S.: Investigation, Methodology, Writing—original draft; D.D.: Investigation; N.Ł.: Data curation, Visualization, Writing—original draft; A.R.M.: Methodology, Writing—review & editing; N.M.R.: Methodology; A.M. (Adam Macierzanka): Writing—original draft; Writing—review & editing; A.M. (Aleksandra Markiewicz): Conception, Software, Writing—review & editing; C.J.: Conception, Data curation, Supervision, Validation, Writing—original draft, Writing—review & editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Bioethical Committee of Medical Chamber in Rzeszów of Teaching Hospital No 1 (protocol code 15/B/2016, approved 29 January 2016).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are not available from the authors.

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3.3. Publication 3 -A3

Łozińska, N., Maldonado-Valderrama, J., Del Castillo-Santaella, T., Zhou, Y., Martysiak-Żurowska, D., Lu, Y., & Jungnickel, C. (2024). Bile conjugation and its effect on in vitro lipolysis of emulsions. *Food Research International*, *184*, 114255.

3.3.1. The objective of the research

- The main aim of this research was to determine five processes influencing the efficiency of the lipolysis process.
- Set measurable parameters for each process.
- Experimentally measure the changes in each parameter concerning the changing ratio of C/U BS.
- Perform meta-analysis on lipolysis data.
- Determine the most influential process affecting lipolysis efficiency.

3.3.2. Reason for undertaking the research problem

Lack of studies on changing lipolysis parameters concerning changing concentration of conjugated and unconjugated BS.

There is no clear understanding of to what extent changes in lipolysis parameters affect the FFA release from oil emulsion.

MSR is mainly measured for substances not related to the digestion process (PAH, drugs), therefore lipolysis products should be investigated to understand the importance of solubilization properties. MSR may indicate the ability of the BS to incorporate lipolysis products into their aggregates concerning the product type, which is a rate-limiting step influencing the efficiency of lipolysis.

Changes in IT and dilatational modulus for individual BS during the lipolysis process may determine their behaviour at the interphase. For now, the previously performed experiments considered IT changes concerning changes in BS concentration. Therefore, there were very limited results presenting changes in IT measurements at the physiological concentration of BS. Moreover, SU BS was not previously tested, limiting the conclusions only to the behaviour of conjugated forms of BS.

Many data covering the lipolysis experiments exist. However, there are a lot of changing variables that may influence the final result, which makes them hard to compare and uneasy to decide on the most predominant factor. The unification of data and the creation of the lipolysis modelling can give us perspective to foresee the final FFA release by controlling the individual factors of the process. Moreover, it would also allow us to determine the most predominant factor affecting the lipolysis process.

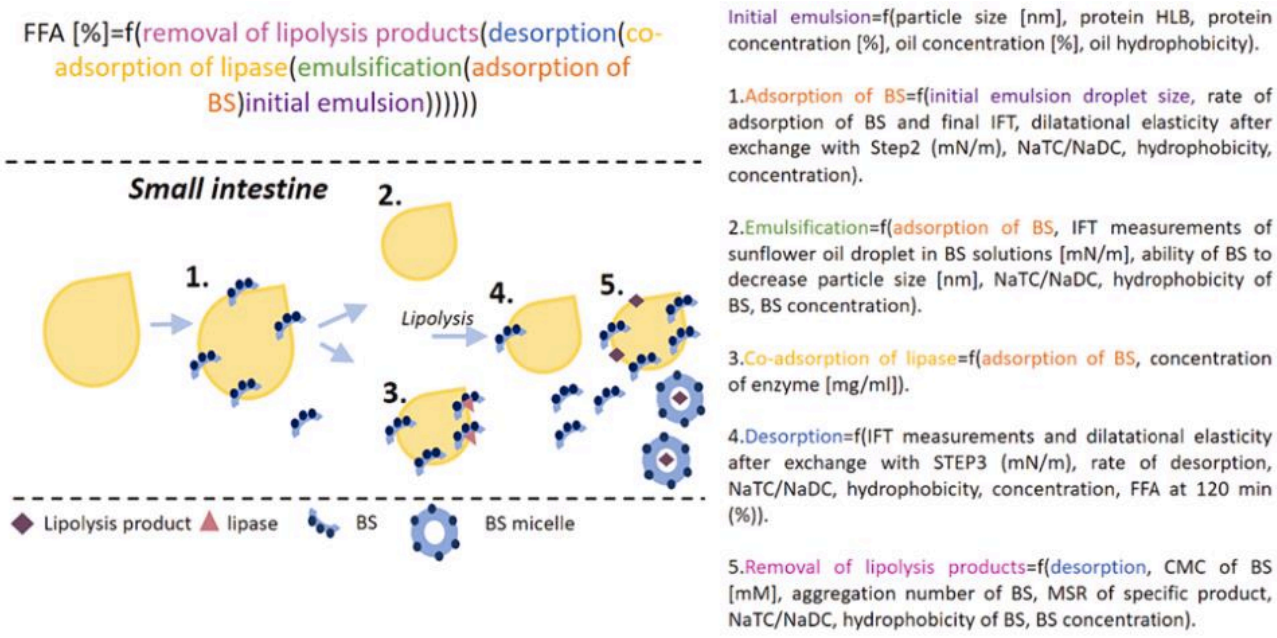
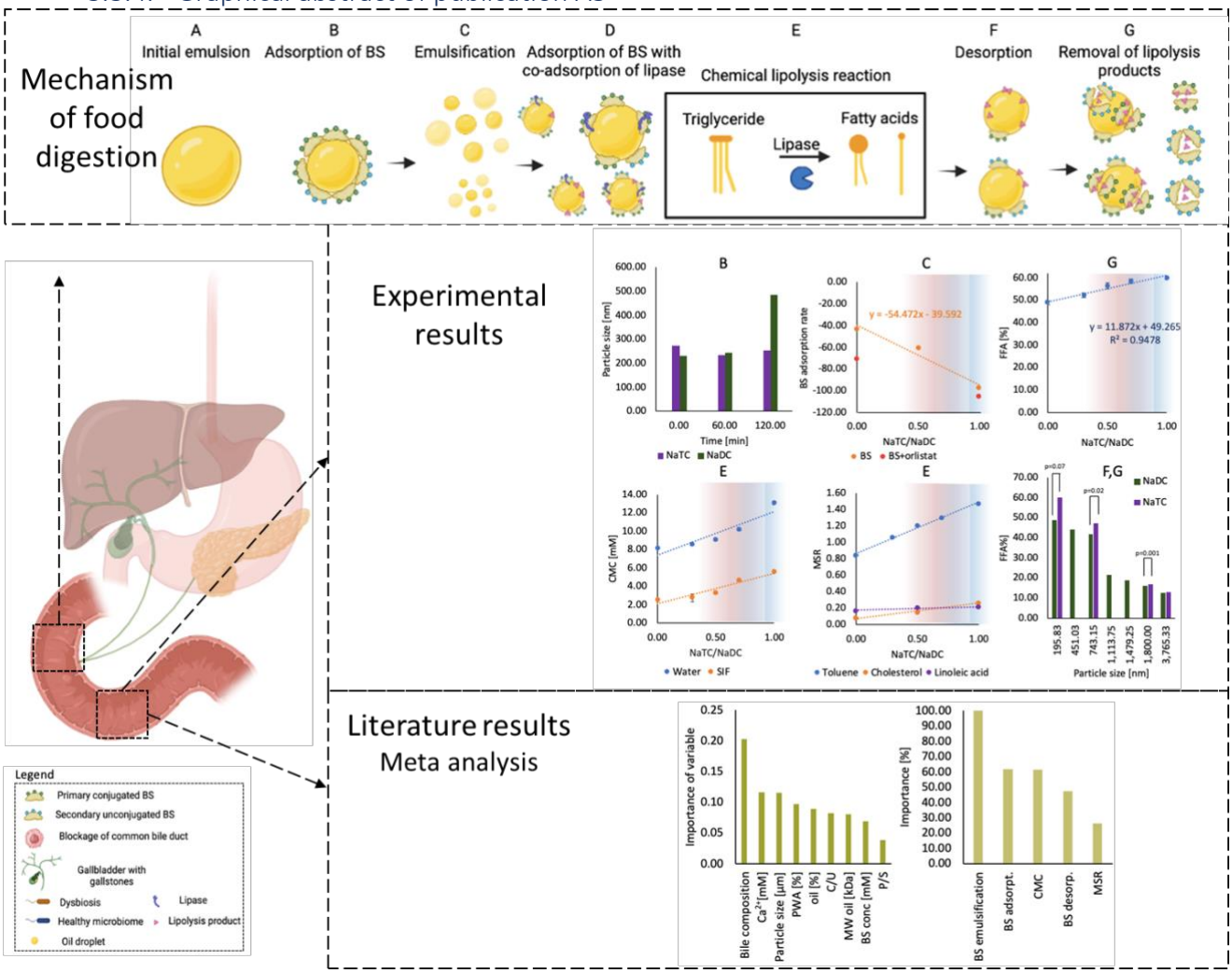


Figure 8 Lipolysis was determined to depend on five dominant processes: 1. Adsorption of BS, 2. Emulsification, 3. Co-adsorption of lipase, 4. Desorption, 5. Removal of lipolysis products. Each of the processes was described as a mathematical function, with parameters influencing its efficiency. Each of the parameters can be experimentally measured concerning the form of the BS. BS – bile salts, IFT – interfacial tension, NaTC – sodium taurocholate, NaDC – sodium deoxycholate, HLB – hydrophilic lipophilic balance, FFA – free fatty acids, CMC – critical micelle concentration, MSR – molar solubilisation ratio.

3.3.3. Main outcomes and conclusions

- Lipolysis efficiency depends on five processes
- NaDC more significantly reduces particle size during the lipolysis process.
- Increasing the concentration of conjugated over unconjugated BS increases interfacial tension and dilatational modulus during the adsorption and desorption step.
- FFA release is enhanced by increasing the concentration of conjugated BS.
- MSR is not affected by conjugation.
- Emulsification is a rate-limiting step of lipolysis

3.3.4. Graphical abstract of publication A3



Food Research International 184 (2024) 114255

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Bile conjugation and its effect on in vitro lipolysis of emulsions

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ARTICLE INFO

Keywords:
Bile salts
Lipolysis
In-vitro digestion
Lipids
Emulsion
Interfacial tension
Adsorption
Desorption

ABSTRACT

Bile Salts (BS) are responsible for stimulating lipid digestion in our organism. Gut microbiota are responsible for the deconjugation process of primary conjugated to secondary unconjugated BS. We use two structurally distinct BS and characterize the rate of lipolysis as a compound parameter. A static in-vitro digestion model as well as *meta*-analysis of literature data has been performed to determine the most influential factors affecting the lipid digestion process. The results demonstrate that lipolysis of emulsions using conjugated BS (NaTC, FFA = 60.0 %, CMC in SIF = 5.58 mM, MSR of linoleic acid = 0.21, rate of adsorption = -0.057 mN/m.s) enhances the release of FFA compared to deconjugated BS (NaDC, FFA = 49.5 %, CMC in SIF = 2.49 mM, MSR of linoleic acid = 0.16 rate of adsorption = -0.064 mN/m.s). These results indicate that conjugation plays an important role in controlling the rate of lipolysis in our organism which can be in turn, tuned by the microflora composition of our gut, ultimately controlling the rate of deconjugation of the BS.

1. Introduction

Elucidating the influence of gut microbiota on the human digestion process is an important goal for the development of ideas to control the lipid digestion process. Bile acids (BA), which support these processes, are bio-surfactants, synthesized from cholesterol in the liver in hepatocytes. Primary BA (cholic and chenodeoxycholic acid) are conjugated with either glycine and taurine to form primary conjugated BAs taurocholic, glycocholic, taurochenodeoxycholic and glycochenodeoxycholic acid and are stored in our gallbladder (Boyer, 2013). These conjugated BA are transported to the small intestine, becoming primary conjugated bile salts (BS) (here we refer to the bile acid-bile salts transition to occur at the ampulla of Vater) (Di Ciaula et al., 2017). Secondary unconjugated bile salts including deoxycholic acid and lithocholic acid, are created through the process of deconjugation, which removes amino acid residues from the primary conjugated BS. The formation of secondary unconjugated BS is catalyzed by a bacterial enzyme known as bile salt hydrolase (BSH). Gram-positive and gram-negative intestinal flora such as *Lactobacillus*, *Enterococcus*, and *Bacteroides spp* possess BSH activity (Urdaneta & Casadesús, 2017). A resulting excessive deconjugation would generate a higher concentration of secondary unconjugated BS, which will elicit a cellular toxic response (De Boever et al., 2000). Alterations in the concentration of secondary unconjugated BS may lead to the development of diseases such as gallstone formation, cholangiocarcinoma, and pancreatic neoplasm (Krupa et al., 2021). Additional effects may be diarrhea, mucosal inflammation, or colon cancer, due to disruption of the gut microbiota composition (Salminen, 1996). It should be noted that BSH activity controls microbiota composition, whereas microbiota regulates BS pool size. Therefore, disruption of the BS-microbiota homeostasis may lead to the development of pathogenicity (Cox, Lundgren, Nath, & Thaiss, 2022).

The administration of antibiotics in adults reduces gut microbial diversity, which may promote the growth of pathobionts, such as toxins from *Clostridioides difficile* and *Enterobacteriaceae* as well as reduce the level of *Bifidobacterium* and butyrate-producing species (Palleja et al., 2018). It is crucial to point out that the usage of antibiotics eliminates

Abbreviations: BS, bile salts; C, conjugated; DI, deionized; IFT, interfacial tension; MICE, multiple imputation by chained equations; MSR, molar solubilization ratio; NaDC, sodium deoxycholate; NaTC, sodium taurocholate; PCA, principal component analysis; U, unconjugated.

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https://doi.org/10.1016/j.foodres.2024.114255
Received 9 January 2024; Received in revised form 15 March 2024; Accepted 18 March 2024
Available online 19 March 2024

bacteria that are sensitive to them and increases the number of antibiotic-resistant bacteria (Duvallet, Gibbons, Gurry, Irizarry, & Alm, 2017). Therefore, antibiotics by themselves do not decrease the overall number of bacteria but decrease their diversity. *Bifidobacterium* is one of the many species possessing BSH activity. Thus, antibiotic use will lead to changes in BS composition (Wei et al., 2020).

There are five transformation mechanisms of conjugated BS driven by intestinal bacteria: (1) dihydroxylation, (2) dehydration, (3) epimerization, (4) deconjugation, and the most recently reported (5) amide conjugation of the cholate backbone with the amino acids phenylalanine, tyrosine, and leucine (Quinn et al., 2020). 7α/β-dehydroxylation converts primary bile salts into secondary bile salts (deoxycholic and lithocholic acid), which occurs mainly in the small intestine. The deconjugation process is the most well-studied transformation and is a rate-limiting process for further BS transformation.

BS differ from standard amphiphilic surfactants, by displaying a bi-facial amphiphilic structure, in which convex and concave surfaces are located on the opposite sides of the four steroid rings. The convex side displays methyl groups and the concave side consists of 1–3 hydroxyl groups (C-3, C-7, C-12) and amino groups (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). Conjugation reduces the pKa from 6 for primary unconjugated BS to 4.5 for glycine-conjugated BS and 1.5 for taurine-conjugated BS (Goto, Mano, & Goto, 2004). This process allows BS to exist in the fully ionized form at physiological pH, which enhances the amphiphilic function of BS. Moreover, the ionized form of conjugated BS prevents nonionic passive absorption of the BS in the small intestine and ensures absorption by an active transport system after the completion of their roles. Unconjugated forms of primary conjugated BS show similar pKa values (Goto et al., 2004). Conjugated BS are more hydrophilic than their unconjugated forms, due to

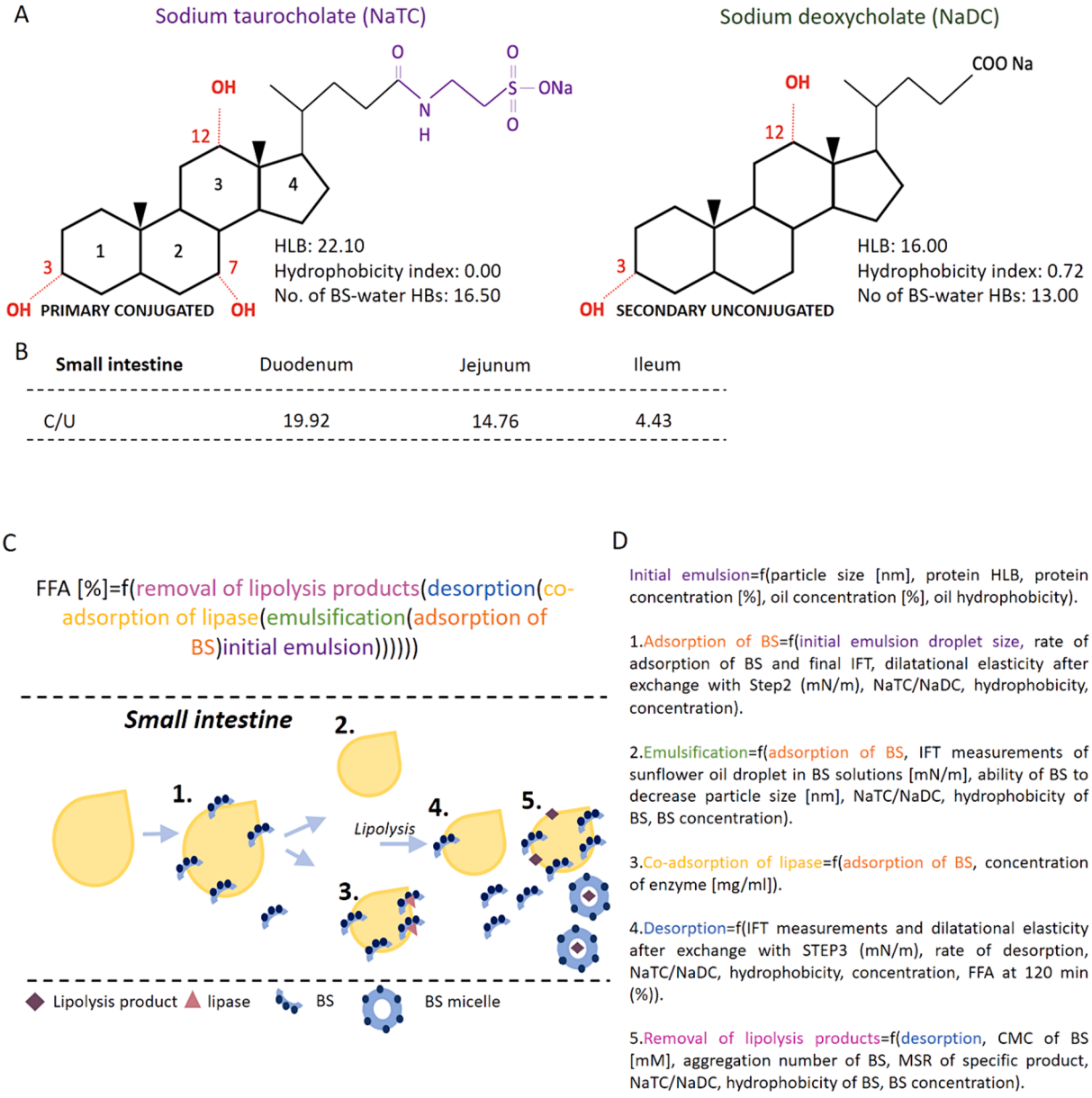

Fig. 1. A. Structure and properties of sodium taurocholate (NaTC), and sodium deoxycholate (NaDC) (Heuman 1989; Łozińska and Jungnickel, 2021). The BSH-mediated deconjugation process increases the hydrophobic character of SC BS, by losing the –OH group at the 7α position. HLB (hydrophilic-lipophilic balance), hydrophobicity index, and BS-water HBs taken from, (Heuman 1989), and (Mustan et al., 2015) respectively. B. The ratio of conjugated to unconjugated BS (C/U) during various segments of the small intestine (Łozińska and Jungnickel, 2021). C. Lipolysis depends on five dominant processes. Lipolysis efficiency is measured by FFA(free fatty acids) release D. Each of the processes can be described as a function of several measured parameters. In this paper, we have analyzed each process (1–5), in terms of the measured parameters. Bile salts (BS), critical micelle concentration (CMC), interfacial tension (IFT), and molar solubilization ratio (MSR).

additional hydroxyl groups at position 7 (Fig. 1A). The higher hydrophilic character results in a higher critical micelle concentration (CMC) of primary conjugated BS. The deconjugation process enhances the formation of aggregates at a lower concentration (in this paper we are only reporting primary micelles formation as secondary BS micelles formed from primary micelles held together by H-bonding occur at biologically irrelevant concentrations (Euston, 2017)). The planar polarity of the BS results in two main biological functions: 1. Formation of small mixed aggregates responsible for transportation of the lipolysis products in the small intestine, and 2. Interface modification such as emulsification of the lipid droplet, facilitating adsorption of the lipase/colipase complex, and displacing protein and lipolysis products from the interface (Maldonado-Valderrama et al., 2011).

In this paper, we discuss lipolysis as an interfacial process strictly related to the behavior of BS at the oil interface, such as adsorption, co-adsorption of lipase, and desorption, as shown in Fig. 1C. Consequently, the interfacial properties of BS can modulate this lipolysis process. Therefore, investigating these interface properties represents a challenge, due to the dynamic nature of the digestion process, where mimicking the process in a single oil–water interface offers an opportunity to study the effect of the contributions of the different BS mixtures to lipolysis.

The importance of BS conjugation has been widely studied with respect to their physiochemical function such as CMC, molar solubilization ratio (MSR), aggregation number, adsorption, desorption properties, etc (Heuman, 1989b; Maestre, Guardado, & Moyá, 2014; J. Maldonado-Valderrama, Muros-Cobos, Holgado-Terriza, & Cabrerizo-Vílchez, 2014; Mukherjee, Dar, Bhat, Moulik, & Das, 2016; Nagadome, Okazaki, Lee, Sasaki, & Sugihara, 2001). Previous works established the impact of BS conjugation on their physiological functions such as free fatty acid release (FFA) (Bellesi & Pilosof, 2021; Łozińska & Jungnickel, 2021). Although it can be observed that lipolysis is controlled by different parameters acting simultaneously, the rate-limiting factor has not yet been determined. Previous studies have analyzed BS during lipolysis-related processes such as their adsorption/desorption dynamics (Parker, Rigby, Ridout, Gunning, & Wilde, 2014), aggregation properties (Pabois et al., 2021), and final FFA release for different BS concentrations (Pabois et al., 2020), however, these studies have been limited to only conjugated forms of BS. Therefore, the specific effect of deconjugation on these processes and in turn, in lipolysis is not known. To answer these questions, we have separated five distinct processes (Bellesi & Pilosof, 2021; Łozińska & Jungnickel, 2021) that may regulate lipolysis as shown in Fig. 1C: emulsification, adsorption of BS/co-adsorption of lipase/desorption at the oil–water interface, and solubilization of lipolytic products.

Each process is dependent on the number of measurable parameters, as shown in Fig. 1D. It should be noted that previously published research by Bellesi et al. (Bellesi & Pilosof, 2021) limited the lipolysis process to three key complex steps but these were not linked to experimental values.

The aim of the paper, therefore, is to determine the change in the five processes that modulate the lipolysis process with changing ratios of conjugated and unconjugated bile salts. We hypothesize that NaTC and NaDC (due to their different H-bonds, hydrophobicities, etc.) will have varying effects on each of these five processes. We aim to determine the most influential and rate-limiting factor resulting in different final FFA releases from emulsions. To aid the experimental conclusions, we will compare the experimental data with a *meta*-analysis of lipolysis data. This will allow us to determine the importance of deconjugation in the overall lipolysis process and design future strategies to control lipolysis by regulating the conjugation of BS.

2. Methodology

2.1. Materials

NaN_3 (S2002), florisil (46385), NaDC (D6750), NaTC (86339), linoleic acid (LO7949), cholesterol (C8667), diazotized procaine (P9879), pancreatin (P1750) were purchased from Sigma Aldrich (Schnelldorf, Germany), toluene was purchased from Ośrodek Badawczo-Rozwojowy Przemysłu Rafineryjnego, Płock, Poland.

SIF was prepared according to Brodkorb et al. (Brodkorb et al., 2019) and was composed of 0.5 M KCl, 0.5 M KH_2PO_4, 5 M NaCl, and 0.15 M $MgCl_2{\cdot}6H_20$ with ionic strength of 0.13 mM. All chemicals were purchased from Sigma Aldrich (Schnelldorf, Germany).

2.2. Emulsion preparation

Emulsions were prepared according to Łozińska et al. (Łozińska & Jungnickel, 2021). Oil in water (O/W) emulsion (oil to water 20:75 % (w/w), or 10:85 % (w/w), (Emulsions S_2, S_7)) were prepared by dissolving whey protein isolate ((WPI) JE051-9–420, Le Seur, USA, concentration of 0.5 % (w/w) in saline buffer (150 mM NaCl (POCH, Gliwice Poland) and 0.02 % (w/w) NaN_3). Sunflower oil (bought at a local market), which was previously purified with florisil, as described in previous studies (Del Castillo-Santaella, Sanmartín, Cabrerizo-Vílchez, Arboleya, & Maldonado-Valderrama, 2014) was used as the oil phase. The mixture of purified sunflower oil and protein dispersion was vortexed for 3 min or homogenized for 2 min to obtain a pre-emulsion. The pre-emulsion was sonicated with an ultrasound generator (Sonics VCX 500, Sonics & Materials Inc., Newtown, CT, USA) with a titanium probe (outer diameter 0.13 cm). Lipolysis experiments were carried out on split samples, one-half for each BS. The conditions for emulsions' preparation are presented in Table S1.

To prepare emulsion S_1 the high-speed d (M133/1281–0, Biospec Products Inc., Basel, Switzerland) was used for two minutes. A membrane homogenizer (external pressure type micro kit from SPG Technology Co. Ltd, Japan) with a 4 μm pore diameter was used. The emulsion has passed three cycles of 5000 kPa and 10 cycles of 10000 kPa (Torcello-Gómez, Maldonado-Valderrama, Martín-Rodríguez, & McClements, 2011). The emulsions were prepared in duplicates. The stability of the emulsions over 48 h has been examined by using Turbiscan LabExpert. PDI around 0.9 indicates a highly polydisperse emulsion (Karmakar, 2019).

2.3. Emulsion droplet size measurement

The mean particle diameter and particle size distribution of the emulsion were measured using a laser light scattering instrument (Metasizer 2000, Malvern Instruments Ltd, Malvern, UK). The absorbance value of the oil droplets was 0.001 (refractive index of 1.467) (Micic et al., 2015). The results of particle size were recorded as the Z-average mean diameter, which is calculated from the particle size distribution (Li et al., 2019).

2.4. Lipolysis of emulsions

A modified INFOGEST *in-vitro* lipolysis model (Brodkorb et al., 2019) was used to simulate the environmental condition of the duodenum. Specifically, 0.8 mL of the SIF and 0.375 mL of the emulsion were added to the thermostatted vessel. After mixing with a magnetic stirrer (1500 rpm), 0.3 mL of 10 mM BS (NaTC or NaDC) and 3 μL of 0.3 M $CaCl_2$ were pipetted, and the pH was set to 7.0 using 0.1 M HCl. Finally, with the addition of 1.0 mL of freshly prepared pancreatin (75 mg at 80 U/mg) the titration was started.

The reaction vessel was continuously stirred and thermostatically controlled to maintain 310.15 K. The extent of the lipolysis was measured by continuous titration with an autotitrator (Cerko Lab

System CLS/M/07/06, Gdynia, Poland) of free fatty acids (FFA) with 0.1 M NaOH. All lipolysis experiments were carried out in duplicate. Exemplary results are shown in Fig. 2D. The rate of lipolysis/surface area (SA) was calculated by dividing the slope from lipolysis experiments within the first 3 min by the total emulsion surface area. The surface area was calculated assuming a spherical shape of the emulsion particle. The size of the emulsions was previously determined. The number of emulsion particles was determined by the volume and the composition of each emulsion.

2.5. *In-vitro* lipolysis experiments

In-vitro lipolysis of adsorbed protein layers at the oil–water interface was measured in OCTOPUS by sequential adsorption comprising three steps: Step1- protein, Step2- lipolysis: BS, BS + lipase or BS + lipase + inhibitor, and Step 3- desorption: replacement of bulk solution by SIF (Maldonado-Valderrama et al., 2014). Briefly, OCTOPUS is a pendant drop surface film balance where a normal capillary tip was substituted by an arrangement of two coaxial capillaries allowing a fully automated subphase exchange of the drop content (Cabrerizo-Vílchez, Wege, Holgado-Terriza, & Neumann, 1999). OCTOPUS is computer-controlled by the software DINATEN (University of Granada). The pendant drop is formed into the oil phase inside a glass cuvette (Hellma, Jena, Germany), which is kept in an externally thermostated cell at 310.15 K for all of the experiments, simulating body temperature. The interfacial tension is recorded at a constant interfacial area (23 mm^2) throughout the adsorption and desorption cycles and/or until the interfacial tension reaches a stable value.

The schematic representation of the sequential lipolysis is presented in Fig. 2A and B with an exemplary experimental output of sequential adsorption (for Step2) present in in Fig. 2C. First, a droplet of 0.1 g/L WPI was formed and the adsorption of the protein layer was recorded for one hour at constant interfacial. Once the protein layer had stabilized, the bulk solution was exchanged by Step2 70 times to ensure complete replacement (Maldonado-Valderrama et al., 2014). The interfacial tension was recorded during the exchange with Step 1 and one additional hour to allow for the stabilization of the lipolytic interfacial layer. Replacement by lipolytic media (Step 2) causes a decrease in the interfacial tension owing to interfacial adsorption and lipolysis while replacement with SIF causes an increase in the interfacial tension owing to desorption caused by the concentration gradient between the interface and the bulk solution (Fig. 2B).

Experiments were performed in duplicate. The OCTOPUS recorded the interfacial tension every 2–3 s. Experiments, which had a drop interfacial tension at time 140—160 s of 18.3 ± 0.5 [mN/m] were classified as repeatable. To determine the final interfacial tension of each of the phases, the average of the last 30 points was used. As shown in Fig. 2C, the lipolysis began with a reduction in the interfacial tension, which could be observed in all BS systems during Step2.

In-vitro lipolysis at the interface was also recorded in the presence of lipase inhibitor orlistat in order to provide a negative control of lipolysis, adsorption of BS in the presence of lipase, etc. Orlistat (60 mg, GlaxoSmithKline, London, UK) is used as an inhibitor of lipase activity (Del Castillo-Santaella et al., 2021, 2015). Orlistat forms a covalent bond with the active site of lipase, with serine, which prevents the lipid digestion process (Kondrashina et al., 2023).

Due to small inter-measurement variations in the initiation of the subphase change, the time of the measurements for each experiment has been normalized as shown in the supporting information Fig. S1A and B. Adsorption rate and desorption have been calculated during Step2 and Step3 exchange respectively for NaTC/NaDC measurements ranging from 0.00 to 1.00 for BS, and BS + lipase NaTC/NaDC indicates the

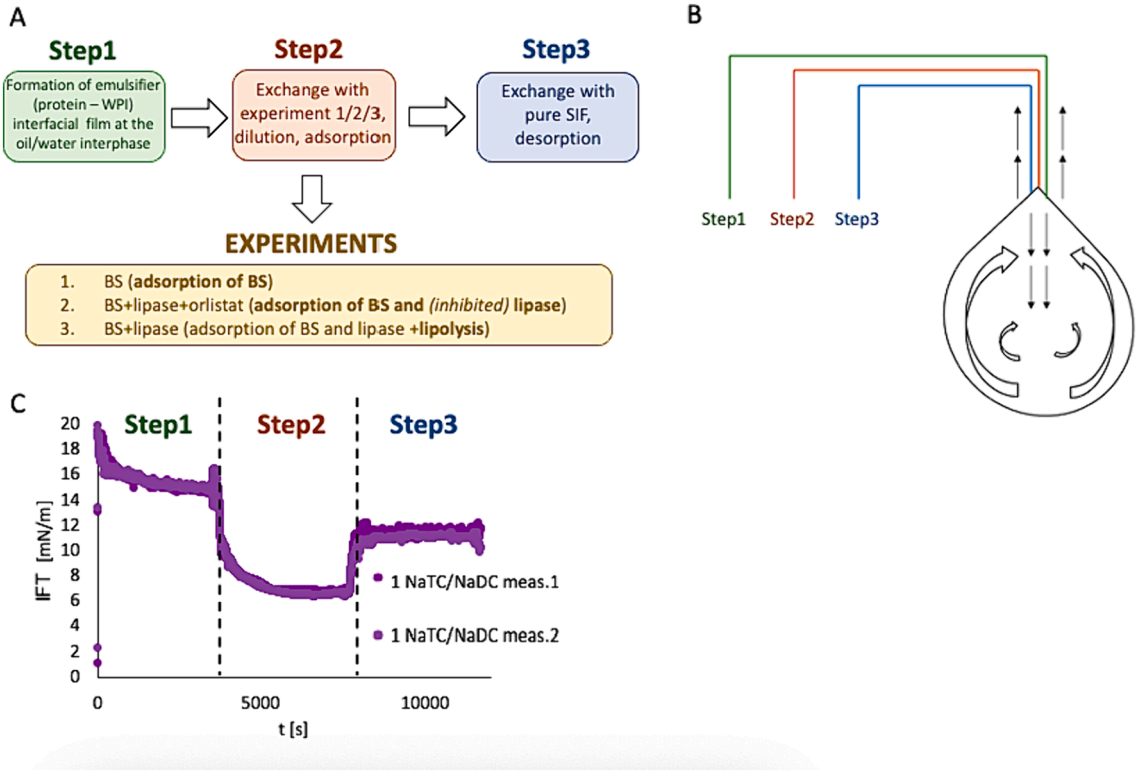

Fig. 2. A. The schematic representation of OCTOPUS experiments. First, the protein (whey protein isolate (WPI)) adsorbs onto the oil–water interface, and then the liquid inside the drop (subphase) is exchanged with three types of fluids (experiments 1–3). B, finally the liquid inside is exchanged by Step2 and Step3. The progress of the experiment is monitored by the changes in interfacial tension of the drop. C. Representative curve for the experimental output of in vitro lipolysis with bile salts (BS) + lipase performed on OCTOPUS. Simulated intestinal fluid (SIF), Interfacial tension (IFT), time (t), sodium taurocholate (NaTC), sodium deoxycholate (NaDC), measurement (meas.).

fraction composition of the total BS concentration in a solution for a given experiment. Rates were calculated from the slope of the interfacial tension versus time obtained for periods of 3786 – 3830 s and 7812–7869 s for adsorption and desorption phases respectively, divided by surface area. Complete interfacial tension profiles of *in-vitro* lipolysis of interfacial layers performed with the OCTOPUS using BS, BS + lipase are presented in Fig. S2.

The interfacial tension of the purified sunflower oil–water interface was checked before every experiment obtaining values of 25.5 ± 1.5 mN/m at 310.15 K.

2.6. Critical micelle concentration (CMC)

The CMC in water and SIF solution of the BS NaDC, NaTC, and their ratios at physiological temperature (310.15 K) were assessed according to Łozińska et. al (Łozińska & Jungnickel, 2021) by using conductivity measurements using an autotitrator equipped with a microconductivity electrode (Eurosensor EPST-2ZAM, Gliwice, Poland). The temperature was maintained using a thermostatic water bath (PolyScience 9106, Niles, USA).

2.7. Static interfacial tension measurements

Additional static interfacial tension measurements were done by using a drop shape analyzer (Krüss Drop shape analyzer DSA 10, Hamburg, Germany). The measurements have been performed to determine the impact of NaTC/NaDC ratio on the reduction of the interfacial tension of oil. These values were aimed to correlate with the potential of specific BS to reduce drop size during lipolysis. The measurements were performed as described previously by Łozińska et al. (Łozińska & Jungnickel, 2021). An oil drop of purified sunflower oil was formed in a measuring cell, filled with 10 mM BS solution, ranging from 0.00 to 1.00 NaTC/NaDC. Interfacial tension of the equilibrated drop was measured after 10 min at a constant temperature of 310.15 K.

2.8. Molar solubilization ratio

Molar solubilization ratio (MSR) for different BS ratios for linoleic acid as a representative high molecular volume FFA; cholesterol as a small molecular weight solubilizate; and toluene as a commonly tested and thus comparable reference standard, were measured. Solutions of specific BS ratio in SIF, in the range of 10–20 mM were prepared for each solubilizate. Each was added in excess. Solubilization was carried out in vials sealed with Teflon septa and mixed thoroughly (1000 rpm, 24 h, 298.15 K) using a horizontal shaker (IKA VIBRAX VXR Basic, Sigma-Aldrich) to allow for incorporation of extra phase into the biosurfactant micelles. Subsequently, the excess phase was separated by centrifugation at 12,298 g for 15 min (MPW-350 Med. Instruments, Poland). MSR was determined according to. Lee et al. (Lee, Porter, & Boyd, 2013). Column: 4.6 × 75 mm Waters Symmetry® C18 (3.5 μm), mobile phase: 90:10 acetonitrile: water, retention time: 4.6 min, run time: 8 min. The solubility of linoleic acid in BS solution was measured by using High-Performance Liquid Chromatography (Agilent Technologies HPLC System 1200 Series, Santa Clara, CA, US)- evaporative light scattering detector (Sedere LT-ELSD Sedex 90, Alfortville, France.). The solubility of cholesterol in BS solutions was quantified according to Mashkour (Muthana Saleh Mashkour, Naser A. Naser, 2017), where 1 mL of BS in SIF solution was mixed with 2 mL of 0.01 M of the diazotized procaine hydrochloride solution, 2 mL of 2 M NaOH, the volume was made up to 10 mL with distilled water. The resultant derivative was measured using a spectrophotometer (Varian Cary 50 UV/VIS spectrophotometer, Palo Alto, USA) at a wavelength of 558 nm. The solubility of toluene in BS solutions was measured at 255 nm using the spectrophotometer as above.

2.9. Lipolysis modeling and statistical analysis

A *meta*-analysis has been performed on experimental data to determine the most influential factors in the lipolysis process. 14 datapoints from experimental work and 190 data points from the literature have been collected from 34 scientific articles ranging from 2001 to 2022, where the "Google Scholar "(Martín-Martín, Orduna-Malea, & Thelwall, 2018) was used with the following key-words: "bile salts", "lipolysis". 17 descriptors were obtained from the papers: BS type and concentration [mM], the concentration of cholesterol, phospholipids, and calcium ions [%], type of digestion model, source of enzyme, enzyme concentration [mg/mL], and activity [U/mg], type and concentration of oil, protein, and emulsifier [%], moment of particle size determination method, particle size [μm]. Type of BS, type of digestion model, source of enzyme, and type of oil have been classified by one-hot encoded columns. Conjugated/unconjugated BS ratio (C/U) and primary/secondary BS ratio (P/S) have been calculated by an averaged numerator and denominator ratio. The concentration of BS, enzymes, phospholipids, cholesterol, calcium ions, oil, protein, and emulsifiers have been recalculated into a percentage composition of the total volume of the system. The molecular weight of the oil has been obtained by using molinsipiration (Jarrahpour et al., 2012). The collated lipolysis data is presented in Table S1. Bile composition consists of bile acid, cholesterol, and phospholipids (Fracchia et al., 2001). For porcine and bovine bile extract data the averaged composition of BS, cholesterol, and phospholipids have been calculated from collected scientific articles. The composition of bovine and porcine bile extract has been shown in Supporting Information Table S2. Categorization was according to the source of BS. Enzyme concentration corresponds to the final enzyme concentration in the reaction vessel. The source of the enzyme has been classified into two types: lipase and pancreatic lipase. Pancreatic lipase differs from pure lipase by compositions of other enzymes such as amylase, trypsin, protease, lipase, and ribonuclease (Hur, Decker, & McClements, 2009). Oil was classified into medium and long-chained according to Takeuchi et al. (Takeuchi, Sekine, Kojima, & Aoyama, 2008) FFA release after 60 min [%] has been chosen as the dependent variable and was recorded from papers numerically, or graphically. It quantifies the total amount of release of lipolysis products during the intestinal digestion process. Datapoints of dynamic models of lipolysis have been excluded from the analysis. The workflow has been shown schematically in Fig. 3.

Multivariate Imputation by Chained Equations (MICE) has been used to estimate the missing data (Łozińska & Jungnickel, 2021). To estimate the error of the replaced missing data by using the MICE, tests that contained no missing data were taken into account. Principle component analysis (PCA) was used to reduce the number of descriptors originally collected. The results of the PCA analysis are shown in Fig. S3.

The evolutionary algorithm to determine the most influential descriptors on the lipolysis process was implemented by using GenexPro (Hanandeh, 2022). Common operators (addition, subtraction, multiplication, division, exponential, logarithm, root, and power functions) were selected. The data was split 80:20 into training and validation sets. Each analysis was composed of five runs. Each analysis was repeated ten times with a new randomized sample of 80:20 training and validation sets. The presented results are an average of the 10 repetitions of five runs of each analysis.

The contribution of each descriptor to each model was determined by 'importance' and sensitivity. Importance was determined by the normalized difference in R^2 between the original model and that with a randomized input variable as determined by GeneXPro (Salgotra & Gandomi, 2021). Sensitivity analysis has been performed manually. The percentage difference between the dependent variable (of the original function) and the dependent variable (when the parameter in question was altered by +/-0.001) for each descriptor has been calculated. Statistical analysis has been performed to determine the significance of the results.

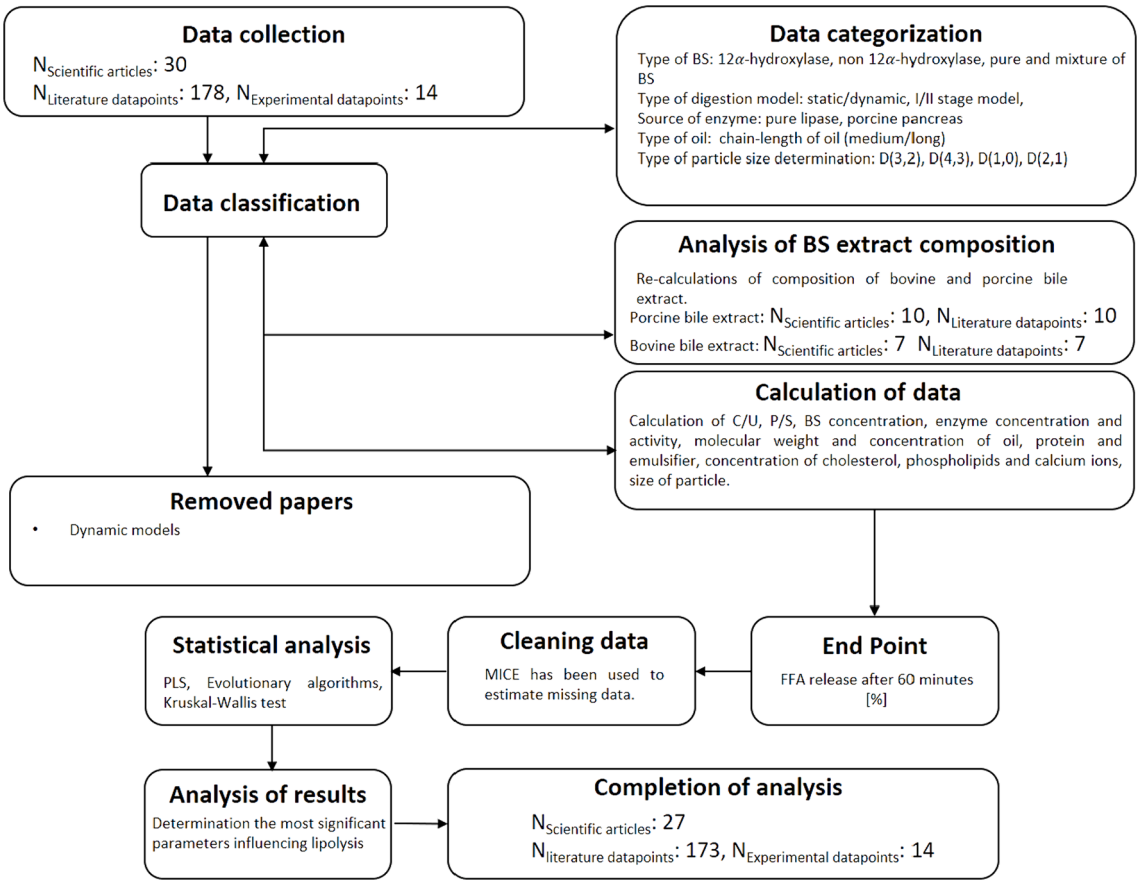

Fig. 3. The workflow of the methodology of lipolysis modeling. Number (N), Multivariate Imputation by Chained Equations (MICE), bile salts (BS), conjugated/unconjugated (C/U), primary/secondary (P/S), partial least square (PLS), free fatty acids (FFA).

The importance of each of the parameters has been calculated by using Equation (2).

$$\left|\frac{v_1 - v_2}{v_1}\right| \times 100\% = \Delta v \tag{2}$$

where v_1 and v_2 were the values for a given parameter for NaTC and NaDC respectively. The most significant parameter, with the highest value of Δv, was then equaled to the value of 100 %. The rest of the parameters have been analogically re-calculated.

The statistical significance for all results was calculated by using a two-tailed *t*-test.

3. Results and discussion

The results are presented in the order of the lipolysis process as shown in Fig. 1C. Sections are divided according to the mentioned lipolysis parameters. Section 3.1 covers the topic of initial emulsion, discussing the importance of particle size during the lipolysis process. In section 3.2 we discuss the ability of different BS to adsorb on the O/W interface, including the importance of co-adsorption of lipase in section 3.4. Section 3.3 refers to the ability of BS to emulsify oil droplets. Subsequently, the desorption process of BS from the surface of the lipid is presented in section 3.4 Finally, the ability to form small aggregates by BS and their capacity to transport lipolysis products are discussed in section 3.5.

3.1. Initial particle size

The importance of particle size in the lipolysis process is well established (González et al., 2020), as is reflected by our results which showed that FFA release for NaDC and NaTC increases with decreasing particle size, as shown in Fig. 4A. A smaller surface area of the droplet can accelerate the lipolysis process, due to greater space for adsorption of BS/lipase complex and faster break-down of droplets (Macierzanka, Torcello-Gómez, Jungnickel, & Maldonado-Valderrama, 2019). However, Fig. 4A shows also that lipolysis with primary conjugated NaTC releases a significantly higher amount of FFA than using secondary unconjugated NaDC for the same size of emulsion. These new findings suggest that lipolysis efficiency is directly linked with BS type and its properties. A higher hydrophobic character of NaDC than NaTC may enhance the emulsification process via greater adsorption. However, the longer residence time of NaDC on the oil droplet may reduce the removal of lipolysis products from the interface and disturb further lipolysis process resulting from lower FFA release. As shown in Fig. 1C the FFA release is a function of five processes occurring during digestion. Fig. 4B shows that decreasing particle size increases the difference of FFA release between NaDC and NaTC, which indicates the significance of the conjugation of BS. Pabois et al. (Pabois et al., 2020) performed lipolysis experiments for two different BS: NaTC and sodium taurodeoxycholate with respect to two different concentrations: 10 mM and 50 mM. The results have shown to be statistically significant between different concentrations for the same BS type and between themselves only for 50 mM. Moreover, a higher amount of FFA released was yielded for BS at 50 mM, underlying that a high concentration of BS significantly increases lipolysis efficiency. FFA release in respect to different emulsion size is shown in Fig. S3A for NaDC and Fig. S3B for NaTC.

FFA release from the oil droplet was shown (Fig. 1C) to depend upon five dominant processes during the lipolysis process. In the following sections, we will discuss the effect of NaTC/NaDC ratio on each of the processes, to determine the most dominant factor during digestion.

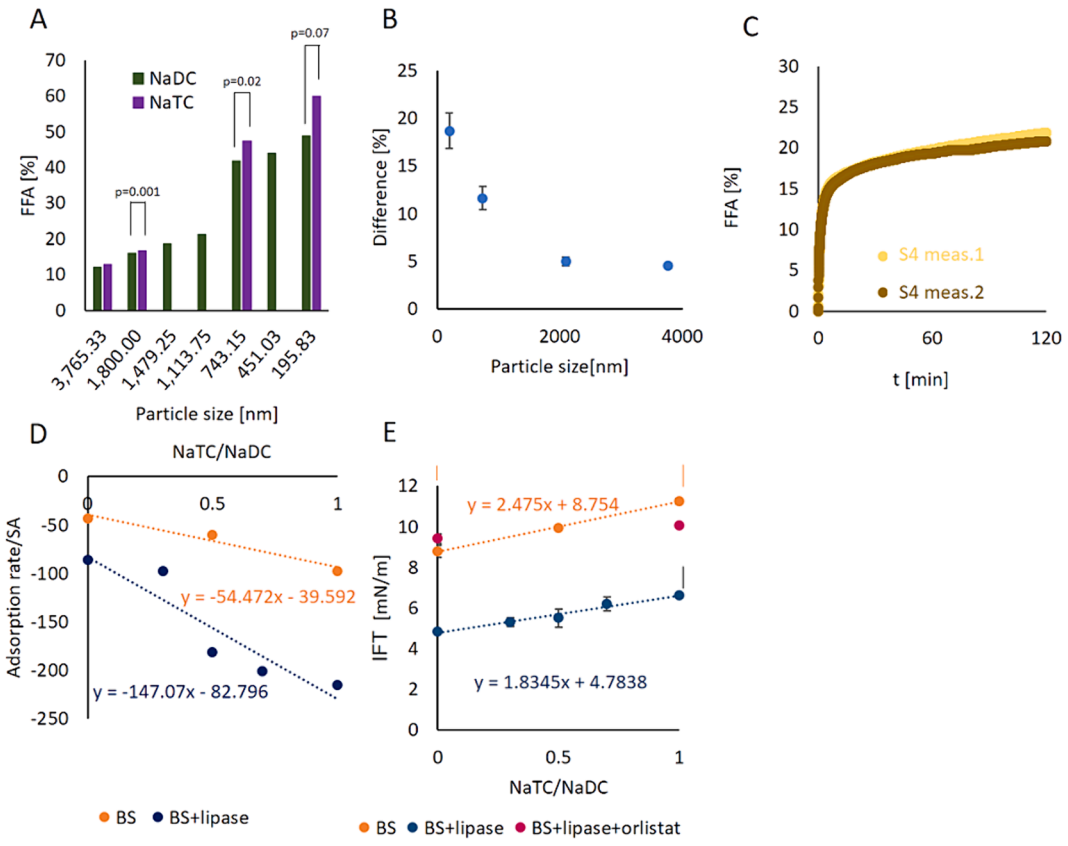

Fig. 4. A. FFA release after 2 h of in-vitro digestion process for whey protein isolate (WPI)-stabilized emulsion of two different types of bile salts (BS): sodium taurocholate (NaTC) and sodium deoxycholate (NaDC)at 10 mM and 310.15 K for different particle size [nm] B. Percentage difference of free fatty acids (FFA) release between NaTC and NaDC obtained for different initial emulsion droplet size. C. Representative curve of in-vitro lipolysis of S4 emulsion using NaTC. All lipolysis other results are presented in Fig. S4. D Measurements of lipolysis in OCTOPUS (Fig. 2C, Step 2) using different ratios of BS in Step2 (BS and BS + Lipase). The adsorption rate is calculated as the slope of the interfacial tension versus time obtained in Step 2 (Fig. 2C) divided by the surface area. E. Interfacial tension after stabilization of Step 2 (Fig. 2C).

3.2. Adsorption of BS onto oil–water interface

The ability of lipase to adsorb onto the lipid droplets in emulsions is assisted by BS, and therefore the BS adsorption kinetics onto the oil droplet interface play an important role (Łozińska & Jungnickel, 2021). The ability of BSs to adsorb onto the oil–water interface describes their potential to remove the surface materials and facilitate lipase adsorption. There are two hypothesized scenarios for the adsorption of BS onto the oil–water interface. In the first scenario, BS adsorbs perpendicular to the interface (Small, 1971). In this case, the sterol ring penetrates into the oil and the charged end remains at the aqueous phase. The second scenario assumes that BS can adsorb horizontally to the interface, as supported by the experimental work performed by Del Castillo-Santaella et al. (Del Castillo-Santaella, 2023). A stronger adhesion of the BS may facilitate the adsorption of the lipase/co-lipase to the surface of the lipid, which may promote lipolysis. However, reduction of the time of BS at the interface can decrease the adhesion of the lipase/colipase but at the same time can facilitate displacement of lipolysis products from the surface.

Fig. 4D-E shows the results from OCTOPUS experiments for Step 2 (according to Fig. 2C), i. e. exchange with Step2 using different rations of BS. The adsorption rate of BS and BS + lipase increases with the concentration of primary conjugated NaTC as shown in Fig. 4D. The adsorption rate is determined by several factors: (1) aggregation number (2) and CMC of BS (detailed description in Section 3.6.1). The aggregation number is an indirect measure of the size of the BS micelle, and diffusion through water (and thus adsorption) is dependent on the size of the object. Similarly, the CMC (a measure of the concentration at which micelles form), indicates if the BS molecules are diffusing as single molecules or micelles. Parker et al. (Parker et al., 2014) have demonstrated that the adsorption behavior follows the micellization properties of the BS. In all performed experiments concentration of BS was 10 mM (physiologically relevant) (Alan F. Hofmann, 1988), therefore concentration for both BS, the primary conjugated NaTC and secondary unconjugated NaDC is above its CMC. Meta-analysis performed by Łozińska et al. (Łozińska & Jungnickel, 2021) has shown that primary conjugated NaTC has a smaller aggregation number (2.60 ± 0.11) than secondary unconjugated NaDC (5.91 ± 0.03) (Coello, Meijide, Rodríguez Núñez, & Vázquez Tato, 1996). The smaller micelles of primary conjugated NaTC allow for faster free diffusion, therefore resulting in a faster rate of adsorption. The addition of the lipase showed a decrease in the rate of adsorption, which indicates that lipase significantly supports the first step of lipolysis.

Fig. 4E shows the interfacial tension curves recorded after 1-hour adsorption of protein for NaTC/NaDC for pure BS, BS + lipase (lipolysis), and BS + lipase + orlistat (inhibited lipolysis). The results show that interfacial tension is significantly lower for BS + lipase than for pure BS experiments. This is a result of the presence of lipolytic products which are surface active and therefore contribute to reducing the interfacial tension. Moreover, the results demonstrate that the final interfacial tension in all cases, increases with increasing concentration of NaTC. Del Castillo-Santaella et al. (Del Castillo-Santaella, 2023) have mentioned that the number and position of the hydroxyl groups affect the adsorption of BS at the interface. NaTC has three hydroxyl groups at positions 3, 7, and 12, as shown in Fig. 1A, which makes parallel adsorption of BS at the oil interface more likely. NaDC, however, lacks

the OH group at position 7, which may enhance greater perpendicular orientation at the oil interface. Results shown here suggest that the planar orientation of NaTC favors lipolysis to a greater extent as quantified by the lower interfacial tension reached as the amount of NaTC increases. The orlistat results did not deviate from the BS interfacial tension, which indicates the ability of orlistat to inhibit lipase function in agreement with previous works reporting lipase inhibition by up to 90 % (Del Castillo-Santaella et al., 2021, 2015). Greater rate of adsorption and interfacial tension for NaTC than NaDC, as shown in Fig. 4D and E which follows the pattern of greater FFA release obtained with conjugated NaTC. The results indicate that lipolysis efficiency is linked with the interfacial properties of BS.

3.3. Emulsification

The ability of the BS to reduce the size of the lipid droplets during lipolysis has been documented (Łozińska & Jungnickel, 2021; Pabois et al., 2020; Wilde, Garcia-Llatas, Lagarda, Haslam, & Grundy, 2019). However, we present here for the first time the results of a coalescence initiated by the unconjugated BS with time, as shown in Fig. 5A-D.

Previously published results by Łozińska et al. (Łozińska & Jungnickel, 2021) have shown the ability of primary conjugated and secondary unconjugated BS to reduce the interfacial tension of an oil droplet. The linear relationship in Fig. 5AC presents the interfacial tension of aqueous solutions with different NaTC/NaDC ratio. The increasing ratio of secondary unconjugated NaDC within NaTC:NaDC systems decreased the interfacial tension (Fig. 5G), which may indicate better emulsification properties during the lipolysis process.

The ability of BS to emulsify the droplets of the emulsion for three different sizes of emulsions is presented in Fig. 5B-D in terms of the time evolution of the droplet diameter. Controls, shown in Fig. 5 C-D, have been stable over the process which is in agreement with other work performed by Calvo-Lema et al. (Calvo-Lerma, Fornés-Ferrer, Heredia, & Andrés, 2019). Larger particles of the emulsion are not easily influenced by the BS type, as can be observed in Fig. 5B Conversely, BS type has a strong influence on emulsions with lower particle size, as observed in Fig. 5C and D. During the first 60 min, secondary unconjugated NaDC significantly reduces the particle size of the emulsion (as shown in Fig. 5B), and after that promotes coalescence of the droplets. This result can be connected with the higher hydrophobic character of NaDC (conjugated NaTC loss hydroxyl group at position 7), which increases the hydrophobicity of the oil droplet.

3.4. Co-adsorption of lipase

To better understand the influence of the type of BS on the lipolysis process, the conformational structural changes of lipase and colipase introduced by BS were investigated, which may further reflect the potential of lipase adsorption. Colipase is a protein cofactor of pancreatic lipase, that assists in the breakdown of lipids (Rathelot, Julien, Canioni, Coeroli, & Sarda, 1976). The molecular dynamic simulation performed by Haque et al (Haque & Prakash Prabhu, 2018) revealed the alterations in the interfacial activity of pancreatic lipase. The binding of NaTC to porcine pancreatic lipase resulted in changing the structure of the lipase. Moreover, this interaction prevented the closed conformation and induced the open conformation of lipase, where the open conformation assists the lipase to stay active without the co-lipase. Thus, the interaction between BS and lipase complex will induce conformational

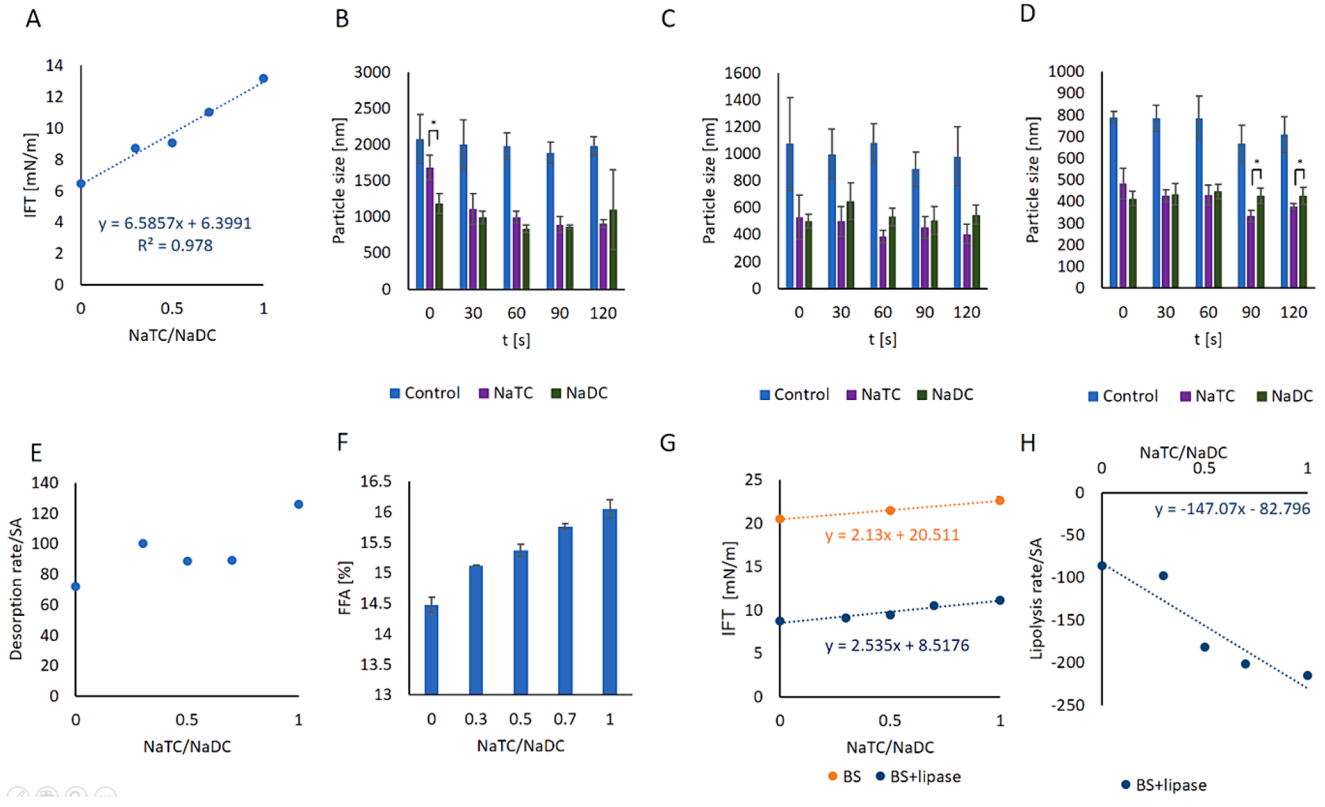

Fig. 5. A Static interfacial tension (sunflower oil) of 10 mM of bile salts (BS) in simulated intestinal fluid (SIF), performed on drop shape analyzer. Interfacial tension for 0.00 sodium taurocholate (NaTC)/sodium deoxycholate (NaDC) and 1.00 NaTC/NaDC were published previously by Łozińska et al (Łozińska and Jungnickel, 2021). The ability of NaTC and NaDC to reduce droplet size for B. 3000 nm C. 800 nm D.500 nm. Controls for experiments C-D were performed according to the description in section 2.4, without the presence of BS. E. Desorption rate measured as the change in interfacial tension due to desorption after lipolysis in OCTOPUS (Fig. 2C, Step 3), divided by surface area, for different BS ratios. F. FFA release from emulsions for different BS ratios after 2 h. The particle size was 1300 nm. G. Interfacial tension obtained after desorption (Fig. 2C, Step 3) with different BS ratios for BS and BS + lipase in OCTOPUS. Values are taken from an average of the last 30 points. H. Lipolysis rate measured as the change in interfacial tension versus time in OCTOPUS results (BS + lipase) for different BS ratios, divided by surface area.

changes in the lipase, and thus influence the lipase activity. How the conjugation of BS influences these conformational structures is not yet known, but due to the different intermolecular bonds, we can assume that some differences should be observable. We show that conjugation affects the adsorption of lipase, since Δinterfacial tension (calculated by IFT of BS + orlistat + lipase – IFT of pure BS ± SD) for 1.00 NaTC/NaDC was −1.18 ± 0.22 mN/m, compared to 0.64 ± 0.52 for 0.00 NaTC/NaDC, and −0.60 ± 0.48 for 1.00 NaTC/NaDC. Hence, NaTC displays a higher interfacial tension than NaDC, as shown in Fig. 4E, and a faster rate of adsorption (Fig. 4D) which may facilitate lipase adsorption to a higher extent than NaDC.

3.5. Desorption of BS

The ability to desorb from the lipid surface plays a crucial role in the lipolysis process and the adsorption–desorption process is a rate-limiting step controlling the rate of lipolysis (Maldonado-Valderrama et al., 2014).

Fig. 5E-H shows the outputs from OCTOPUS experiments and *in-vitro* digestion of emulsions. The increasing concentration of NaTC speeds up the rate of desorption as can be observed in Fig. 5E. The rate of desorption of different BS ratios correlates with FFA release obtained in the lipolysis of emulsions (Fig. 5F). NaTC shows the highest ability to desorb from the interface, as reflected in the higher interfacial tension reached after subphase exchange with SIF and in agreement with its higher hydrophilicity. Moreover, the higher desorption rate correlates with faster removal of lipolysis products from the interface, therefore enhancing lipolysis. Fig. 5G shows the final interfacial tension of the desorption profile (Step 3, Fig. 2C) from interfacial films formed by sequential adsorption in Figs. 4D and E. These are composed of protein + BS or protein + BS + lipase and obtained after (Step3, Fig. 2C). In the absence of Lipase, the interfacial tension after desorption reaches a close value to that of the bare oil–water interface (Fig. 5G). This means that BS had displaced protein upon sequential adsorption and they practically fully desorb from the interface as a response to the concentration gradient imposed as the subphase is depleted. Conversely, the interfacial tension reached after desorption in the presence of Lipase (Fig. 5G), just increases slightly with respect to that shown before the exchange in Fig. 4E. This suggests that BS desorbs but lipolytic compounds remain anchored at the interface.

Moreover, increasing the concentration of NaTC increases the interfacial tension of the interfacial film after desorption in the presence and absence of lipase (Fig. 5G). This agrees with the hydrophilic character of NaTC compared to NaDC which promotes desorption.

The lipolysis rate, presented in Fig. 5H, follows the results of FFA release from Fig. 4C. Again, the increasing concentration of primary conjugated NaTC enhances the rate of lipolysis in emulsions.

3.6. Removal of lipolysis products

3.6.1. Micellization properties

As shown by Maestre et al. (Maestre et al., 2014) BS planar polarity plays a crucial role in the self-assembly of BS into small aggregates and influences their structure. The limitation in the number of hydrophobic interactions in one BS molecule results in formation of the smaller micelles than spherical micelles of classical amphiphilic surfactants (Euston, 2017). Carey and Small (Carey, 1972) hypothesized that in the liquid phase, BS forms primary micelles by hydrophobic interactions, subsequently self-associate into secondary micelles held by hydrogen bonding between hydroxyl groups. Kawamura et al. (Kawamura, H., Murata, Y., Yamaguchi, T., Igimi, H., Tanaka, M., Sugihara, G., & Kratohvil, 1989) suggested a disk-like structure consisting of hydrophobic sides of BS directed toward the center of the micelle with hydrophilic sides oriented on the outer surface. Oakenfull and Fisher (Oakenfull, D. G., & Fisher, 1997) proposed the formation of the layers of dimers, formed by BS, held by hydrophobic interactions between them. Towards the outside of the micelle, the hydrophobic end was directed, while Na^+ ions occupied the central cavity of the micelle.

CMC presented in Fig. 6A correlated with the results of other authors (Maestre et al., 2014; Mukherjee et al., 2016), i.e. increasing the concentration of primary conjugated NaTC increases the CMC, in both water and salt environments, which due to the change in hydrophilicity of the BS systems. Secondary unconjugated NaDC BS have been shown to create small aggregates within the lower concentration than primary conjugated NaTC. CMC in SIF has shown to be significantly lower than in water, due to the usual shielding of the charges on the BS.

3.6.2. MSR

Molar solubilization ratio (MSR) is the ability of the surfactant to incorporate compounds into their mixed micelle. In terms of our research, MSR is important in determining the efficiency of the individual BS to remove specific lipolysis products from the interface. Results from a *meta*-analysis performed by Łozińska et al. (Łozińska & Jungnickel, 2021) have revealed that conjugated BS possessed a greater ability to incorporate products into their aggregates. Moreover, the $\log K_{oW}$ and molecular volume are crucial in influencing the potential of the individual compound to solubilize inside BS micelles. Interestingly, reduction of the MSR of cholesterol in BS has been shown to be a cause of cholelithiasis(Krupa et al., 2021). Reduced BS concentration promoted development of the gallstone disease, due to reduced cholesterol solubility. Wiedmann et al. (Wiedmann & Kamel, 2002) stated that BS with more hydroxyl groups have a higher cholesterol solubilization potential. More hydrophilic conjugated BS have shown higher solubilization properties towards all tested compounds as shown in Fig. 6B, which suggests that reduced levels of NaTC may result in lower cholesterol solubility and may potentially increase the risk of gallstone formation. Previous experiments have shown that the MSR for cholesterol of conjugated BS lies between 0.23 and 0.46 (Neiderhiser & Roth, 1968), which is in agreement with our results. Linoleic acid (as our representative FFA) does not show a significant change in MSR with the degree of conjugation. We can therefore conclude that conjugation of the BS has a limited influence on the ability of the BS to remove the lipolysis products from the interface.

3.7. Lipolysis-modeling and the importance of conjugation of BS

Bile composition was the most influential parameter in the lipolysis process, as shown in Fig. 6C. Sensitivity analysis has shown that increasing the diversity of BS increases the FFA release. Specific types of BS reveal different properties that may diversely influence the individual steps of lipolysis. Diversity of BS affect lipolysis efficiency due to differences in the physiochemical properties of the BS (Hofmann & Roda, 1984). Additionally, components of BS such as phospholipids and cholesterol, may impact lipolysis. It was previously shown that the molar ratio of phospholipid to BS influences the size and structure of micelles (Mazer, N. A., Benedek, G. B., & Carey, 1980), and phospholipids-BS aggregates prevent inhibition of digestion by removal of accumulated lipolysis products from the interface (Macierzanka et al., 2019).

The second most influential factor, as shown in Fig. 6C is Ca^{2+} concentration, which positively correlates with lipolysis, as it helps to precipitate the FFA, as shown by Zangenberg et al. (Zangenberg, Müllertz, Gjelstrup Kristensen, & Hovgaard, 2001). Particle size is one of the three most influential factors. The sensitivity analysis has revealed that decreasing the particle size increases lipolysis efficiency. The smaller the particle size, the larger the surface area of the lipid droplet available for BS and lipase to adsorb. protein weight average (PWA) has shown a positive contribution towards FFA release. A greater concentration of protein increases emulsion stability and provides a smaller particle size, which may be easier and faster digested (Grundy, Wilde, Butterworth, Gray, & Ellis, 2015).

Increasing oil concentration decreases the efficiency of FFA release.

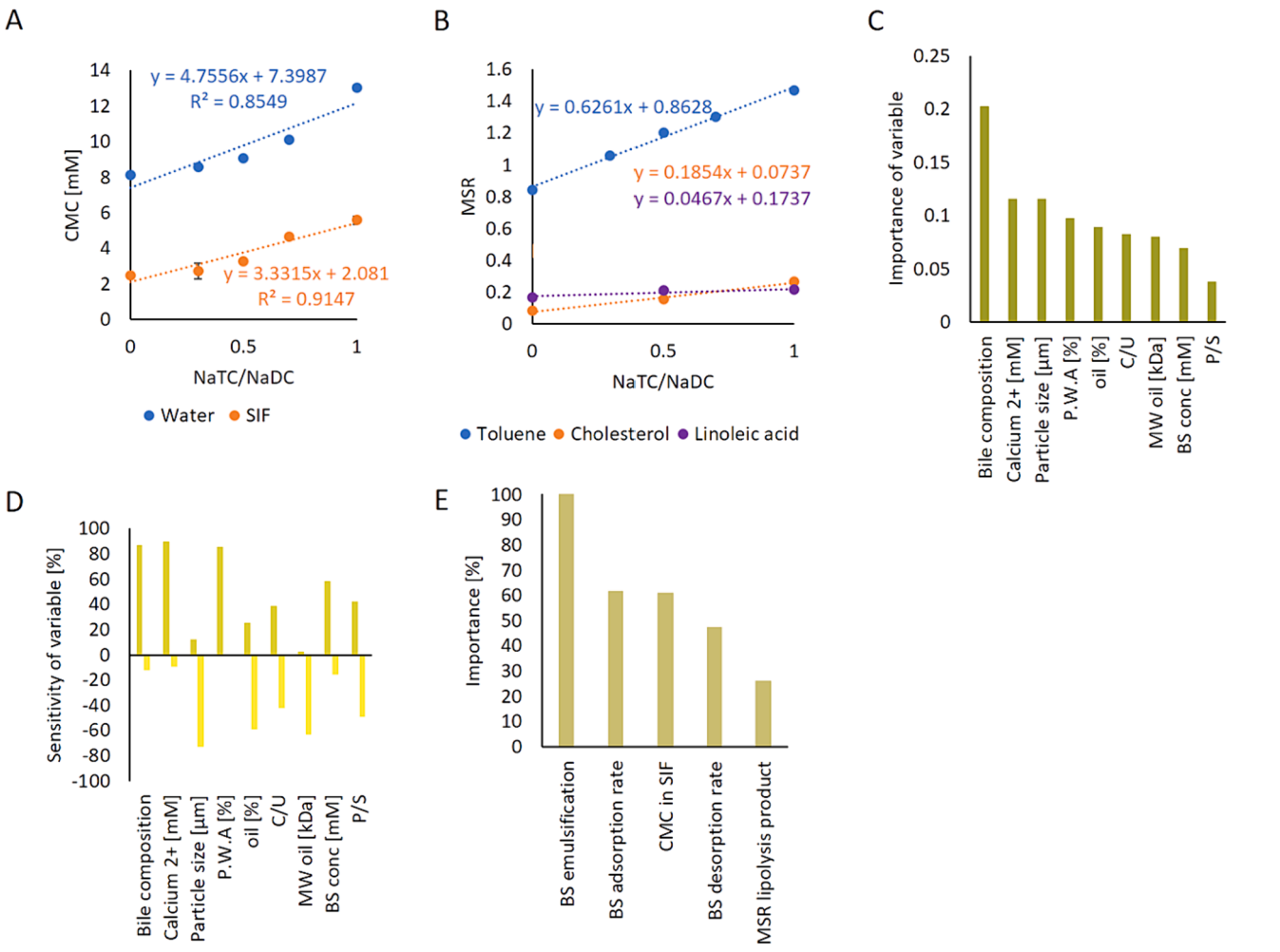

Fig. 6. A. Critical micelle concentration (CMC) of sodium deoxycholate (NaDC), sodium taurocholate (NaTC), and their ratios at 310.15 K in water and SIF. CMC of 0.00 NaTC/NaDC and 1.00 NaTC/NaDC in water was previously published by Łozińska et al. (Łozińska and Jungnickel, 2021). B. Molar solubilization ratio (MSR) for two different types of BS: NaTC and NaDC for different compounds: toluene, linoleic acid, and cholesterol. MSR of toluene was calculated to be 0.45 for sodium cholate (Kolehmainen, 1985). C. Sensitivity analysis has shown that the most important variables are: BS mix composition, Calcium ions, and particle size.D. The sensitivity analysis shows the magnitude of the impact of each variable. BS mix components and calcium concentration have been shown to have a positive impact on the lipid digestion process. Increasing particle size has shown to have a negative contribution towards free fatty acids (FFA) release E. BS conjugation has shown to mostly affect the emulsification step during the lipolysis process. Conjugated forms of BS reduce droplet size to a higher extent than their unconjugated forms therefore enhancing the lipolysis process. BS adsorption rate and CMC have shown around 60 % of importance. Primary/Secondary (P/S), protein weighted average (P.W.A), Molecular weight (MW), concentration (conc).

High oil concentration promotes the formation of bigger particle sizes and leads to re-solubilization of the substrate. Sensitivity analysis has also revealed that increasing the molecular weight of oil decreases lipolysis efficiency (Ji, Shin, Hong, & Lee, 2019). The C/U and P/S ratios (as molecular descriptors) were insignificant in the *meta*-analysis. Even though this might be surprising initially, the C/U and P/S influence other (phenomenological) parameters such as emulsification, and BS adsorption/desorption, as the C/U and P/S are not orthogonal parameters, and are part of the composition variable.

However, the type and form of BS may indirectly affect the lipolysis process by modulating its parameters. A higher C/U ratio would enhance the adsorption process (Fig. 4E), which may facilitate adsorption of co-lipase. Therefore, it may enhance the potential to reduce the droplet size, due to a greater emulsification ability of the conjugated BS (as shown in Fig. 5A). Moreover, the predominant concentration of primary conjugated over secondary unconjugated (P/S) BS may promote faster removal of the lipolysis process by their higher desorption ability. Conjugated BS possessed the ability to create aggregates under lower concentrations than unconjugated BS (Fig. 6A). Therefore, the prevalent ratio of C/U BS may result in higher FFA release (as shown in Fig. S3).

As stated in the aim, we aimed to determine the dominant process that can be affected by the degree of conjugation, as shown in Fig. 1C and D. Our analysis, as presented in Fig. 6E showed that the emulsification, (initial droplet size, interfacial tension of sunflower oil droplet) process of BS was the most significant. The emulsification, as shown in Fig. 1C is the second process taking place during lipolysis. This appears logical, as the size of the emulsion is a phenomenological parameter governing and affecting all other processes such as adsorption/desorption, and thereby the lipolysis process. The second most important was shown to be the BS-adsorption process, as this process will further influence the other process downstream. The CMC, as the first non-process parameter, was shown as the third most important, highlighting the importance of micelle formation of the BS in the removal of lipolysis products.

3.8. Limitations of the research

We decided to examine the influence of conjugated and unconjugated BS in pure systems rather than in complex mixtures for several reasons. Complex mixtures contain components, such as cholesterol,

phospholipids, or bilirubin which may mask the action of BS. Moreover, as shown by our analysis (Table S2) porcine and bovine bile extracts differ in the composition of BS and other components, either among or between themselves. Therefore, complex systems would present too many compounding factors and variations of component concentrations which may directly or indirectly influence the lipolysis process, and direct elucidation of the effect of conjugation would be hindered. Accordingly, we decided to present pure BS systems, to aid the understanding of the impact of conjugation on the lipolysis process.

4. Conclusions

Here we have demonstrated the influence of two BS with different conjugation types on the lipolysis of emulsions. The impact on lipolysis is modulated by five factors which are in turn influenced by the nature of BS; adsorption of BS, emulsification of the lipid droplets, co-adsorption of lipase, desorption of BS, and finally removal of lipolysis products. We have found that emulsion droplet size is the dominant factor determining lipolysis. The effect of conjugation was more evident at smaller droplet sizes. Additionally, a higher adsorption rate was observed for BS systems with a higher level of conjugation while increasing the quantity of unconjugated BS showed improved emulsification of the lipid droplets. The higher hydrophilic character of primary conjugated BS promotes faster desorption from the oil–water interface, resulting in faster and improved removal of lipolysis products.

Meta-analysis of *in-vitro* lipolysis results revealed the importance of several factors in lipolysis. The presence of unconjugated BS in a BS mixture was shown to be the most significant factor in reducing the FFA released from oil-in-water emulsion. NaDC has been shown to have a lower rate of adsorption and desorption than NaTC. However, it was shown to more efficiently reduce the size of the oil droplet than NaTC, therefore promoting the emulsification process, but only at the beginning of the process. Later on NaDC promotes coalescence which reduces the efficiency of emulsification.

We have shown that deconjugation has a positive influence on the rate of lipolysis. This effect is due to the change in particle size caused by the BS during lipolysis, as we have shown by analyzing the effect of conjugation on each of the five processes mentioned above. The adsorption rates of BS and CMC have shown almost equal importance.

The deconjugation process of BS plays a crucial role in our body ensuring the function of our organism. Its disruption, even to a small extent, may impact our organisms. A change in the C/U ratio of BS may influence the absorption of essential nutrients, dysregulate our hormonal receptors, affect BS synthesis, and in consequence promote the development of disease. For the first time we have shown how two different BS forms affect lipolysis by modulating five different processes, and we have shown the importance of each of the processes.

Future analysis comparing *in-vitro* with *in-vivo* data would be crucial in understanding the importance of the BS in our small intestine.

CRediT authorship contribution statement

Natalia Łozińska: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Julia Maldonado-Valderrama:** Writing – review & editing, Methodology. **Teresa Del Castillo-Santaella:** Writing – review & editing, Methodology. **Yanija Zhou:** Investigation. **Dorota Martysiak-Żurowska:** Methodology. **Yuanqi Lu:** Writing – review & editing. **Christian Jungnickel:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Julia MALDONADO-VALDERRAMA reports financial support was provided by Ministry for Science and Innovation, Spain. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.].

Data availability

Data will be made available on request.

Acknowledgments

JM-V. acknowledges support from the Spanish Ministry of Science, Innovation and Universities MCIN/AEI/10.13039/501100011033, project PID2020-116615RAI00 and research group FQM-115, and JM-V is a participant of the INFOGEST international network on Food Digestion.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114255.

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3.3.6. Publication A3 Supporting Information

Bile conjugation and its effect on in vitro lipolysis of emulsions

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1. Emulsion preparation

Table S3 The conditions for emulsion preparation, where H states of homogenized, working time 2 minutes and V for vortex, working time 3 minutes. PDI of emulsions varies from 0.18-0.98. PDI – polydispersity index, SD – standard deviation.

Emulsions	H/V	Pulse on/off [s]	Amplitude [%]	Time [min]	Average of particle size [nm] ± SD	PDI
S_1	H	-	-	-	200 ± 3	0.18 ± 0.93
S_2	H	50/30	50	2.5	450 ± 38	0.60 ± 0.66
S_3	H	2/3	70	5	740 ± 35	0.84 ± 0.18
S_4	H	2/3	50	10	1300 ± 300	0.70 ± 0.29
S_5	V	10/30	80	2	1500 ± 140	0.98 ± 0.02
S_6	V	2/5	80	1	2100 ± 180	0.74 ± 0.11
S_7	V	2/5	80	1	3800 ± 300	0.91 ± 0.2

2. Standard and normalized time for IFT measurements at The OCTOPUS.

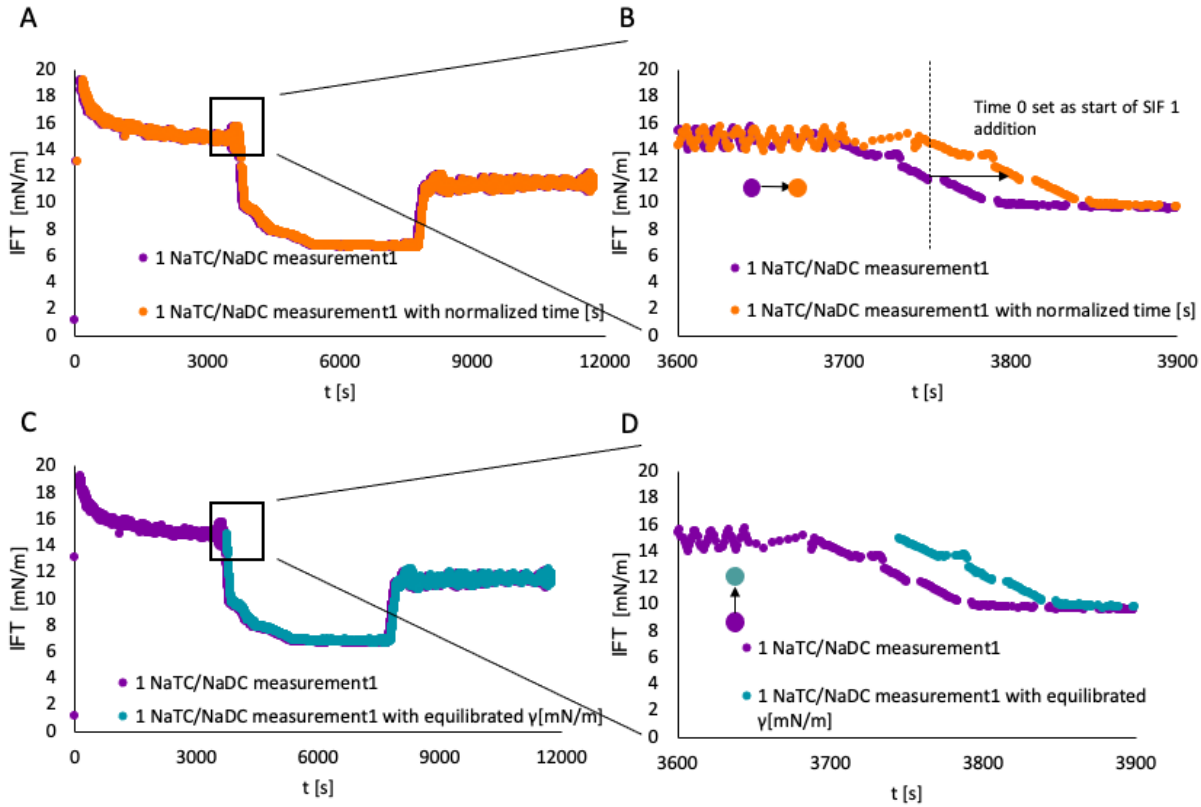

Figure S1 Exemplary graphs for in-vitro lipolysis results from OCTOPUS equipment A. The results for primary conjugated sodium taurocholate (NaTC) for standard time and normalized time. B. The black arrow indicates the distance between standard time and normalized time for NaTC of simulated intestinal fluid (SIF) I results. The starting point when the exchange of the protein layer with SIF I began was 3745.1 s and 7776.16 s when SIF II was exchanged. C. The results for PC NaTC for standard IT and equilibrated IT. D. The black arrow indicates the distance between standard IT and equilibrated IT for NaTC of SIF I results. The starting point for SIF I exchange was 15mN/m. Sodium deoxycholate (NaDC). Interfacial tension (IFT)

The time and IFT of all the experiments have been normalized, to allow the calculation of comparable adsorption and desorption rates.

3. **Interfacial Tension (IFT) measurements with OCTOPUS: sequential adsorption of WIP and BS (+lipase) and desorption cycles at the sunflower oil-water interface.**

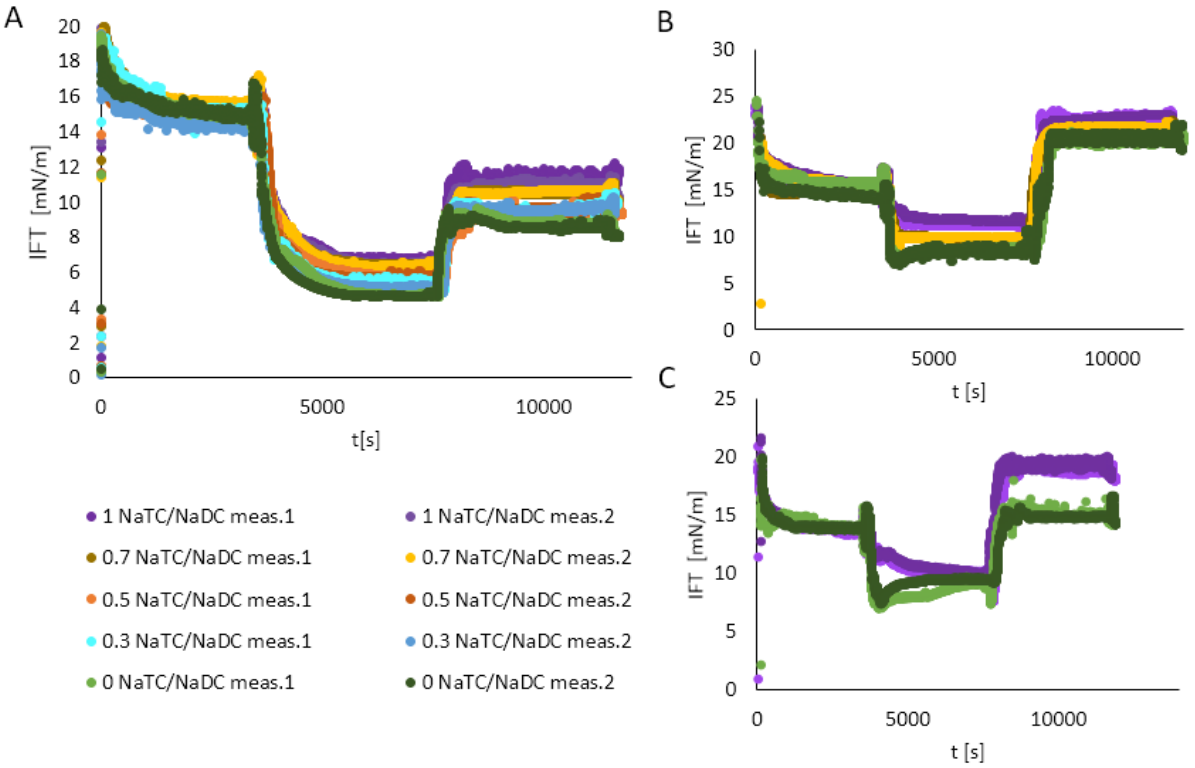

Figure S2 Interfacial tension (IFT) measurements of in-vitro digestion process performed on OCTOPUS device for different ratios of sodium deoxycholate (NaDC) and sodium taurocholate (NaTC) for A. bile salts (BS) + lipase B. BS and C. BS+lipase+orlistat measurements. Time (t)

IFT measurements presented on Fig. S1 A-B consist of three phases. Step 1: protein adsorption, Step 2: lipolysis, subphase exchange with BS or BS+lipase (rapid decrease of IFT) and Step 3: desorption, subphase exchange by SIF (rapid increase of IFT). All measurements were done in duplicate.

4. Principal component analysis (PCA) results

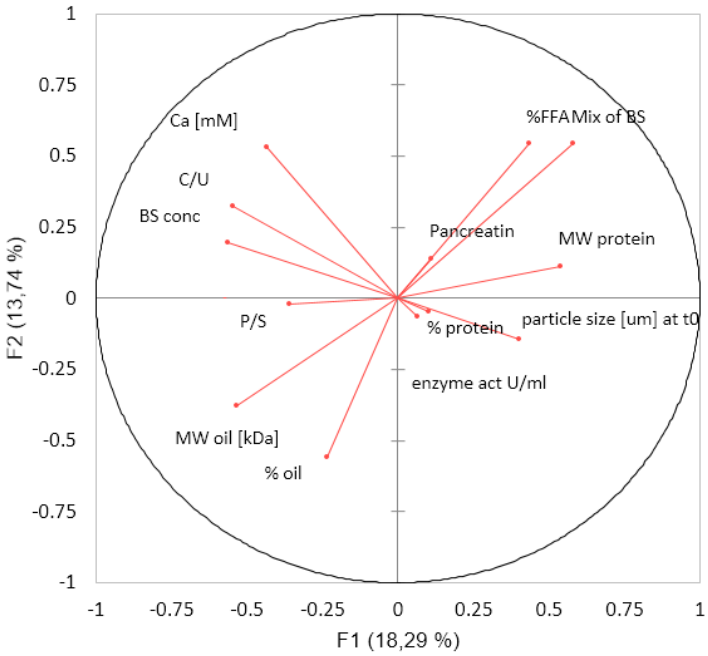

Figure S4 PCA analysis was used to reduce the number of descriptors.

Figure S3 FFA release in respect to PDI of emulsion S_1-S_7 for two different BS: NaDC and NaTC. Increasing PDI was shown to reduce FFA release for both NaDC and NaTC. PCA – principal component analysis, BS – bile salt, C/U – conjugated/unconjugated, Ca – calcium, FFA – free fatty acids, MW – molecular weight, act – activity, t0 – time 0 [min]

5. *In-vitro* lipolysis of emulsions: Free fatty Acis (FFA) release

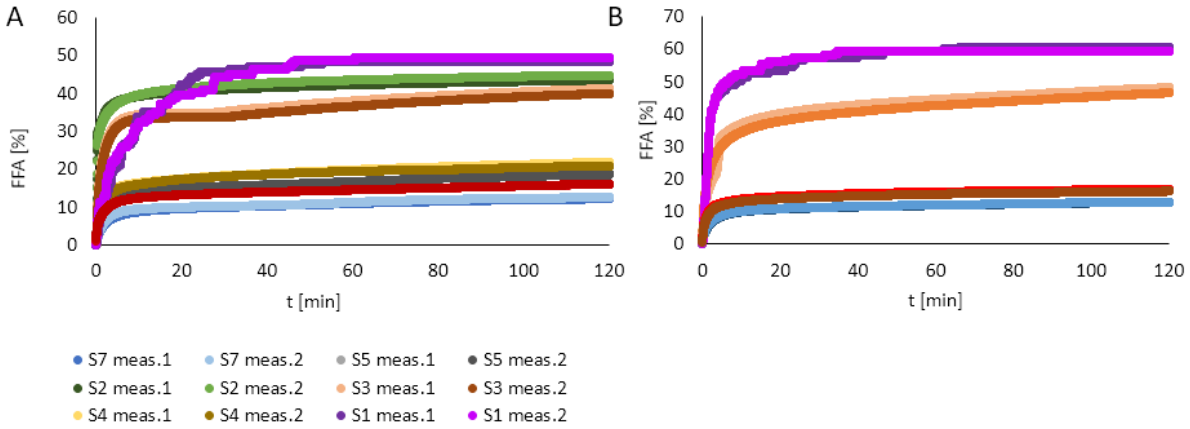

Figure S3 Free fatty acids (FFA) release from whey protein (WP)I-stabilized emulsions (S1-S7) at 10mM under physiological conditions at 310.15 K. for A. sodium deoxycholate (NaDC) and B. sodium taurocholate (NaTC). All measurements were done in duplicate

FFA release from 7 emulsions with different particle sizes for NaDC and NaTC. NaTC experiments have shown to have higher FFA release than NaDC for the same emulsion. For emulsion S_7, with the greatest particle size (3800nm) the difference between the FFA release of NaTC,12.93% ± 02, and NaDC, 12.34% ± 0.27, was not significant. Decreasing the particle size of emulsions increased the difference of FFA release for NaDC and NaTC. FFA release from the smallest emulsion S_1 (200nm), for NaTC was 60.04% ± 0.93 and for NaDC was 48.82% ± 0.98, which indicates the importance of particle size of emulsion and type of BS during the lipolysis process

Table S1 Data of meta-analysis for literature data and experimental results. Please note that the original table, with the complete data is available with the paper at https://www.sciencedirect.com/science/article/abs/pii/S0963996924003259 Exp – experimental work, D1 – D(2,1), D2 –(D(3,2), D3 – (D4,3), D4- (D(1,0), P0 – particle size [um] at time 0 [min], P – protein [%], O – oil [%], FFA – free fatty acids [%], Ca – calcium [mM], P.W.A.O- protein weight average of oil [%], MW O – molecular weight of oil [kDa], E.A – enzyme activity [U/ml], Pan – pancreatin, M BS – Mixture of BS, BS – bile salts [mM].

P/S	C/U	BS	MBS	Pan	E.A	MW O	O	P	P.W. A.O	D1	D2	D3	D4	P0	Ca	FFA	Ref
4	2	10	1	0	120	867	36	4	86	1	0	0	0	1	5	44	[1]
4	2	10	1	0	120	867	36	4	86	1	0	0	0	1	5	50	[1]
1	10	10	0	1	96	867	2	1	45	0	1	0	0	2	10	13	[2]
1	10	10	0	1	96	867	2	1	45	0	1	0	0	2	10	12	[2]
1	10	10	0	1	96	867	2	1	45	0	1	0	0	2	10	12	[2]
1	10	10	0	1	96	867	2	1	45	0	1	0	0	2	10	11	[2]
1	10	10	0	1	96	867	2	1	45	0	1	0	0	2	10	10	[2]
1	3	10	1	1	600	215	1	0	0	0	1	0	0	0	3	7	[3]
1	3	10	1	1	600	320	1	0	0	0	1	0	0	0	3	15	[3]
10	10	10	0	1	27	867	8	0	30	0	0	0	1	4	10	5	[4]
10	10	50	0	1	27	867	8	0	30	0	0	0	1	4	10	20	[4]
0	10	10	0	1	27	867	8	0	30	0	0	0	1	4	10	6	[4]
0	10	50	0	1	27	867	8	0	30	0	0	0	1	4	10	15	[4]
0	0	10	0	1	2000	867	3	0	45	1	0	0	0	2	0	14	[5]
10	10	10	0	1	2000	867	3	0	45	1	0	0	0	2	0	16	[5]
1	3	11	1	1	100	867	4	0	0	1	0	0	0	0	1	54	[6]
1	3	11	1	1	100	867	4	0	0	1	0	0	0	0	1	45	[6]
1	3	11	1	1	100	867	4	0	0	1	0	0	0	0	1	43	[6]
1	3	11	1	1	100	867	4	0	0	1	0	0	0	0	1	33	[6]
1	3	20	1	1	113	241	1	1	45	1	0	0	0	0	10	94	[7]
1	3	20	1	1	113	241	1	1	256	1	0	0	0	0	10	86	[7]
1	3	20	1	1	113	241	1	1	69	1	0	0	0	0	10	86	[7]
1	3	0	1	1	6	215	4	1	69	0	1	0	0	0	0	8	[8]
1	3	1	1	1	6	215	4	1	69	0	1	0	0	0	0	14	[8]
1	3	3	1	1	6	215	4	1	69	0	1	0	0	0	0	23	[8]
1	3	5	1	1	6	215	4	1	69	0	1	0	0	0	0	28	[8]
1	3	13	1	1	6	215	4	1	69	0	1	0	0	0	0	42	[8]
1	0	12	0	0	480	492	11	0	11	0	0	1	0	19	0	23	[9]
1	0	12	0	0	480	492	11	0	33	0	0	1	0	20	0	22	[9]
1	0	12	0	0	480	492	11	0	5	0	0	1	0	15	0	24	[9]
1	0	12	0	0	480	492	11	0	30	0	0	1	0	27	0	17	[9]
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1	0	12	0	0	480	492	11	0	10	0	0	1	0	17	0	18	[9]
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4	2	12	1	0	13500	867	3	1	86	0	0	1	0	1	7	49	[10]
4	2	12	1	0	13500	867	3	1	171	0	0	1	0	1	7	52	[10]
4	2	12	1	0	13500	867	3	1	256	0	0	1	0	0	7	81	[10]
4	2	12	1	0	13500	867	3	1	86	0	0	1	0	1	7	60	[10]
4	2	12	1	0	13500	867	3	3	171	0	0	1	0	1	7	61	[10]
4	2	12	1	0	13500	867	3	1	256	0	0	1	0	1	7	68	[10]
4	2	12	1	0	13500	867	3	1	86	0	0	1	0	1	7	50	[10]
4	2	12	1	0	13500	867	3	1	171	0	0	1	0	1	7	53	[10]
4	2	12	1	0	13500	867	3	1	256	0	0	1	0	1	7	81	[10]
4	2	12	1	0	13500	867	3	1	86	0	0	1	0	1	7	56	[10]
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1	3	5	1	1	590	421	1	0	78	0	0	1	0	0	1	49	[11]
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1	3	5	1	1	590	349	1	0	78	0	0	1	0	2	1	38	[11]
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1	3	26	1	1	762	249	3	0	69	0	0	1	0	1	0	26	[18]
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1	3	4	1	1	2447	339	0	0	444	0	0	1	0	65	1	77	[20]
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1	3	4	1	1	1791	339	0	0	444	0	0	1	0	65	1	65	[20]
1	3	4	1	1	3023	339	0	0	444	0	0	1	0	50	1	56	[20]
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1	3	4	1	1	1441	339	0	0	103	0	0	1	0	10	1	73	[20]

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4	2	4	1	1	867	282	5	0	69	0	0	1	0	9	0	21	[21]
4	2	4	1	1	867	339	3	1	50	0	0	1	0	9	0	45	[21]
1	3	5	1	0	133	241	0	0	58	0	1	0	0	37	5	73	[22]
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1	3	5	1	0	133	241	0	0	46	0	1	0	0	7	5	59	[22]
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1	3	20	1	0	600	241	0	0	58	0	1	0	0	37	10	73	[22]
1	3	20	1	0	600	241	0	0	39	0	1	0	0	4	10	95	[22]
1	3	20	1	0	600	241	0	0	46	0	1	0	0	7	10	83	[22]
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1	3	20	1	0	600	241	0	0	46	0	1	0	0	7	20	35	[22]
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4	2	4	1	1	44	249	2	0	0	0	0	1	0	22	1	39	[23]
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1	3	24	1	0	1140	867	2	0	86	0	1	0	0	18	10	38	[24]
1	3	24	1	0	1140	867	2	0	50	0	1	0	0	3	10	37	[24]
1	3	24	1	0	1140	867	2	0	1	0	1	0	0	18	10	29	[24]
1	3	12	1	1	480	241	2	0	50	0	1	0	0	0	0	61	[25]
1	3	12	1	1	480	241	2	0	18	0	1	0	0	0	0	58	[25]
1	3	12	1	1	480	241	2	0	18	0	1	0	0	0	0	58	[25]
1	3	12	1	1	480	807	3	0	40	0	1	0	0	0	0	58	[26]
1	3	12	1	1	480	807	3	0	40	0	1	0	0	0	0	36	[26]
0	0	10	0	1	2000	867	3	0	45	1	0	0	0	2	0	15	Exp
1	1	10	0	1	2000	867	3	0	45	1	0	0	0	2	0	15	Exp
2	2	10	0	1	2000	867	3	0	45	1	0	0	0	2	0	16	Exp
0	0	10	0	1	2000	867	1	0	45	1	0	0	0	0	0	48	Exp
10	10	10	0	1	2000	867	1	0	45	1	0	0	0	0	0	59	Exp
10	0	10	0	1	2000	867	1	0	45	1	0	0	0	0	0	50	Exp
0	10	10	0	1	2000	867	1	0	45	1	0	0	0	0	0	54	Exp
0	0	10	0	1	2000	867	3	0	45	1	0	0	0	4	0	11	Exp
10	10	10	0	1	2000	867	3	0	45	1	0	0	0	4	0	12	Exp
0	0	10	0	1	2000	867	3	0	45	1	0	0	0	1	0	17	Exp
0	0	10	0	1	2000	867	3	0	45	1	0	0	0	0	0	52	Exp
0	0	10	0	1	2000	867	1	0	45	1	0	0	0	1	0	41	Exp
10	10	10	0	1	2000	867	1	0	45	1	0	0	0	1	0	44	Exp
0	0	10	0	1	2000	867	3	0	45	1	0	0	0	1	0	20	Exp

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Table S2 Composition of porcine and bovine extracts. P – phospholipids, Ch – cholesterol, C -cholate, G – glycocholate, T – taurocholate, CD – chenodeoxycholate, TCD – taurochenodeoxycholate, GCD – glycochenodeoxycholate, C – deoxycholate, GD – glycodeoxycholate, TD – taurodeoxycholate, GL – glycolitocholate, TL – taurolitocholate, H – hyocholate GH – glycohyocholate, TH - taurohyocholate, HD – hyodeoxycholate, GHD – glycohyoxcholate, THD – taurohyodeoxycholate, UD – ursodeoxycholate, GUD – glycoursodeoxycholate.

Porcine bile extract [%]																				
C	G	T	CD	TCD	GCD	D	GD	TD	GL	TL	H	GH	TH	HD	GHD	THD	UD	GUD	P	Ref
0	0	0	49	44	46						14	15	20	36	39	36				[1]
	30	40					15	7						5						[2]
			1	13	20				0	1	1	3	2	0	35	24				[3]
						4	13	6												[4]
			8	17										6	21	8				[5]
5						18	39	24						13					2	[6]
	1			3	31							13			48	4				[7]
1	13	6				4								3						[8]
					35												16			[9]
Bovine bile extract [%]																				
C	G	T	CD	TCD	GCD	D	GD	TD	GL	TL	H	GH	TH	HD	GHD	THD	UD	GUD	P	Ref
	42	38		4	3		6	8												[4]
																				[10]
8	15	35				5	8	5												[11]
0	25	43	1	2	3	1	8	9	0	0					0	0		0		[12]
1	36	41	0	2	2	0	8	9												[13]
60	15	35					8	8												[14]
3	14	20					2	5												[8]
	18	15	0	10	2	9														[9]

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4. Additional results

Additional results concerning the action of two other BS were also examined: sodium glycochenodeoxycholate (NaGCDC) and sodium glycodeoxycholate (NaGDC). NaGCDC is the PC BS formed in an alternative pathway, as shown in Figure 3, and NaGDC is the secondary conjugated BS formed after the deconjugation process. Our research aimed to determine the impact of the deconjugation process on lipolysis efficiency, therefore only two BS (NaTC and NaDC) have been chosen as representatives, as their concentration in the small intestine exceeds other BS.

However, NaGCDC differs from NaTC by an additional hydroxyl group, which due to our presented research, may be of great importance in affecting lipolysis efficiency. Moreover, examination of the effect of NaGCDC expands our research of information about the effect of the action of secondary conjugated BS on the lipolysis process.

The aim of performing these additional results was to check if their action has a significant impact in comparison to previously chosen BS: NaTC and NaDC in two main experiments: in-vitro lipolysis of emulsion and in-vitro digestion.

4.1. Methodology

4.1.1. Dilatational rheology

The dilatational rheology on the interfacial layer is measured at the end of each phase by subjecting the droplet to 10 cycles of periodic deformation by injection/extraction volume at 0.1 Hz of measurement frequency (v). The dilatational modulus is calculated from the response of the interfacial tension to the deformation by the following equation: $E = E' + iE'' = \epsilon + iv\eta$ (1)

E' is the storage modulus, which accounts for the elasticity of the interfacial layer (ε), E'' is the loss modulus, which accounts for the viscosity (η) of the interfacial layer, and v is the angular frequency of the applied oscillation. The amplitude of the applied oscillation was set up to < 5% to avoid excessive perturbation of the adsorbed interfacial layer (del Castillo-Santaella et al. 2015). At this oscillation frequency, the interfacial layer displays a mostly elastic response obtaining E'>>E'' in all cases. Hence, only the values of the complex modulus will be reported here and discussed as dilatational elasticity.

4.1.2. In-vitro lipolysis of emulsion

A modified INFOGEST *in-vitro* lipolysis model (Brodkorb et al. 2019) was used to simulate the environmental condition of the duodenum. Specifically, 0.8 mL of the SIF and 0.375 mL of the emulsion were added to the thermostatted vessel. After mixing with a magnetic stirrer (1500 rpm),0.3 mL of 10mM BS (NaTCorNaDC) and 3 µL of 0.3M $CaCl_2$ were pipetted, and the pH was set to 7.0 using 0.1 M HCl. Finally, with the addition of 1.0 mL of freshly prepared pancreatin (75 mg at 80 U/mg), the titration was started. The reaction vessel was continuously stirred and thermostatically controlled to maintain 310.15 K. The extent of the lipolysis was measured by continuous titration with an autotitrator (Cerko Lab *N.* System CLS/M/07/06, Gdynia, Poland) of (FFA) with 0.1 M NaOH. All lipolysis experiments were carried out in duplicate. Experiments were performed according to Łozińska et al. (Łozińska et al. 2024)

4.1.3. In-vitro lipolysis

In-vitro lipolysis of adsorbed protein layers at the oil-water interface was measured in OCTOPUS by sequential adsorption comprising three steps: Step1- protein, Step2- lipolysis: BS, BS + lipase or BS + lipase + inhibitor, and Step 3- desorption: replacement of bulk solution by SIF (Maldonado-Valderrama et al. 2014; Łozińska et al. 2024).

4.2. Results&Discussion

4.2.1. Dilatational modulus

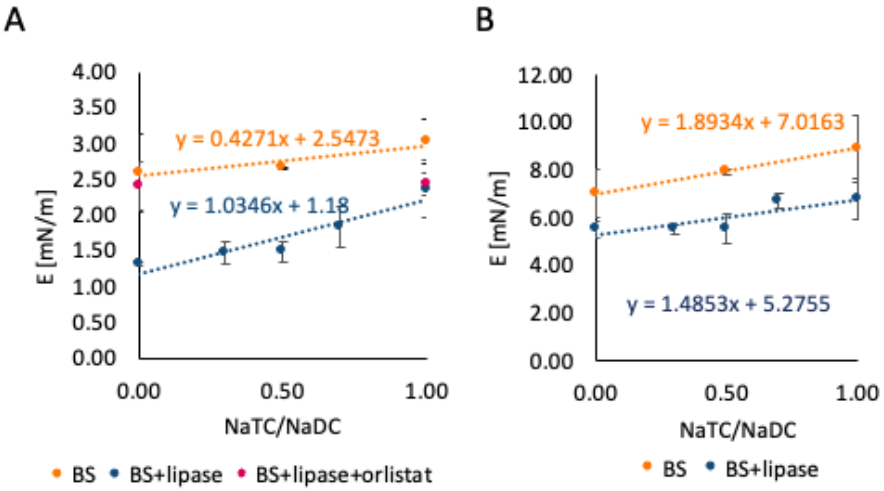

Figure 9 A. Dilatational complex moduli of interfacial layers after an exchange of the adsorbed protein at the interface with one of the following solutions: BS, BS+lipase, BS+lipase+orlistat.Average taken from 10 records + SD. B. Dilatational complex moduli of interfacial layers after an exchange of the previous solution with simulated intestinal fluid. BS – bile salts, NaTC – sodium taurocholate, NaDC – sodium deoxycholate, E - dilatational modulus.

In the presence of lipase, the dilatational modulus increases slightly with the concentration of BS (as shown in *Figure 9* A, again supporting the increased presence of lipolytic products at the interface which is enhanced as the concentration of BS increases. The dilatational modulus of desorption (*Figure 9*B) follows the trend from adsorption results

4.2.2. In-vitro static lipolysis

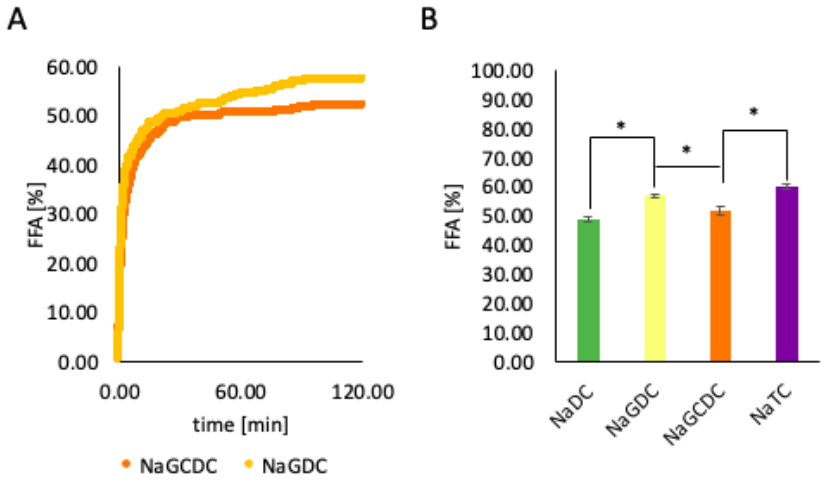

Figure 10 A. In-vitro static digestion experiments of PC NaGCDC and SC NaGDC. B. T-sample t-test was used to calculate the statistical significance of FFA release between Na GCDC and NaGDC. NaGDC has been shown to have statistically higher FFA release (FFA=57.07%) than NaGCDC (FFA=51.79%). NaGCDC – sodium glycochenodeoxycholate, NaGDC – sodium glycodeoxycholate, NaTC- sodium taurocholate, NaDC – sodium deoxycholate, FFA – free fatty acids

The results presented in Figure 10 show that SC NaGDC have greater potential to enhance FFA release during the lipid digestion process than PC NaGCDC. The greater lipolysis efficiency of NaGDC is a result of its greater desorption potential from the oil droplet during the lipolysis process, as shown in Figure 11B. The space left after the removal of lipolysis products from the oil interphase ensures the continuous process of lipid digestion by adsorption of NaGDC and lipase/co-lipase complex. The FFA release differs significantly for PC NaTC and NaGCDC, which means that the conjugation ratio with cholic and chenodeoxycholic has an impact on the lipolysis process. Moreover, the results indicated that SC NaGDC has greater potential to enhance FFA release than SU NaDC, showing the importance of the conjugation process after the action of BSH. As it was shown in our research FFA release from emulsion is connected with interfacial processes.

4.2.3. In-vitro lipolysis

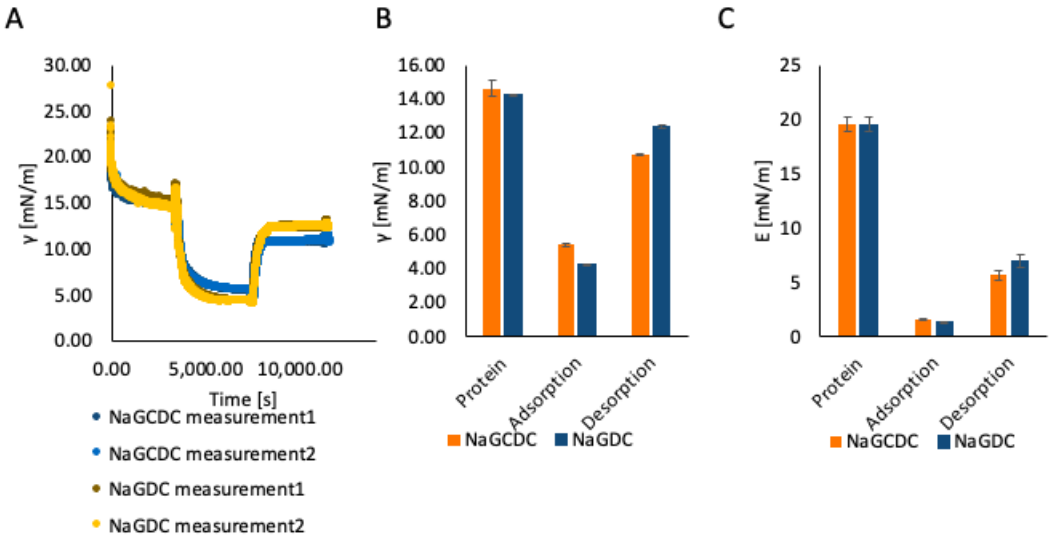

Figure 11 A. FT measurements of in-vitro digestion process performed on OCTOPUS device for different BS: SU sodium glycodeoxycholate (NaGDC) and PC sodium glycochenodeoxycholate (NaGCDC). B. Final (average of last 30 points) IFT have shown that NaGCDC has greater adsorption potential, which means it can more effectively adsorb to the surface of the oil droplet which enhances the adsorption of lipase/co-lipase complex during the lipolysis process. However, NaGDC has shown a greater potential to desorb from the soil surface, which can ensure the effective removal of accumulated lipolysis products from the oil interphase. C. Young modulus of NaGCDC and NaGDC during adsorption and desorption steps. NaGCDC – sodium glycochenodeoxycholate, NaGDC – sodium glycodeoxycholate, γ – interfacial tension. E - dilatational modulus.

The results presented in Figure 11 B show that NaGCDC have a greater ability to adsorb to oil interphase, therefore enhancing lipase/co-lipase complex to adsorb to the oil droplet and start the digestion process. However, the results also indicate that NaGDC faster desorbs from the oil droplet, which plays an important role in ensuring the removal of lipolysis products from the oil interphase, which gives greater space for lipase/co-lipase complex for further adsorption on the lipid droplet and continue lipolysis process.

5. Conclusions

The results of the PhD dissertation revealed the potential of BS to control the lipolysis process. The deconjugation process was shown to be crucial for the efficiency of the lipolysis process. Two predominant forms of BS in the small intestine: PC-NaTC and SU-NaDC were chosen for further analysis concerning lipolysis efficiency.

In the first stage of the research the meta-analysis of phenomenological parameters: was performed to indicate the importance of the concentration of conjugated BS (Łozińska and Jungnickel 2021). The performed analysis of research results aimed to assess the ability to create micelles, responsible for incorporating lipolysis products into their structure, removing them from the oil interphase and further transporting them to our organism (Pabois et al. 2021). The collected results showed that conjugated forms of BS require lower concentrations and fewer molecules than unconjugated BS to form micelles. Moreover, micelles created by PC BS showed a greater ability to incorporate components into their structure. Performed experiments of in-vitro digestion of emulsion of conjugated and unconjugated systems of BS showed the importance of conjugation. For the first time, it was presented that concentration of conjugated BS can modulate lipolysis efficiency.

In the next stage of the research, the analysis of BS composition concerning specific diseases has been performed (Krupa et al. 2021). The development of disease was shown to significantly alter the ratio of conjugated and unconjugated BS. Blockage of the common bile duct reduced the flow of the BS from the gallbladder to the small intestine which resulted in the alteration of the BS synthesis (Dai et al. 2011). Since the composition of BS stimulates BS synthesis, their reduced concentration during re-absorption from the small intestine promotes BS synthesis by an increased activation of the Cholesterol 7-α hydroxylase providing to formation of the excessive concentration of conjugated BS (Kok et al. 2003). Development of disease was indicated as factors directly altering the composition of BS and indirectly modulating the efficiency of the lipolysis digestion process.

The following stage of the research (Łozińska et al. 2024)for the first time revealed that the lipolysis process is modulated by five processes and their efficiency is controlled by the form of BS. Each of the processes was presented as a mathematical function of measurable variables. The rate of lipolysis was shown to be controlled by the conjugation form of BS. Meta-analysis of in-vitro lipolysis experiments was performed revealing the significance of individual factors in the lipid digestion process. Unconjugated forms of BS, NaDC, have been shown to reduce the size of the droplet to a higher extent than conjugated NaTC providing greater efficiency of the emulsification process at the beginning of the lipolysis, later on, NaDC contributes to decreasing efficiency of emulsification, by promoting coalescence of the droplet. NaTC promotes FFA release during the lipolysis process by a greater rate of adsorption and desorption than NaDC. The experimental results and meta-analysis of the lipolysis process allowed us to determine the adsorption process and formation of the micelles as predominant factors influencing lipolysis.

The results of my PhD dissertation revealed the importance of the deconjugation process of BS. Conjugation concentration, for the first time, was shown to regulate the rate of lipolysis and the results have shown the controlled way to modulate lipolysis efficiency by modulating five different processes. The importance of the development of diseases, as a factor disturbing the lipolysis process, was shown to alter the BS composition in our body and dysregulate their function as lipolysis agents.

6. Limitations

6.1. **In-vitro digestion studies** One-compartment static digestion models, that were used in publication A1 and publication A3 are good for determining the endpoint of the digestion process, however, the main limitation of the research is that the experiments performed in the static digestion model do not reflect and include the kinetics and physiology of digestion such as absorption, the response of hormones, no effect of gastric emptying and peristaltic movements (Wang et al. 2021). Moreover, the titration reaction is not specific for lipid digestion. There is no possibility to differentiate between digestion products in the case of a complex food matrix, consisting of proteins or starch, which are also neutralised by an alkaline solution (Zhou et al. 2021). This kind of model also does not consider the conditions and processes in the stomach. The gastric and intestinal phases can be performed separately by using a two-compartment model, however, it also requires pre-conditioning as the results obtained from the gastric phase have to be manually transferred (Huang et al. 2021).

6.2. **Digestion conditions**: Another limitation of performed research is its general focus on the BS function. The in-vitro digestion experiments were performed only in the presence of BS, however, the human intestinal digestion of lipids takes place in the presence of a complex matrix of cholesterol, phospholipids, and a greater number of BS diversity, such as PC–sodium glycocholate, sodium glycochenodeoxycholate, sodium taurochenodeoxycholate, SU – sodium lithocholate and secondary conjugated – sodium glycodeoxycholate, sodium taurodeoxycholate (Sensoy 2021). The performed experiment didn't consider the effect of phospholipids, which also influence final FFA release.

6.3. **Modulation of emulsion:** Digestion studies were performed by using an emulsion of the same type of oil and stabilised by WPI. The studies were limited to the interaction of BS with only one type of emulsion. The effect of the composition and structure of emulsion on the lipolysis extends by decreasing fat absorption or increasing the bioavailability of nutrients (Pabois et al. 2020)was restricted in performed research.

6.4. **BS action:** The BS play an important role in the digestion process, but they are also crucial components as receptor regulators (Da Silva et al. 2013). The performed studies don't cover the influence of changes in BS composition apart from the digestion process. For example, changes induced in the regulation of FXR or functioning of BS synthesis.

6.5. **Digestion conditions** Experiments measuring CMC of BS used a simple and non-invasive micro-titration technique, however, its sensitivity allowed only to determine one CMC, whereas the current more advanced techniques allow for more detailed measurements of primary and secondary BS micelles (Mukherjee et al. 2016).

7. Future perspectives

Future perspectives should focus on increasing the potential to understand the mechanism of lipolysis and to control the lipid digestion process

7.1. **In-vitro digestion studies:** During the digestion process in our organism, there are a lot of factors acting simultaneously, that influence the final rate of digestion (Bauer et al. 2005; Bellesi et al. 2018; Macierzanka et al. 2019), therefore, In-vitro semi-dynamic and dynamic models should be used considering: (a) bioaccessibility of nutrients and passive absorption of digestion products (for example ESIN or ARCOL system), (b) interaction of nutrients and delivery of functional food, (c) action of gut microbiota (for example TIM-1 system providing complex high-density microbiota of animal or human origin), (d) digestive secretion (DGM system) should be used in future studies. Moreover, in-vivo studies should be performed.

7.2. **Digestion conditions:** To expand the opportunity for performing lipolysis under controlled conditions in-vitro digestion studies with the presence of phospholipids, cholesterol and complex BS compositions should be performed.

7.3. **Modulation of emulsion:**: The interaction of BS emulsifiers is considered a key factor in modulating lipolysis (Naso et al. 2019). Therefore, microscopic examination, such as confocal microscopy technique, microscope laser light scattering spectroscopy, of lipid droplets during lipolysis for individual BS and different digestion conditions should be investigated to determine the impact of the interaction of BS with emulsion on final fat digestion efficiency and possible mechanism to control lipolysis rate. Moreover, the effect of more complex emulsions and their effect on final FFA release via interaction with BS should be studied to determine the possible mechanism of interaction of emulsion with BS. The results may give a perspective to design an emulsion that would be digested in a controlled way and would modulate the lipolysis process by enhancing or suppressing FFA release from the emulsion.

7.4. **BS action:** The effect of BS towards BS synthesis via controlling FXR should be more deeply studied to understand the possible perturbation of the lipolysis process from the molecular side. Controlling the BS synthesis appears to be a key solution to reducing the obesity problem (Haeusler et al. 2016). Therefore, uncovering the potential to modulate it would give great value to future studies in finding a solution to the obesity epidemic.

7.5. **Digestion conditions:**: Studies of the activity of BSH should be performed, by using non-invasive methods such as bioluminescent imaging (Khodakivskyi et al. 2021), as the BSH controls the C/U ratio of BS in the small intestine (Bourgin et al. 2021). The increasing activity of BSH would affect in formation of excessive conc of SU BS, which were considered as agents contributing to the development of colon cancer, fat malabsorption and obesity. Moreover, the influence of exogenous parameters, such as antibiotics, should be examined, as they are correlated with the development of disease, decreasing BS composition and diversity and reducing BSH activity (Kronman et al. 2012; Daly et al. 2021). Also, the effect of probiotics and prebiotics should be examined, to check their desirable properties as agents increasing BSH activity.

7.6. **In-vitro digestion studies**: BS are responsible for delivering the essential components to our organism, the disturbance of this process is of great importance and may also be connected with the development of a disease state, such as malabsorption(Montoro-Huguet et al. 2021). Therefore, the absorption potential of digestion end products and BS should be measured, by using the CaCo2 cells - hd29 model, animals' cell lines from piglets or rats, human cell lines or using chambers (ex-vivo models) that use intestinal tissue.

8. Other scientific achievements

8.1. Research internships

POWR.03.05.00-00-Z044/17, 27.03-2022-27.06.2022. University of Granada, Faculty of Science, Department of Applied Physics. Supervisor: Julia Maldonado Valderrama, Associate Professor

BIP (Blended Intensive Programme) at L'Institut Agro - Institut national d'enseignement supérieur pour l'agriculture, l'alimentation et l'environnement (16.06.2023-23.06.2023)

8.2. Other research internships

Internship at Dezhou University, China (08.2018-09.2018)

8.3. Conferences

7th International Conference on Food Chemistry & Technology (FCT 2021), Paris, France 8-10.11.2021, oral presentation.

4th Food Structure and Functionality Forum Symposium 2021, 19-20.10.2021, poster presentation

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