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Crosstalk and interaction between gut microbiota and bile acids

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Zusammenfassung

Hintergrund

Die Interaktion zwischen Gallensäuren und der Gesamtheit der Darmmikrobiota ist Gegenstand jüngster Forschung. Mikrobiota sind im Kolon ständig den von der Leber sezernierten Gallensäuren ausgesetzt. Diese Interaktion hat physiologische Konsequenzen, da Mikrobiota in hohem Maße den Stoffwechsel des menschlichen Körpers beeinflussen und eine Dysbiose (pathologische Abweichung der Zusammensetzung des Mikrobioms von der Norm) mit Erkrankungen wie Adipositas, Diabetes Mellitus Typ 2 oder chronischer Cholestase assoziiert ist. Im Sinne einer Bilateralität beeinflusst das Mikrobiom das Gallensäureprofil des Organismus und *vice versa*. Gallensäuren sind daher eine wichtige Determinante der Funktion und Zusammensetzung des Mikrobioms.

Methodik

Im Sinne einer Übersichtsarbeit wurde die Interaktion zwischen Gallensäuren und Mikrobiota anhand von *in vitro* Experimenten untersucht. Dazu wurden zwei verschiedene Nährmedien (LB und GIM) hergestellt, die mit konjugierten, freien und synthetischen Gallensäuren angereichert wurden. Mikrobiota aus dem Caecum von weiblichen, männlichen, SHAM und BDL Mäusen wurden gewonnen und mittels dieser Medien unter aeroben und anaeroben Bedingungen kultiviert. Der Einfluss der Gallensäuren auf das bakterielle Wachstum wurde anhand der CFU/ml und OD600 bestimmt. Weiterhin bestimmten wir die Aktivität der bakteriellen Enzyme „bile salt hydrolase“ (BSH) und 7α-Dehydroxylase anhand der Generierung von unkonjugierten (freien) und dehydroxylierten (sekundären) Derivaten der zugesetzten Gallensäuren mittels LC-MS. Ferner untersuchten wir, ob bakterielle Stoffwechselprodukte in den Kulturen entstanden sind, die die Expression von Genen des Gallensäurestoffwechsels in Hepatozyten modulieren. Dazu wurden HepG2 Zellen mit Überständen der bakteriellen Kulturen kultiviert und die Expression von Cyp7a1 und Shp in diesen Zellen mittels RT-PCR gemessen.

Ergebnisse

Gallensäuren hemmen das Wachstum der Darmmikrobiota in unterschiedlichem Maße. CA zeigte die stärkste Wachstumshemmung sowohl in LB als auch in GIM, wo kein Wachstum beobachtet wurde. Die konjugierten Gallensäuren TCA und TUDCA zeigten einen Trend zur milderen Hemmung bakteriellen Wachstums. Die synthetische Gallensäure CHS zeigte in GIM eine sehr starke Hemmung, nicht jedoch in LB. Ferner hemmten CA und TCA in LB das Wachstum von Bakterien der SHAM-Tiere stärker als das Wachstum von Bakterien der BDL-Tiere. TUDCA zeigte diesen Effekt jedoch nicht.

Weiterhin zeigte sich die bakterielle Biotransformation von Gallensäuren *in vitro* im ersten Schritt, der Dekonjugation (BSH-Aktivität), suffizient unter aeroben und anaeroben Bedingungen. Der zweite Schritt, die Generierung von sekundären Gallensäuren (7α-Dehydroxylase-Aktivität) war weder unter aeroben, noch unter anaeroben Bedingungen festzustellen.

Ferner konnten wir zeigen, dass Stoffwechselprodukte bakterieller Kulturen in Gallensäureselektionsmedien Einfluss auf hepatische Genexpression haben, Gallensäuren als solche jedoch nicht (außer CDCA). Die Expression von Cyp7a1 wurde unter CA, TCA und UDCA reprimiert. Die Expression von Shp wurde unter CA und TCA induziert.

Schlussfolgerung

Die Interaktion zwischen Gallensäuren und Darmmikrobiota ist komplex. Gallensäuren und ihr Metabolismus durch das Mikrobiom tragen zur intestinalen und hepatischer Homöostase bei.

Abstract

Background

Research on the interaction between bile acids and microbiota is currently experiencing a zestful renaissance. Microbiota are exposed heavily to bile acids in the colon. This interaction has physiologic implications as the gut microbiota clearly impact on the host's metabolism. Moreover, dysbiosis (a pathologic deviation from a normal microbiota composition) is strongly associated with obesity, type 2 diabetes or chronic cholestasis. In the sense of bilateralism, the host's bile acid profile is modulated by gut microbiota and *vice versa*. Hence, bile acids are an important determinant of microbiota diversity and function.

Methods

The interaction between bile acids and microbiota has been investigated with *in vitro* experiments. For this purpose, two different growth media (LB and GIM) have been prepared, which were supplemented with conjugated, free and artificial bile acids. Microbiota from the caecum of female, male, SHAM and BDL mice have been obtained and cultured in an aerobic and anaerobic environment. The impact of bile acids on bacterial growth was determined via CFU/ml and OD600. Furthermore, the activity of the bacterial enzymes bile salt hydrolase (BSH) and 7α-dehydroxylase has been investigated. Therefore, the formation of free and dehydroxylated (secondary) bile acid derivatives in the cultures was measured via LC-MS. Moreover, we investigated, whether the cultures yielded bacterial metabolites which are capable of modulating hepatic expression of genes of the bile acid metabolism. HepG2 cells were incubated with supernatants of the bacterial cultures and the expression of Cyp7a1 and Shp was quantified via RT-PCR.

Results

Bile acids inhibit bacterial growth of gut microbiota to a varying extent. CA showed the strongest effect of growth inhibition in LB and GIM. The conjugated bile acids TCA and TUDCA showed attenuated growth inhibition. The semi-synthetic bile acid CHS showed a strong suppression of bacterial growth in GIM, but not in LB.

Moreover, CA and TCA in LB showed a stronger growth suppression of bacteria from SHAM mice than of bacteria from BDL mice. TUDCA did not show this effect. Furthermore, bacterial biotransformation of bile acids *in vitro* is possible, but only the first step, bile acid deconjugation (BSH activity), was performed sufficiently in both, aerobic and anaerobic conditions. Consequent generation of secondary bile acids (7α-dehydroxylation) was neither observed in aerobic nor in anaerobic conditions.

Additionally, we were able to show a transactivation of hepatic genes, by metabolites of bacterial cultures in bile acid media. Cyp7a1 expression was repressed under CA, TCA and UDCA. Shp expression was induced under CA and TCA. Bile acids alone do not affect hepatic gene expression (except for CDCA).

Conclusion

The interaction between bile acids and gut microbiota is complex. Bile acids and their metabolism through gut microbiota contribute to intestinal and hepatic homeostasis.

1. Introduction

1.1. The gut microbiome

1.1.1. Composition and species

The human gut microbiome consists of approximately 10^{13} to 10^{14} single microorganisms. It was long believed that these microbiota would outnumber the body cells of a normal human individual by one order of magnitude. Although recently new studies suggested that this ratio is closer to 1:1, the sheer number of microbes in our gut is still inconceivable and adds up to a weight of 1.5-2.0 kg of sole bacteria (1–3).

Though these microbes are widely colonizing the entire digestive system, stomach and small intestine only contain a few bacterial species owing to the acidic environment and the vermicular movement. Generally, bacterial colonisation becomes denser towards the distal parts of the intestines. Peak densities are reached in the caecum and colon where one gram of intestinal content can contain up to 10^{12} cells. The vast majority of these bacteria are obligate anaerobes and the intestinal community of a normal individual contains 300 – 500 bacterial species. Moreover, the entirety of genes encoded by the bacterial genome exceeds that encoded by the human genome by a factor of 500 (3,4).

The members of the human intestinal community belong primarily to two divisions of bacteria: *Firmicutes* (51%) and *Bacteroidetes* (48%). Similarly, in mice, these two divisions are also the most abundant, although *Firmicutes* are found more frequently (60-80%) than *Bacteroidetes* (20-40%). Important genera in the group of *Firmicutes* are *Clostridium*, *Lactobacillus*, *Ruminococcus*, *Eubacterium*, *Roseburia* and *Fecalibacterium*. Important genera in the group of *Bacteroidetes* include *Bacteroides*, *Prevotella* and *Xylanibacter*. Especially the genus *Bacteroides* seems to be of particular importance. Its species account for 30% of intestinal bacteria alone (3,5,6).

Other phyla occurring as minor constituents in the intestinal lumen are *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia*. Interestingly, *Escherichia coli* (a facultative anaerobe, belonging to the phylum of *Proteobacteria*), can be found

in almost every human individual. *Archaea*, *Eukarya* and viruses can also be detected, though their role in intestinal physiology needs to be examined more closely (7).

In a continuing effort to characterize intestinal microbes, Arumugam et al. recently introduced the concept of “enterotypes”. Despite the sheer abundance of species residing in the gut, after analysing 39 metagenomes of individuals from Europe, America and Japan, the authors were able to define three robust clusters of bacteria, each characterized by the predominance of either *Bacteroides*, *Prevotella* or *Ruminococcus* respectively. These clusters were independent from age, gender or nationality. Though this concept needs to be reviewed thoroughly, as subsequent studies have failed to show the same distribution and others state conceptual problems, it may provide an interesting approach in the understanding of our microbial symbionts in health and disease (8–10).

1.1.2. Factors shaping the microbiome

In the course of trying to establish frameworks for characterizing the gut microbiome, it became evident that this community is a highly diverse and variable ecosystem. It is subject to constant alteration and dynamically adapts to given circumstances. Major differences within the populations of different individuals may be recognised depending on origin, genetics, environmental factors, lifestyle and diet (7,10).

Age and mode of delivery

Infants were believed to have sterile intestines when they are born. Recently, this belief has been challenged, as several studies suggested an already-in-utero-colonization of the foetus. Jimenez et al. were able to detect bacteria belonging predominantly to the genera *Enterococcus* and *Staphylococcus* in the meconium of 21 healthy new-borns (11). Another study yielded signs of microbial communities in the placenta and the amniotic fluid (12). However, other authors pointed out that these studies need to be seen cautiously, as the provided evidence is based on rather inadequate methods and lacks important parameters of validity (13).

Therefore, at the present time, the conception of prenatally sterile neonates may be assumed with clear conscious. Primary colonization is acquired when the infant

is exposed to a broad spectrum of environmental bacteria postpartum. It has become clear that the mode of delivery is very much decisive for the composition of the first gut commensals as the acquired gut flora resembles the habitat, the infant had contact with during birth. Therefore, infants who are born vaginally, harbour organisms which are inherent to the mother's vagina, while caesarean delivery results in colonization with organisms belonging originally to the mother's skin. However, after 6 months, these initial differences vanish (14–16).

The microbial complexity at the beginning of life is less-marked and rather unstable. In the first months and years of the infant's life, the intestinal community becomes more stable and gains robustness against environmental influences. Eventually the diversity increases, and the bacterial composition converges towards an adult condition consisting of predominantly *Bacteroides* and *Firmicutes*. At three years of age, microbial colonization resembles the one of an adult (7,10,14).

Given an unchanging selective pressure of environmental conditions and the absence of extensive changes in dietary habits, nor frequent usage of antibiotics, the bacterial composition of one individual remains relatively stable throughout adulthood. In older age, the diversity of the microbial community decreases again (14,17).

Diet

Dietary changes seem to have the most profound impact on microbiome composition. Both, short- and long-term dietary interventions may deeply modulate bacterial proportions in the gut and herewith the ensemble of genes these bacteria are expressing (18).

A study conducted in germ-free mice, prepared with human gut microbiota to mimic the human intestinal ecosystem, displayed rapid compositional alteration of faecal bacteria and altered microbiome gene expression within 24 hours after switching from a low-fat, high fibre, plant-based diet to a high fat, high sugar, "western" diet (19).

Consistent with this, Wu et al. analysed faecal samples from 10 individuals and found a considerable change in microbiota composition 24 hours after initiating a high-fat, low-fibre, or low-fat, high-fibre diet. However, this dietary transition was not able to permanently alter basal compositions as represented by the

Bacteroides or *Prevotella* enterotype respectively. Dietary habits though do indeed have an impact on enterotypes, but this effect becomes manifest only in the long-term. The *Bacteroides* enterotype is typically associated with a diet high in protein and animal fat, whereas individuals consuming high levels of dietary carbohydrates have the *Prevotella* enterotype (20).

Generally, a diet high in plant-based components like complex carbohydrates and fibre (as opposed to a diet rich in animal protein and fat), seems to be beneficial on bacterial diversity in the gut. Individuals adhering to a plant-based diet had significantly lower levels of *Bacteroides*, *Bifidobacterium*, *E. coli* and *Enterobacteriaceae* species. However, the consequences of these shifts in composition remain unclear and need further examination (21,22).

Origin

Geography is another meaningful determinant of gut microbiota composition. Several studies provided evidence that individuals from different countries harbour different microbiota. Ou et al. showed that microbiota composition in native Africans, hosting predominantly *Prevotella*, is significantly different to African Americans where *Bacteroides* prevail. This is consistent with the above presented findings from Wu et al. Moreover, total bacteria and short chain fatty acids (SCFAs) were significantly more abundant in native Africans, whereas African Americans had higher levels of secondary bile acids (BAs) matching their higher fat intake (20,23).

A study comparing the microbiota of European children and those of children from a rural village in Burkina Faso also revealed significant differences between the two groups. African children showed significant higher abundancies of *Bacteroidetes*, especially genera *Prevotella* and *Xylanibacter*, and a cutback in *Firmicute* levels. Interestingly, European children were lacking *Prevotella* species (belonging to phylum *Bacteroidetes*) at all. Furthermore, African children had significant more SCFAs than European children (24).

Yatsunenko et al. also found profound differences in the microbial collective when they compared faecal samples of 531 individuals from the USA, Venezuela and Malawi. The authors observed a remarkable difference especially between Americans and individuals from Venezuela and Malawi (10).

However, differences in microbiota compositions may be difficult to ascribe to geographical factors alone. As dietary habits in the examined groups vary widely, it is more likely that the observed compositional alterations are consequence of different local nutrition (24).

Lifestyle

Certain lifestyle factors may also have microbiota shaping potential. Clarke et al. linked high levels of physical activity and high protein intake to highly diverse gut microbiota. Comparing professional athletes to size-matched obese (BMI > 30 kg/m^2) and age/gender-matched lean (BMI < 25 kg/m^2) controls, the authors found significant more phyla in the athlete group. Athletes had 22 distinct phyla, whereas in samples of the low and high BMI group (lean and obese controls), only 11 and 9 phyla respectively could be detected. Frequent exercise may therefore be beneficial on intestinal health and homeostasis (25).

Furthermore, as the understanding of the brain-gut axis advances, it has become clear that stress and associated hormonal signals have an impact on gut microbiota composition (26). O'Mahony et al. observed significant changes in microbial diversity when they compared rats which were subject to maternal separation early in life (a strong stressor), to non-separated rats (27). Consistent with this, one study using infant monkeys exposed to early maternal separation, revealed a transient decrease in quantity of gut microbiota, especially *Lactobacilli*, leaving those monkeys more prone to disease (28).

Another study investigating the effects of smoking on gut microbiota in individuals with active Crohn's disease (CD) revealed significant shifts in microbiota composition in the smokers group. Smokers with CD had significant higher levels of *Bacteroides-Prevotella* (38.4%) as against to non-smokers (28.1%). This may indicate a potential impact of smoking on gut microbiota composition (29).

1.1.3. Physiologic implications

Host and gut microbiota coexist in a mutualistic relationship which is essential for the maintenance of the host's intestinal physiologic functions. The microbial community is key to nutrient and xenobiotic metabolism. It helps to preserve the structural integrity of the intestinal mucosal layer and prevents pathogens from spreading. Moreover, important immunologic functions are imparted on the host's

immune system (30). Interestingly, in opposition to microbial profiles on the genera level, which may be highly divergent between individuals (as discussed above), the functional gene catalogue encoded by the microbial ensemble does not deviate substantially inter-individually, but rather is fairly similar. These, among many individuals equally expressed genes, can be seen as a common core metagenome (31,32).

Metabolic functions

Non-digestible dietary components or components which escape proximal uptake are substrates for colonic bacteria. These include dietary fibre (complex carbohydrates), indigestible oligosaccharides, bile acids and xenobiotics. Bacterial fermentation of complex carbohydrates and oligosaccharides, leads to generation of short chain fatty acids (SCFAs) which are an important energy source for colonocytes and also have signalling properties (33). They account for 6-10% of the daily required caloric intake of western people (34). However, this amount might be dependent on the present diet, since members of African communities who consume a higher amount of dietary fibre, produce more SCFAs (24).

In the human intestines, mainly three types of SCFAs arise from fermentation: Butyrate, propionate and acetate. Butyrate is the major energy source for colonocyte metabolism, propionate is metabolized in the course of hepatic gluconeogenesis and acetate plays a role in appetite regulation, lipogenesis and muscle cell metabolism (4,33).

The generation of SCFAs in the intestinal lumen correlates positively with the diversity of gut microbiota and the amount of dietary fibre consumed. A highly diverse intestinal community is able to generate multiple types of SCFAs in decent abundancies, whereas low microbiota diversity impairs the metabolic output of SCFAs and may result in the predominance of only one kind of SCFA or depletion of SFCAs in general (33). SCFAs are also substrates for the G-protein-coupled-receptors (GPCRs) GPR41 & GPR43 and exert important signalling functions in the host. In mice, there is evidence that activation of GPRCs in intestinal epithelial L-cells leads to inhibited gut motility and increased caloric uptake via the enteroendocrine hormone peptide YY (PYY) pathway. Moreover, pancreatic function and central regulation of appetite is modulated by increased secretion of glucagon-like-peptide 1 (GLP1) (3).

In bile acid metabolism, gut microbiota also play an important role. Synthesized in the liver from cholesterol and secreted into the duodenum, bile acids traverse onto the distal parts of the gut and undergo structural remodelling by the gut microbiota in the colon. Deconjugation and dehydroxylation of primary bile acids result in the formation of secondary bile acids, which are not only detergents for lipid solubilisation, but also potent signalling molecules involved in various metabolic processes in intestines and liver (3,33,35). Bile acids and their intestinal transformation will be discussed in particular later in this work.

Maintenance and Protection of the mucosal layer

Maintenance of the structural integrity of the mucosal layer is also an essential function of gut microbiota. SCFAs are able to regulate expression of cell-to-cell adhesions like tight junctions. Moreover, certain bacterial taxa indirectly exert influence on the expression of desmosomes or on cytokines involved in the apoptotic process of enterocytes. Also, SCFAs are known to be potent stimulators of epithelial cell growth and differentiation as well as inducers of expression of mucin genes in intestinal goblet cells, further fortifying the mucosal barrier (4,30,35,36).

Protection of the mucosal layer is ensured by dense growth of the bacterial lawn which inhibits the attachment and reproduction of invading pathogens and prevents infiltration of the mucosal layer. There is evidence that germ-free mice are more susceptible to intestinal infections than their colonized counterparts, suggesting this “barrier effect” to be crucial on host physiology and health. Multiple mechanisms help protecting the mucosal layer. Physiologic adherent bacteria occupy attachment sites in the epithelial brush border to prevent growth of pathogenic microbes. There is a steady-state in supply and demand of nutrition, maintained by the host to prevent an overabundance. In addition to that, some bacterial taxa produce bacteriocins to curb the growth of other potentially pathogenic taxa (4).

Immunomodulation

Recent evidence confirms the symbiotic relationship between host and gut microbiota to be essential for the development of both, the innate and the adaptive immune system. The total surface of the gut's mucosal layer is roughly 200m^2 and unsurprisingly, as these tissues have immediate contact with the host's direct environment, they contain a multitude of immunocompetent cells. Analysing the mucosal layer of germ-free mice, the impact of gut microbiota on immune competence becomes evident: These mice have an impaired mucosal immune system. They have fewer lymphoid cells, smaller mucosal lymphoid follicles, fewer circulating IgA secreting plasma cells, ergo lower concentrations of immunoglobulins, and depleted populations of submucosal T cells. However, when exposed to microbial components in the intestinal lumen, lymphoid cell numbers increase and follicles grow, suggesting microbial colonization to be a driving force for the development of the immune system (4,6,37).

Both, bacterial metabolites and bacterial antigens respectively, trigger immune responses. SCFAs induce hematopoietic and non-hematopoietic cell lines via activation of GPCRs and can inhibit histone deacetylases (HDACs). Inhibition of HDACs has an anti-inflammatory effect by downregulating pro-inflammatory factors like tumour necrosis factor (TNF) or nuclear factor-κB (NF-κB). Additionally, the expansion of peripheral regulatory T cells (T_{reg}) is promoted via inhibition of HDACs by SCFAs. Moreover, certain bacterial taxa itself are potent inductors of T_{reg} or T-helper cells (T_H) and anti-inflammatory cytokines (e.g. IL-10). These processes result in an ongoing low level immune activation which keeps pathogenic organisms at bay (36,38).

Furthermore, microbial antigens have the potential to activate the innate immune system via toll-like-receptors (TLRs). These receptors are so-called pattern-recognition receptors (PRRs) that are sensible to preserved microbial antigens (pathogen associated molecular patterns – PAMPs), such as lipopolysaccharide (LPS) or flagellin. In host physiology, they play a crucial role in the detection of microbial invasion, thereby maintaining intestinal homeostasis. Interestingly, microbial products like LPS are also expressed by non-pathogenic commensals. However, at the present time it is not clear whether or how physiologic colonization is spared from the host's immunologic response. Activation of TLRs leads to multiple processes involved in the homeostasis of the mucosal layer, e.g. repair of

damaged intestinal epithelium through a MyD88-dependent process, increased proliferation of intestinal epithelial cells or generation of antimicrobial proteins (37,39).
The described processes indicate the significance of microbial influences on the development and homeostasis of the adaptive and innate immune system.

1.1.4. Pathophysiologic implications

As the characteristics of the gut microbiome in states of health and physiology are investigated thoroughly and the understanding advances, it has become clear that states of diseases in turn, are associated with a disruption of these normal characteristics and might manifest themselves among other things in a so-called state of “dysbiosis”. Defined by PubMed as “Changes in quantitative and qualitative composition of Microbiota. The changes may lead to altered host microbial interaction or homeostatic imbalance that can contribute to a disease state often with inflammation.”
Remarkably, many systemic diseases such as obesity, metabolic syndrome, autoimmune diseases and atopy, but also of course local gastrointestinal diseases like Crohn’s disease (CD) or Ulcerative Colitis (UC) are reflected in altered composition of our gut commensals. Intestinal bacterial species and their metabolites interact with the host’s organs and cells and may either trigger aberrant processes or maintain the physiologic status. To the present time though, researchers still face the question, if dysbiosis is the cause and if so, to what extent, or the consequence of states of disease (40).

Obesity and metabolic syndrome

The pandemic spreading of obesity and metabolic syndrome may be one of the biggest challenges in modern day medicine. Recent studies in both, mice and human, linked obesity to a deviation of the two prevalent phyla in the gut. Phylum *Bacteroidetes* was suppressed, whereas phylum *Firmicutes* was augmented in obese individuals (41–43). In mice, 7-week administration of subtherapeutic doses of antibiotics, led to similar significant shifts in gut microbiota composition with a predominance of *Firmicutes* over *Bacteroidetes*. Intriguingly, antibiotic-treated mice had higher percentages of body fat, further providing evidence for the linkage between biodiversity in the gut and obesity (44).

Opposite results came from Schwiertz et al., who were also investigating the *Bacteroidetes/Firmicutes* ratio in relation to obesity. In a study with a total of 98 lean, overweight and obese individuals, they found phylum *Bacteroidetes* to outweigh phylum *Firmicutes* in overweight and obese individuals (45). Another study examining stool samples from obese and lean individuals, also contradicted the results of Ley et al. The authors could not find any evidence at all that the relative proportions of *Bacteroidetes* and *Firmicutes* correlate in some way with the host's weight (46).

However, the close linkage between the host's weight or weight gain and his microbiota is undeniable. Germ-free mice, when fed a western-style diet high in fats and sugars, do not gain weight significantly as opposed to their colonized counterparts having the same diet (47). Turnbaugh et al. showed in an elegant study pointing in the same direction that genetically obese mice harbour a microbiome, more efficient in extracting energy from the diet, than the microbiome of their lean littermates. Astonishingly, germ-free mice, when colonized with the microbiota of these obese individuals, gained significantly more weight and body fat, than GF-mice colonized with the microbiota of the lean individuals (48). Similar results were again provided by Bäckhed et al., who treated GF-mice with gut microbiota of conventionally raised mice. Within 14 days, the animals showed a 60% increase in total body fat and a decreased sensitivity to insulin, despite consuming around 25% less of standard chow diet (49).

A study, which included 292 non-obese and obese Danish individuals was able to identify two groups within the population. One group showed high intestinal bacterial richness (i.e. high gene count), as opposed to the other group with low bacterial richness (i.e. low gene count). The latter group (23% of the collective), displayed significantly pronounced adiposity, decreased insulin sensitivity, dyslipidaemia and an imbalance of pro- and anti-inflammatory bacterial taxa in the gut, promoting continuous low-grad inflammation. These findings suggest a linkage between depleted bacterial diversity and a metabolic syndrome-associated phenotype (50).

In conclusion, it seems reasonable to attribute a pivotal role to the entirety of gut microbiota in obesity and weight gain.

Diabetes

Diabetes is strongly associated with obesity and metabolic syndrome. It is therefore hardly surprising that individuals who suffer from diabetes also show an altered microbiota profile when compared to healthy controls. In a recent study including 36 male individuals of which 18 were diagnosed with type 2 diabetes, the authors witnessed significant shifts in microbial proportions. In the diabetic group, levels of phylum *Firmicutes* (here within especially levels of class *Clostridia*), were significantly reduced. Interestingly, the level of class *Betaproteobacteria* belonging to phylum *Proteobacteria* (which is only represented to a small extent in the healthy gut), was significantly increased in the diabetic group compared to the non-diabetic group. Notably, an expansion of *Proteobacteria* might be associated with dysbiosis and chronic intestinal inflammation (51,52).

A Danish study with 277 subjects showed a positive correlation between gut microbiota derived serum branched chain amino acid levels (BCAAs) and insulin resistance. The microbiome of insulin resistant individuals showed a disequilibrium in biosynthesis and elimination of BCAAs mainly caused by two species, *Prevotella copri* and *Bacteroides vulgatus* respectively. Moreover, the authors demonstrated that *Prevotella copri* in mice led to an induction of insulin resistance and worsened glucose intolerance (53).

Karlsson et al. shotgun-sequenced microbiomes of 145 European women with normal, impaired and diabetic glucose metabolism. The authors observed significant compositional alterations in the different microbiomes. Based on these observations, a mathematical model was established, which was able to accurately identify “diabetic metabolisms” among individuals with insulin resistance (54).

These studies suggest the important role of gut microbiota in the context of diabetes and may also provide potent new approaches in the treatment of this disease.

Inflammatory bowel disease

Further important challenges of modern-day medicine are inflammatory bowel diseases (IBDs) such as Crohn’s disease (CD) or ulcerative colitis (UC). The aetiology of these diseases is not yet fully understood, but it is believed that a complex combination of environmental factors, genetic predispositions and

bacterial influences eventually leads to the onset of the disease and results in ongoing activation of immune mechanisms targeting luminal antigens and thus harming intestinal tissues. Although determining differences in microbiota composition in IBDs is presumably biased by different techniques and sites of specimen collection across various studies, dysbiosis seems to be tightly associated with onset and development of IBDs. Both, increase and depletion of several bacterial taxa have been observed. Still, it remains unclear if alteration in gut microbiota is causally responsible for IBDs or just simple consequence (55,56). In a study with 57 IBD patients compared to 46 healthy controls, Ott et al. found significantly reduced bacterial diversity in both, CD and UC patients. CD patient's diversity was reduced by 50% and UC patient's diversity by 70% compared with controls. The authors ascribed this loss of diversity to a depletion of anaerobic bacteria such as phyla *Bacteroides*, *Eubacterium* and *Lactobacillus* (57). Similar evidence is provided by Takahashi et al., who analysed stool samples from 10 CD patients in remission and compared them to 10 healthy subjects. The abundance of the genera *Bacteroides*, *Eubacterium*, *Faecalibacterium* and *Ruminococcus* was significantly lower in CD patients compared to the healthy controls. Furthermore, on the species level there was a marked reduction of butyrate-producing bacterial species like *Faecalibacterium prausnitzii* or *Clostridium lavalense* (58). Another metagenomic approach from Manichanh et al. showed a significant decrease in diversity within the phylum *Firmicutes* among CD patients when compared to healthy controls. In the control group, 43 distinct bacterial taxa were identified, whereas in CD patients only 13 could be detected (59).
Another study conducted with twin pairs discordant in UC also revealed dysbiosis in the UC group. Affected individuals presented lower bacterial diversity but an increment in *Actinobacteria* and *Proteobacteria*. Furthermore, the UC group had atypical aerobes and was lacking potentially protective bacterial species (60).

Cardiovascular disease

Several studies examined the relationship between gut microbes and cardio-vascular diseases like atherosclerosis, myocardial infarction and stroke. It is proven that specific metabolic products of dietary phosphatidylcholine or L-carnitine, which arise through a gut microbiota dependent manner, promote atherogenesis and subsequently promote cardiovascular events respectively.

Among these metabolic products are trimethylamine N-oxide (TMAO) and choline. In mice with preserved microbial flora (i.e. no antibiotics), administration of ten times the daily choline amount resulted in significantly increased aortic atherosclerosis, whereas in mice, treated with broad-spectrum antibiotics simultaneously to deplete intestinal flora, no such findings could be observed. This is consistent with data from Tang et al., who measured reversible decreased plasma levels of TMAO after antibiotic treatment. Furthermore, administration of a high choline diet in mice with conserved microbial flora led to significantly increased development of foam cells and upregulation of scavenger receptors, but not in mice treated with antibiotics. These findings suggest gut microbes to be central players in the development of phosphatidylcholine mediated atherosclerosis and cardiovascular disease (61–63).

1.2. Physiology of bile acids

1.2.1. Characteristics of bile acids

Bile consists of bile acids (BAs), phosphatidylcholine (PC) and cholesterol. Bile acids make up approximately two thirds of the human bile and are therefore the major component in the bile liquid (64).

Bile acids are the end products of cholesterol metabolism in the liver and consist of 24 carbon atoms fused together. Preserving the steroid skeleton, the molecule has three 6-carbon rings (A, B, C) and one 5-carbon ring (D). Cholesterol is modified enzymatically several times, before the final bile acid molecule arises. Common modifications are hydroxylation at positions 3, 7 and/or 12 of the steroid nucleus and the addition of side chains linked to the head of it. As a result of these enzymatic modifications, the junction between rings A and B is consistently *cis*-configured, i.e. the hydrogen at C5 position is in β-configuration. However, the hydroxyl groups at positions 3, 7 and 12 are commonly in α–configuration. For this reason, human bile acids assume a curved shape, with the α–side being concave and hydrophilic and the β–side being convex and hydrophobic. This accounts for the highly amphipathic properties of bile acids and the formation of small micelles

in aqueous solutions. Features which are crucial for their biological function as intestinal detergents (65). Details are displayed in figure 1.

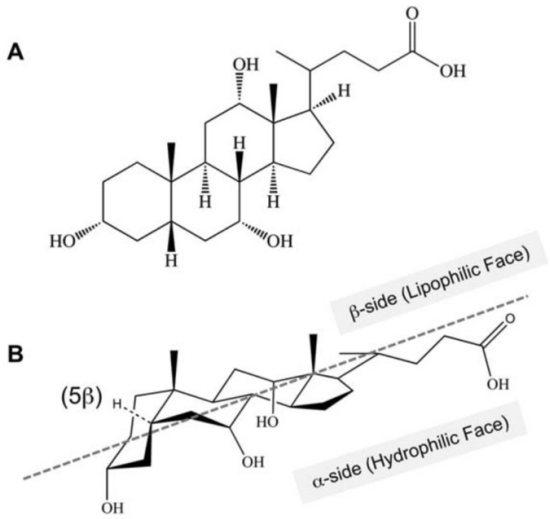

Figure 1. (A) Structure of the primary bile acid cholic acid. (B) Spatial configuration of the cholic acid molecule which underlines its amphipathic characteristic. Modified after (66)*.*

1.2.2. Functions

Bile acids have long been viewed as molecules which are mainly involved in lipid digestion and absorption. In light of recent findings, this conception needs to be reconsidered. Of course, bile acids are excellent emulsifiers of all kinds of lipids, be it dietary fats or vitamins, and we do rely on them for solubilizing and absorbing fats efficiently. The composition of bile is characterised by the simultaneous presence of bile acids, phosphatidylcholine (PC) and cholesterol. These compounds form mixed micelles while stored in the gall bladder. Upon release into the small intestine, PC and cholesterol are eliminated from the conglomerate. The remaining bile acids, due to their amphipathic nature, now act as detergents and break down fat clots into smaller pieces, making them more available to intestinal lipases (67,68).

Bile acids also facilitate transportation of dietary lipids in the aqueous intestinal milieu by solubilizing them in the form of micelles, a process called micellar solubilisation. This increases the overall concentration of lipids in the gut and accelerates enterocytic uptake by a factor of ~100. However, to efficiently

solubilize fat molecules, bile acid abundancy needs to exceed a certain threshold, called the critical micellar concentration (68).

Another important function of bile acids and bile is the excretion of substances that cannot be metabolized or excreted via the urinary tract. This mechanism is called biliary secretion and accounts for the elimination of bile acids (as end products of cholesterol metabolism) and cholesterol itself, as well as bilirubin (end product of heme metabolism) and various metals and xenobiotics and their metabolites (68).

Furthermore, high concentrations of bile acids in hepatic bile canaliculi help generating bile itself. In an ATP-dependent mechanism, bile acids are secreted into the canalicular lumen against high concentration, creating an osmotic pressure. Along this gradient, water and ions are carried into the bile canaliculus, resulting in the formation of bile liquid (65,68). On its way out of the canaliculi, towards the bile ductuli and the major bile duct, the liquid becomes enriched with PC molecules, which protrude from the canalicular membrane towards the lumen, gradually forming mixed micelles. This mechanism also promotes biliary lipid secretion (68).

Bile acids also act as antimicrobial agents and are capable of inflicting severe damage to live cells mainly by altering cell membrane integrity (67).

A study in rat hepatocytes, conducted by Pazzi et al., yielded especially hydrophobic bile acids (e.g. chenodeoxycholic acid – CDCA, deoxycholic acid – DCA) to be toxic on biological membranes. Even in low concentrations, significant leakage of intracellular components was observed. Moreover, electron microscopy of the affected hepatocytes revealed an ultrastructure similar to hepatocytes lysed with detergent Triton X-100 for comparison. Hydrophilic BAs (e.g. cholic acid – CA, ursodeoxycholic acid – UDCA) did not show any signs of toxicity on biological membranes in this study. UDCA in fact, alleviated the toxic effects of DCA (69). In consistency with this, Shekels, Beste and Ho stated that membrane damaging potential depends on hydrophobicity of the bile acids (70).

The severity of membrane damage depends on certain factors, one of which is the bile acid concentration. Biological membranes and connected proteins are subject to instant degradation in high concentrations of bile acids. By contrast, low concentrations of bile acids still inflict damage to biological membranes in humbler ways, like alternating membrane-bound proteins or influencing membrane permeability. Another factor is the intrinsic bile acid characteristic. Conjugated bile

acids (see chapter 1.2.3.), as against unconjugated bile acids, are not capable of penetrating biological membranes in physiological pH milieus due to their ionisation. Unconjugated bile acids though, are able to cross biological membranes in a passive fashion, albeit this feature depends on hydroxylation status of the bile acids: dihydroxy bile acids are more penetrative than trihydroxy bile acids. Lastly and quite conceivable, membrane structure and composition are of importance, regarding the extent of membrane damage. Membrane charge, membrane-bound proteins, sugars and lipids, hydrophobicity and fluidity may determine bile acid associated membrane damage (67).

As bile acids have been a subject of intense research in the last couple of years, it has become clear that they have other specific functions besides the mentioned: Bile acids are signalling molecules with endocrine activity. They play an essential role in diverse metabolic pathways, including lipid and glucose metabolism, energy homeostasis and hepatic gene expression. These effects are mostly mediated by the newly discovered nuclear hormone farnesoid x receptor (FXR) and the membrane-bound G-protein-coupled receptor (GPCR) GPBAR1 also known as TGR5 and will be reviewed more closely in chapter 1.2.6. (71).

1.2.3. Synthesis

The total amount of circulating bile acids in human individuals varies between 3 – 5g. They underlie a highly effective reuptake mechanism to save resources known as enterohepatic circulation. However, 5 – 10% of circulating bile acids are lost due to intestinal elimination. Consequentially, the daily re-synthesis of bile acids is around 200 – 500mg.

Bile acid synthesis requires the precisely orchestrated activity of 17 hepatic enzymes located in the endoplasmic reticulum, cytosol, mitochondria and peroxisomes. Two major pathways account for the synthesis of bile acids. The neutral pathway, i.e. the classic pathway, is in charge of around 90% of *de novo* BA synthesis. The alternative pathway, also known as the acidic pathway because of the formation of acidic metabolites during the process, contributes only a small amount, but might be important in states of liver disease and in neonates (72,73).

These two metabolic routes differ by the sequence of the reactions taking place. The endpoint of both pathways, is the formation of the two primary bile acids in human individuals: Cholic acid (CA) and chenodeoxycholic acid (CDCA). The

classic pathway is characterized by modifications of the steroid skeleton of cholesterol before the oxidation of its side-chain. This order is reversed in the alternative pathway, where cleavage of the side-chain happens before the reconstruction of the steroid nucleus (65,66).

In rodents, the primary bile acids also include CA and CDCA, but additionally, muricholic acids (MCA) are found. Muricholic acids are formed by 6α/β-hydroxylation of CDCA.

The classic pathway is initiated by an enzyme called cholesterol 7-alpha hydroxylase (CYP7A1). Belonging to the group of cytochrome-P450 enzymes, it is the key-enzyme and rate-limiting step of the BA synthesis and can only be found in the liver. At the endoplasmic reticulum, it starts the process by hydroxylation of cholesterol at C7, resulting in 7α-hydroxycholesterol which is then converted to 7α-hydroxy-4 cholesten-3-one by an enzyme called 3β-hydroxy-Δ5-C27-steroid dehydrogenase/isomerase (HSD3B7). The formation of CA requires an intermediate step. Sterol 12α-hydroxylase (CYP8B1) hydroxylates the former 7α-hydroxy-4-cholesten-3-one at the C12 position yielding 7α,12α-dihydroxy-4 cholesten-3-one.

Following several other steps, such as reduction of the $\Delta^{5,6}$ double-bond to form a *cis*-configured A/B ring connection, both 7α-hydroxy-4 cholesten-3-one and 7α,12α-dihydroxy-4 cholesten-3-one are then processed similarly, which ultimately results in the formation of CDCA or CA respectively (65,66,74).

The alternative or acidic pathway is initiated by an enzyme called sterol 27-hydroxylase (CYP27A1). It also belongs to the family of cytochrome-P450 enzymes and initially oxidizes cholesterol to 27-hydroxycholesterol. The alternative pathway mainly accounts for the formation of CDCA (65).

As a last step in synthesis, the side-chain carboxylic acid gets conjugated with either glycine or taurine by a peroxisomal enzyme called bile acid CoA: amino acid N-acyltransferase (BAAT). In men, glycine-conjugated bile acids prevail. The ratio is circa 3:1 but does depend on dietary habits. In mice, mostly taurine (>95%) conjugates occur (65,72,75,76).

Conjugation of bile acids increases hydrophilicity and almost fully ionizes these molecules, thus improving solubility in intestinal pH conditions during digestion. Besides that, conjugated bile acids are more resistant to enzymatic breakdown

and passive absorption in the intestines. Upon fasting, conjugated bile acids are stored at high concentrations in the gall bladder (72,74).

1.2.4. Enterohepatic circulation

Bile acids underlie highly effective reuptake and transportation systems. These mechanisms are summarized as the enterohepatic circulation and ensure a constant flow of bile acids between intestines and liver. Most of the released bile acids are reabsorbed in the distal ileum via active transporters. This applies for approximately 95% of circulating BAs. The remaining portion traverses on to the colon and undergoes bacterial modification (see chapter 1.2.5.) and passive absorption. Some of the BAs will eventually slip through absorption mechanisms and be excreted in the faeces. This amount is compensated by *de novo* synthesis (72).

Active uptake of BAs into ileocytes is performed by an apical transporter called apical sodium-dependent bile acid transporter (ASBT). This transporter is able to cotransport one bile acid molecule along with two sodium ions. Though all types of BAs are taken up by ASBT, conjugated forms are preferred over unconjugated forms. This is consistent with the fact that deconjugation happens mainly in the colon. Furthermore, ASBT takes up dihydroxy BAs (CDCA, DCA) more efficiently than trihydroxy BAs (CA). Once taken up, BAs are complexed to intracellular proteins called ileal bile acid-binding proteins (IBABPs). IBABPs are believed to ensure and facilitate transportation to the basolateral membrane within the cell. Upon reaching the basolateral membrane, BAs are led out of the cell into portal circulation via the heterodimeric Na+ independent organic solute transporter OSTα/β (77,78).

Via the portal blood stream, bile acids, most of them complexed to albumin, reach hepatic tissues. Through the sinusoidal epithelium with its large pores (fenestrae), the bile acids traverse into the space of Disse and therefore reach the basolateral (sinusoidal) surface of hepatocytes. Bile acids now dissociate from albumin and are taken up into the hepatocytes via different transport mechanisms. First pass clearance of conjugated BAs is between 75% to 90% (78–80).

Out of several uptake mechanisms, probably the most important one is the one conducted by the Na+-taurocholate cotransporting polypeptide (NTCP). This transporter shares commonalities with the ileal ASBT and is expressed in the liver

and, as recently discovered, in pancreatic acinar cells. Localized at the basolateral membrane of hepatocytes, it mediates the uptake of BAs against a ten-fold concentration gradient. NTCP has a higher affinity to conjugated BAs, but also unconjugated BAs are transported. Similar to ileal ASBT, transport happens in a Na+ dependent fashion, with two sodium ions being co-transported simultaneously. Additionally, a basolateral hepatic Na+-K+ ATPase maintains an out-to-in sodium gradient, alongside which BAs are transported (78–81).

Also, Na+ independent basolateral BA uptake occurs in the liver. Though especially important for the uptake of unconjugated BAs (as opposed to NTCP which mainly carries conjugated BAs), this pathway plays only a minor role in total uptake quantities and is accomplished by transporters belonging to the group of organic anion cotransporting polypeptides (OATPs). These transporters have high affinity to unconjugated bile salts (yet lower than NTCPs) and also other amphipathic compounds (e.g. bilirubin, organic anions and cations, neutral steroids and small peptides) are substrates for them. Hence, they represent an important transport mechanism for a variety of ligands. Physiological function of OATPs involves exchange with an intracellular anion (e.g. Glutathione or HCO3-) (78,79,82).

1.2.5. Biotransformation

Upon food intake (i.e. fat intake), the gall bladder contracts as a reaction to cholecystokinin and releases bile into the duodenum via the common bile duct. Bile acids are now facing intestinal bacterial colonies and are subject to interaction with them. Bacterial modifications take place in both, the small and the large intestine. However, the major part of these processes takes place in the colon and affects only a minor part of the secreted bile acids (5%) as the enterohepatic circulation is able to reuptake 95% of BAs in the distal small intestine. The major modifications on bile acids executed by colonic bacteria are deconjugation and 7α-dehydroxylation. Further modifications are 7β-dehydroxylation and oxidation and epimerisation of the hydroxyl groups at positions C3, C7 and C12, which results in the formation of *iso*-bile acids (β configuration of the hydroxyl groups). Conclusively, the biliary and faecal bile acid pool differ in terms of composition (65,83,84). Details are displayed in figure 2.

Figure 2. Biliary and faecal bile acid pools. Bacterial transformations are deconjugation, 7α/β-dehydroxylation, oxidation and epimerisation. Modified after (83).

Deconjugation

An obligatory precondition, before further biotransformation can take place, is the enzymatic hydrolysis of the C-24 N-acyl amide bond between bile acids and their glycine/taurine conjugates called deconjugation. A group of bacterial enzymes called bile salt hydrolases (BSHs) catalyses this pivotal step, making bile acids available to a range of other bacterial enzymes. BSHs are widely distributed among gut microbiota, though predominantly gram positive species express the enzyme: *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Clostridium* and *Bacteroides spp.* (85,86)

The biological benefit of bacterial BSHs and bile salt deconjugation remains yet to be determined more precisely. Theories include provision of sources of carbon, nitrogen or sulphur for bacterial species (87), energy sources in form of the amino acids glycine and taurine (88) and detoxification of otherwise highly toxic bile salts (89).

7α-dehydroxylation

Quantitatively the most important bacterial bile acid modification is 7α-dehydroxylation. This step is accountable for the formation of the two main secondary bile acids in men DCA (3α,12α) and LCA (3α). The former arises from CA (3α,7α,12α) and the latter from CDCA (3α,7α) respectively. In human faeces, secondary bile acids predominate. Interestingly only a minority of colonic bacteria, all of which belong to genus *Clostridium*, is able to perform 7α-dehydroxylation (83). In rodents, 7α-dehydroxylation yields hyodeoxycholic acid (6α-hydroxyl group) or murideoxycholic acid (6β-hydroxyl group) (90,91).

Oxidation and epimerisation

Oxidation and epimerisation of the bile salt molecule are further modifications carried out by colonic bacteria. Bacterial enzymes called hydroxysteroid dehydrogenases (HSDHs) are in charge of these reactions, performed at positions C3, C7 and C12. Oxidation goes hand in hand with reduction and is characterised by insertion or extraction of H_2 at the positions mentioned. Epimerisation is a reversible conversion of stereochemistry from the α to the β configuration of the bile acid molecule and *vice versa*. Various bacteria may have different kinds of HSDHs, e.g. one having 7α-HSDH and the other 7β-HSDH. Both types are necessary for epimerisation. 7α epimerisation is responsible for the formation of UDCA from CDCA in human faecal bile acid pools. In rodents, 6β-epimerisation results in the formation of ω-muricholic acid (ωMCA) (65,83,90,92).

1.2.6. Signalling and bile acid targets

Bile acids are ligands for three nuclear receptors which are abundantly expressed in liver and intestinal tissues: The farnesoid X receptor (FXR), the pregnane X receptor (PXR) and the vitamin D receptor (VDR). Additionally, secondary bile acids are ligands for the G-protein coupled receptor GPBAR1 also known as TGR5.

First discovered in 1995, the nuclear farnesoid X receptor FXR and its effects have become well characterized in recent years. FXR is the major intracellular bile acid sensor and binds both, free and unconjugated bile acids with different degrees of affinity. CDCA is the most potent ligand, followed by DCA, LCA and CA. Interestingly UDCA does not activate FXR. FXR is further activated by several other compounds not related to bile acid metabolism, such as cholesterol and its derivatives. The receptor is ubiquitously expressed in the liver, the intestines, the kidney and the adrenal cortex. By regulating various key genes involved in the BA cycle, the receptor plays an essential role in bile acid homeostasis and metabolism. Moreover, it is involved in lipid and glucose metabolism. PXR is relevant in detoxification of bile acids and xenobiotics by inducing the CYP3A group of cytochrome-P450 enzymes. VDR plays a role in vitamin D and calcium pathways and especially regulates LCA metabolism. PXR and VDR are only activated by LCA (72,93–96).

Bile acid synthesis and transport underlie a negative feedback mechanism with FXR being the central regulating element. Activation of hepatic FXR leads to inhibition of the cytochrome-P450 enzymes CYP7A1, CYP8B1 and CYP27A1, which are involved in bile acid synthesis. Via two different FXR dependent pathways, transcription of relevant genes is throttled, and an excess of bile acids is prevented. Activation of FXR is followed by expression of the transcriptional repressor small heterodimer partner (SHP), which in turn negatively influences the activity of the transcription factors liver related homologue-1 (LRH-1) and hepatocyte nuclear factor-4α (HFN-4α). These factors are usually ligands to bile acid responsive elements (BAREs) located in the promoter regions of the Cyp7a1 and Cyp8b1 genes. Repression of these factors leads to repression of bile acid synthesis (65,72,96).

Another way, how bile acids modulate their own synthesis, is via the FXR/FGF19/FGFR4 pathway. Intestinal-derived fibroblast growth factor 19 (FGF19), or in mice, the corresponding orthologue (FGF15), are induced upon activation of FXR. In an endocrine manner, FGF15/19 binds to its hepatic receptor FGFR4 and subsequently downregulates Cyp7a1 mRNA expression via a kinase-signalling pathway. Cyp8b1 expression is not affected by this pathway (72,93,95,97).

Not only does FXR activation inhibit BA synthesizing enzymes, but also, it has a direct impact on BA transport mechanisms. In the liver, FXR activation enhances bile acid conjugation by stimulating the responsible enzyme bile acid CoA:amino acid N-acetyltransferase (BAAT). Also, FXR induces the apical bile salt export pump (BSEP), which excretes BAs into the bile canaliculus and thereby increases bile flow. Moreover, the basolateral BA transporter NTCP is downregulated by FXR. In the intestines, FXR activation leads to suppression of the luminal transporter ASBT, which is the major enterocytic BA carrier. Furthermore, subsequent members of the BA transport sequence are modulated. The expression of the ileal bile acid binding protein (IBABP) is increased, as well as the expression of the basolateral organic solute transporter (OSTα/β). In addition, the intestinal derived fibroblast growth factor (FGF15/19) is induced, which in turn decreases BA synthesis via the FXR/FGF19/FGFR4 pathway as described above. FXR induced stimulation of BA transport systems is therefore necessary to ameliorate the toxic effects of BA in the cells (95,96,98).

2. Aim of the study

The aim of the present study is to investigate the crosstalk and interactions between gut microbiota and bile acids in an *in vitro* experimental setting. We aimed to evaluate the impact of different bile acids on the growth of mouse gut microbiota in different growth media. Moreover, we investigated, if the cultured gut microbiota are able to biotransform the supplemented bile acids into their unconjugated and dehydroxylated derivatives and if bacterial metabolic products further arise from this bile acid microbiota interaction that in turn affect hepatic expression of genes involved in bile acid homeostasis.

We hypothesized that:

- Different bile acids have different impact on gut microbiota growth and conjugation status of the bile acids differently impacts on bacterial growth and host metabolism.
- Gut microbiota cultured in bile acid media are capable of biotransforming bile acids *in vitro.*
- Gut microbiota cultured in bile acid media produce metabolites which impact on hepatic expression of genes of the bile acid metabolism.

3. Materials and methods

3.1. Animal studies

Female and male 9 to 11-week-old C57BL6 mice from the on-site animal facility of the Center for Medical Research Graz were used for all experiments. Animals had access to standard chow diet (Ssniff M-Z) and water ad libitum. They were held at a 12-hour day/night cycle at a room temperature of 24°C. All experiments were approved by the local animal care and use committees in accordance with the National Academy of Sciences.

3.1.1. Bile duct ligation

C57BL6 mice were bile duct ligated as described previously for seven days. The procedure was the same for sham-operated animals, except for the ligation (99).

3.1.2. Sacrificing

Animals were sacrificed at 9:00 a.m. Sevofluran® was used for deep terminal anaesthesia and animals were put to death by cervical dislocation.
The animal was then fixed to the ground in sterile conditions under a laminar flow hood and the abdomen was disinfected. Subsequently, median laparotomy from the sternum to the bladder was performed, laying open the viscera. These were then removed from the body. Special attention was paid to not contaminate the organs with fur or skin parts of the animal. After incising the caecum with a sterile scalpel, the content was removed with a small spatula and stored in a 1.5 ml Eppendorf® tube at -80°C for further use.

3.2. Bacterial cultures

Caecal contents were dispersed in NaCl according to stool weights under sterile conditions in a laminar flow hood and vortexed to generate a homogenous suspension. The suspension was then centrifuged at 1000 rpm^{-1} for 60 seconds to

eliminate solids and insoluble contents. The supernatant was removed and stored in a fresh 1 ml Eppendorf® tube. This tube was used to generate serial dilutions which were used for the bacterial cultures.

3.2.1. Growth media

The experiments were performed using two different culture media. Lysogeny broth (LB) medium was made by dissolving 5g/l yeast, 10g/l tryptone and 10g/l NaCl in distilled water. Gut Microbiota Medium (GIM) was made as described previously (100). For agar plates 15g/l (LB) and 12g/l (GIM) noble agar was added. Culture media were autoclaved and a layer of approximately 0.5cm was poured into 150mm diameter plates under the flame (standard lab burner) to ensure sterility. Afterwards plates and liquid media were let cooled down at room temperature. Culture media and plates were stored at 4°C until further use.

3.2.2. Bile acid stock solutions

Bile acids were purchased from Sigma-Aldrich®. The sodium salt (to ensure better solubilisation in culture media) of the following free bile acids were used: cholic acid (CA), ursodeoxycholic acid (UDCA), taurocholic acid (TCA), tauroursodeoxycholic acid (TUDCA) and cholylsarcosine (ChS), which is a bile acid analogue, resistant to bacterial deconjugation and dehydroxylation during enterohepatic cycling (101). Bile acid stock solutions with concentrations of 50mg/ml (CA, TCA, UDCA, ChS) and 25mg/ml (TUDCA) respectively were made with distilled water. Details are displayed in table 1.

bile acid (sodium salts of the bile acids were used, except for cholylsarcosine)	**molecular weight (g/mol)**	**molar concentration of stock solution**
cholic acid (CA)	430.561 g/mol	116 mM (50mg/ml)
taurocholic acid (TCA)	537.688 g/mol	93 mM (50mg/ml)
ursodeoxycholic acid (UDCA)	414.60 g/mol	120 mM (50mg/ml)
tauroursodeoxycholic acid (TUDCA)	521.689 g/mol	48 mM (25mg/ml)
cholylsarcosine (ChS)	501.64 g/mol	100 mM (50mg/ml)

Table 1. Molecular weights of the used bile acids and concentrations of the stock solutions.

3.2.3. Culturing

Bile acid stock solutions were added to the culture media or agar in a ratio of 1:10 (1:5 for TUDCA), resulting in concentrations of roughly 10mM.

Serial dilutions of the original caecal bacterial suspension were used for plating and liquid cultures depending on the current culture medium. 100µl of bacterial suspension were used for plating on agar and 50µl for inoculation of the liquid cultures. Plating was performed with a Drigalski spatula, and as well as the inoculation process, happened under the flame (standard lab burner), to avoid contamination with airborne germs. For the anaerobic experiments, plating and inoculation was done in the anaerobic chamber. Incubation of both, plates and tubes respectively, was 24 hours at 37°C and 180rpm^{-1} in aerobic conditions (room air) or anaerobic conditions (N_2 80%, CO_2 20%).

The colony forming units (CFU) were determined the next day by manual counting. Optical density of each tube at a wavelength of 600nm (OD600) was determined using a Spectramax photometer. After determining the OD600s, the tubes were centrifuged at 13000rpm^{-1} for 20 minutes. The supernatant was then removed under the flame and let trough a 0.2µm Nalgene™ syringe filter (ThermoFisher

Scientific™) to guarantee sterility. Then brought in a fresh 10ml tube, this sterile supernatant was stored in a standard -20°C freezer until used for cell cultures.

3.3. Cell Cultures and transactivation of FXR signalling

3.3.1. Cell line

For cell culture experiments and subsequent qPCR, the HepG2 cell line was used. It was first established in 1983 from a hepatoblastoma of a 15 year old Caucasian male (102). The cell line was purchased from American Type Culture Collection (ATCC).

3.3.2. Culturing

The HepG2 cell line was cultured in Dulbecco's modified eagle medium (DMEM) (Gibco®, ThermoFisher Scientific™) with an additive of 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (1000U/ml) at 37°C in a 95% O_2 / 5% CO_2 environment.

Experiments were performed in triplicates on a 12 well plate. Cells were incubated in medium containing supernatant from the liquid medium bacterial cultures treated with various BAs (dilution 1:10 in cell culture medium) or directly with BAs including CDCA as positive control for transactivation of FXR.

3.4. Gene expression analysis

3.4.1. RNA isolation

For RNA isolation, cells were lysed the next day using TRIzol reagent as described previously (103). Total RNA concentrations of each sample were measured using NanoDrop™ 2000 Spectrophotometer (ThermoFisher Scientific). Quality of the sample was determined calculating the 260/280 ratio and the 260/230 ratio (reference values were 1.9 – 2.0 and 2.0 – 2.2 respectively).

3.4.2. cDNA synthesis

Complementary DNA was generated by reverse transcription of 1.5µg RNA, 0.4µl random hexamers (Invitrogen™, ThermoFisher Scientific) and filled up to 10µl with sterile distilled H_2O. The cDNA-Mix for the first strand synthesis contained 4µl 5x cDNA synthesis buffer, 2µl 0.1M DTT, 0.3µl 10mM dNTPs, 0.5µl RNAse inhibitor, 0.5µl Superscript™ II (all Invitrogen™, ThermoFisher Scientific) and 2.7µl Aqua dest. Cycling conditions (MyCycler™ Thermal Cycler, Bio-Rad Laboratories) were 65°C for 5 minutes, 25°C for 10 minutes, 42°C for 90 minutes with 10µl cDNA-Mix being added at this step as soon as 42°C would be reached, 70°C for 15 minutes and 4°C until further use. Total reaction volume was 20µl.

3.4.3. Real-time quantitative PCR (qPCR)

cDNA samples were diluted 1:20 with Aqua dest. For the calculation of the standard curve, 3µl of each cDNA sample was pooled into a fresh tube. From this pool, standards were diluted with Aqua dest. in the following concentrations: 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160. Expression of desired genes was analysed using LightCycler® 480 SYBR Green I Master (Roche) and the LightCycler® 480 Real-Time PCR System (Roche). The primers were diluted 1:10 with Aqua dest. Reactions were performed in duplicates on a 384 well plate with a 10µl mixture containing 2µl of diluted cDNA and 8µl mastermix. The mastermix consisted of 5.25µl SYBR Green I Master, 2.65µl sterile Aqua dest., 0.35µl forward primer and 0.35µl reverse primer. Cycling conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Further steps were continuous heating from 55°C to 95°C for melting curve analysis and cooling finally down to 40°C. The LightCycler® 480 Software, Version 1.5 was used for automatically calculating the threshold cycle values.

3.5. Statistics

Statistical analysis was done using parametric *t*-test or un-parametric Wilcoxon-Mann-Whitney-Test. Data are presented as arithmetic means ± standard deviation (SD). A P-value < 0.05 was considered significant.

4. Results

4.1. Bacterial growth

In the course of examining the effects of bile acids on gut microbiota and their growth in different media, we performed several steps and established an experimental setup which allowed us to demonstrate how different and to what extent gut microbiota growth is affected under bile acid selection pressure.

4.1.1. Bacterial growth of directly plated faecal samples

One female and one male C57BL6 mouse were sacrificed. Faecal contents were diluted in 1000µl NaCl per sample, vortexed and centrifuged (1000rpm^{-1} x 60 seconds). Serial dilutions of faecal samples were made and 100µl of a 10^{-4} dilution was plated on LB agar. Only minor differences in growth between the female and the male individual were observed. CFU/ml are listed in table 2.

Mouse No.	Sex	Stool weight (mg)	CFU/ml on LB agar
1	w	255	1.25 x 10^7
2	m	270	3.86 x 10^7

Table 2. CFU/ml on LB agar of one female and one male mouse.

4.1.2. Bacterial growth in bile acid supplemented LB medium

To examine the effects of different conjugated and unconjugated bile acids on bacterial growth, LB medium supplemented with different bile acids was made as described above. One male C57BL6 mouse was sacrificed and faecal content (~220mg) was diluted in 1000µ NaCl, vortexed and centrifuged (1000rpm^{-1} x 60 seconds). 50µl of faecal suspension was inoculated in LB and BA supplemented LB (1:10). Tubes were incubated overnight at 37°C x 180rpm^{-1}. 100µl of each 10^{-5} diluted culture were consecutively plated on LB agar the next day.

Compared to control, both 12α bile acids suppressed bacterial growth. Suppression in CA was strongest, followed by suppression in TCA medium. Cholylsarcosine also suppressed bacterial growth and ranged between CA and

TCA. Only minor differences were observed in TUDCA. CFU/ml are listed in table 3. LB agar plates are displayed in figure 3.

Treatment	CFU/ml
Control	1.304×10^9
CA	2.3×10^8
TCA	7.78×10^8
TUDCA	1.288×10^9
Cholylsarcosine	6.08×10^8

Table 3. CFU/ml on LB agar. Aliquots of cultures in bile acid selection media were plated in volumes of 100µl. Cholic acid, CA; taurocholic acid, TCA; tauroursodeoxycholic acid, TUDCA.

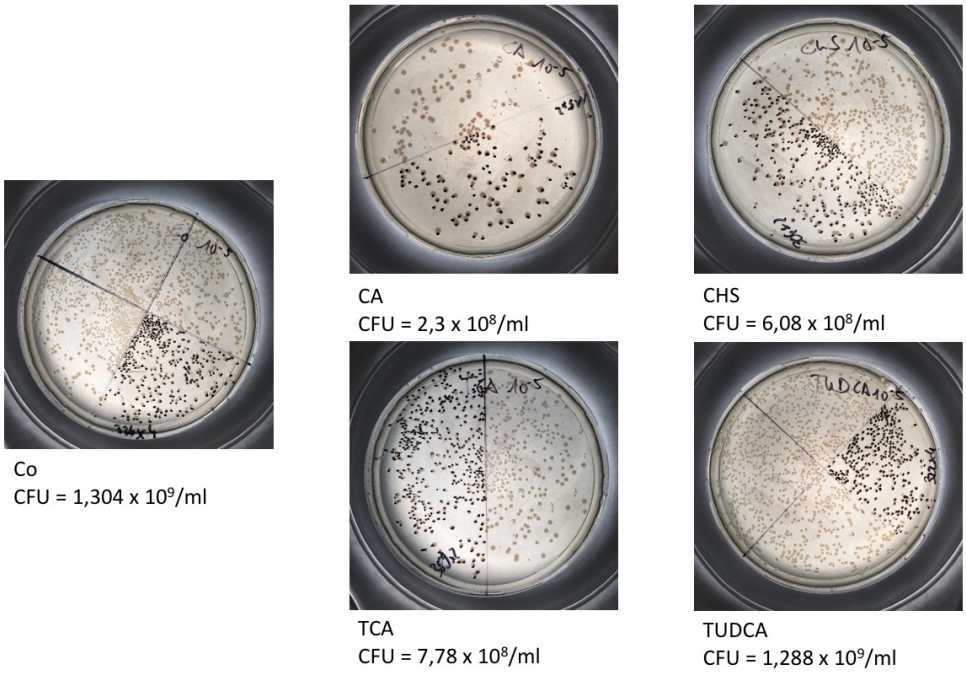

Figure 3. Colonies on LB agar plates. Aliquots of cultures in bile acid selection media were plated in volumes of 100µl. Control, Co; cholic acid, CA; taurocholic acid, TCA; tauroursodeoxycholic acid, TUDCA; cholylsarcosine, CHS.

4.1.3. Differences in haemolytic properties of the bacterial cultures

We further assessed the haemolytic behaviour of the grown colonies. A single colony from each treatment was picked and plated on a blood agar plate. No major differences in haemolytic behaviour were observed among the different treatments. The haemolysis type could not be clarified.

4.1.4. Bacterial growth over time in LB and GIM medium

Originally designed to optimize the growth of *Shigella spp.*, lysogeny broth (LB) is nowadays widely used as a standard growth medium for microbial experiments. The advantages lie especially in the cultivation of *Escherichia choli* strains, but also many kinds of other bacterial strains can be cultivated with LB (104). To examine the microbial growth of intestinal microorganisms under bile acid selection pressure, we aimed to mimic the intestinal environment more closely. Goodman et al. therefore introduced a medium, based on a modified tryptone, yeast, glucose formula, specifically designed to harbour gut microbiota, the gut microbiota intestinal medium – GIM (100).

Both culture media were supplemented with different bile acids as described above. Six 10 to 11 weeks old C57BL6 mice, three females and three males, were sacrificed and faecal content was diluted with NaCl relative to the weight to ensure equal dilution ratios (1:4). Details are listed in table 4.

Mouse No.	Sex	Stool weight (mg)	NaCl dilution volume (µl)	Inoculation volume (µl)
3	w	155	620	35
4	w	255	1020	35
5	w	235	940	35
6	m	407	1628	35
7	m	247	988	35
8	m	319	1276	35

Table 4. Details of three female and three male mice, NaCl diluation volume and inoculation volume.

After vortexing and centrifugation (1000rpm^{-1} x 60s) the samples, 35µl of each suspension were inoculated in bile acid selection LB and GIM and incubated at 37°C x 180rpm^{-1}. After 6, 12 and 24 hours (overnight), aliquots of the control tubes were taken, diluted in series and plated on LB and GIM agar plates in volumes of 100µl. Additionally, OD600 of the aliquots was measured at the stated times. Details are displayed in figure 4 and 5.

LB Medium

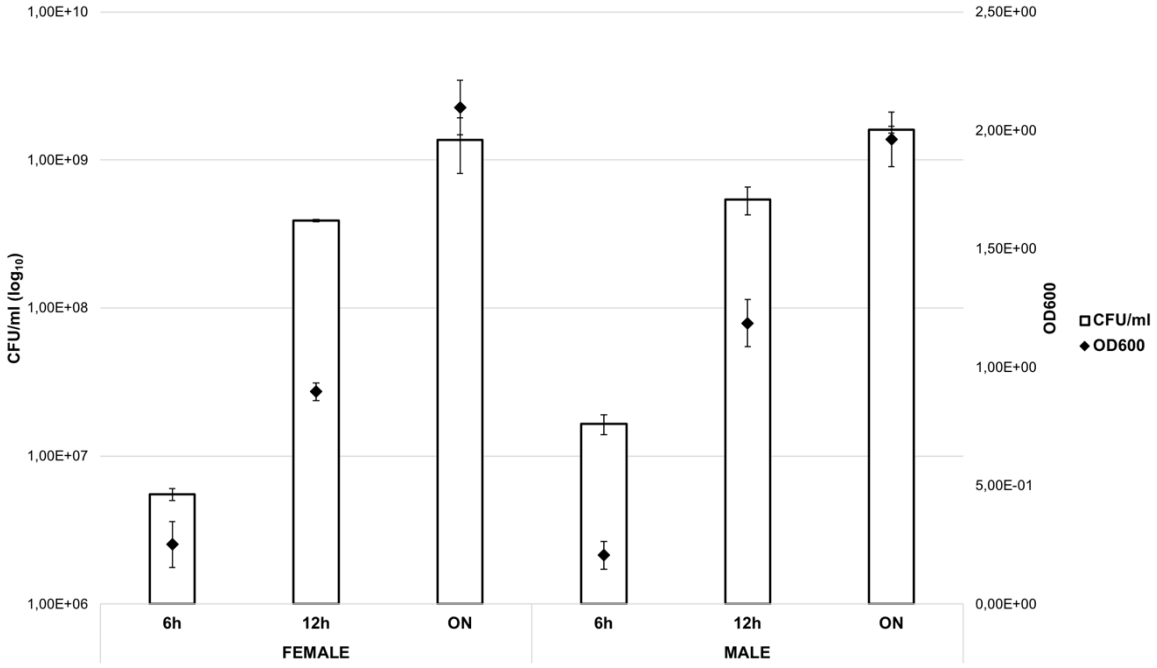

Figure 4. CFU/ml (log_{10}) and OD600 of samples from female and male mice. Aliquots of cultures in LB medium at 6, 12 and 24 (ON) hours were plated in volumes of 100µl on LB agar plates. Data are mean ± SD of 3 mice per group.

In both genders, bacterial growth after 6, 12 and 24 (ON) hours determined by CFU/ml showed the same tendencies. OD600 values corresponded well with CFU/ml. After 24 hours, the stationary phase seems to be approached. In the male group, growth after 6, 12 and 24 hours was greater than in the female group (+300%, +138,46%, +117,07%).

GI Medium

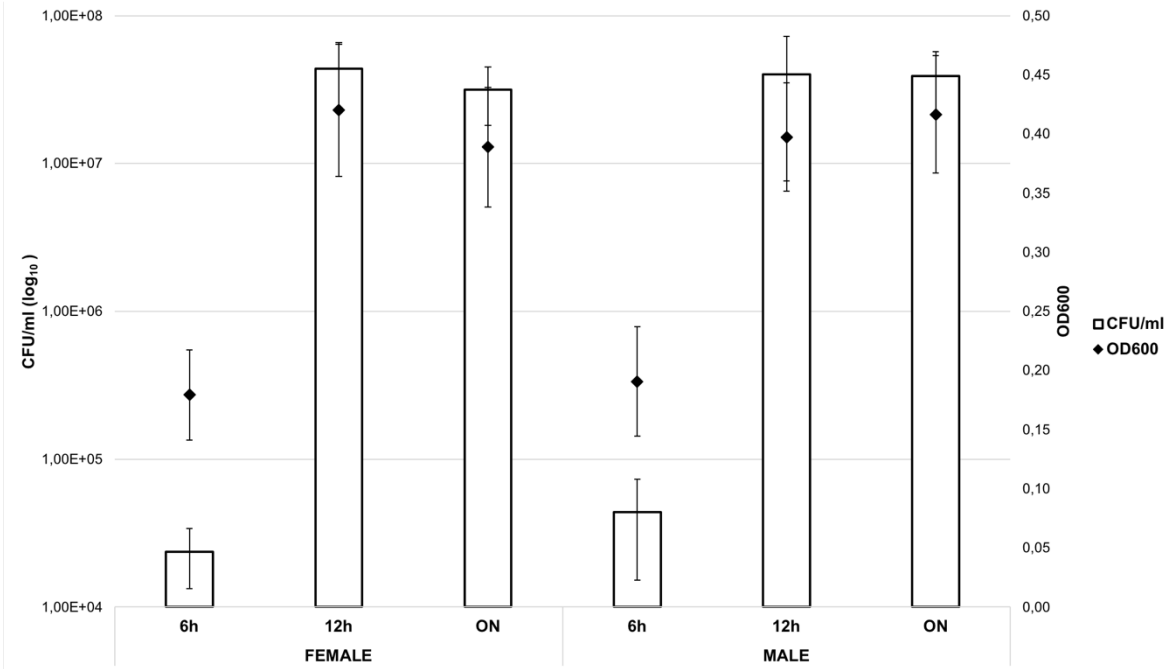

Figure 5. CFU/ml (log_{10}) and OD600 of samples from female and male mice. Aliquots of cultures in GIM medium at 6, 12 and 24 (ON) hours were plated in volumes of 100µl on GIM agar plates. Data are mean ± SD of 3 mice per group.

In both genders, bacterial growth after 6, 12 and 24 (ON) hours determined by CFU/ml showed the same tendencies. OD600 values corresponded well with CFU/ml in the female group. In the male group, OD600 values of 6 hours vs. 12 and 24 hours corresponds well with CFU/ml, but OD600 values of 12 vs. 24 hours show an opposing result, when compared to CFU/ml. Stationary phase seems to be approached already after 12 hours, determined by similar CFU/ml after 12 than after 24 hours. In the male group, growth after 6 hours was stronger than in the female group (+86,2%). Only minor differences in growth after 12 and 24 hours were observed when comparing the female vs. the male group.

Furthermore, when comparing the CFU/ml and OD600 values of LB and GIM, it is noticeable that bacterial growth is weaker in GIM. Highest values of CFU/ml in GIM are roughly two orders of magnitude less than the highest values of CFU/ml in LB. Highest OD600 values in GIM are roughly a fifth of the highest OD600 values in LB.

4.1.5. Bacterial growth in BA supplemented LB and GIM medium

Furthermore, we were aiming to evaluate the results of 4.1.2. After culturing for 24 hours, aliquots of the control and bile acid selection tubes were taken, diluted in series and plated on standard LB and GIM agar plates in volumes of 100µl. Additionally, OD600 of these aliquots was measured. Details are displayed in figure 6 and 7.

LB Medium

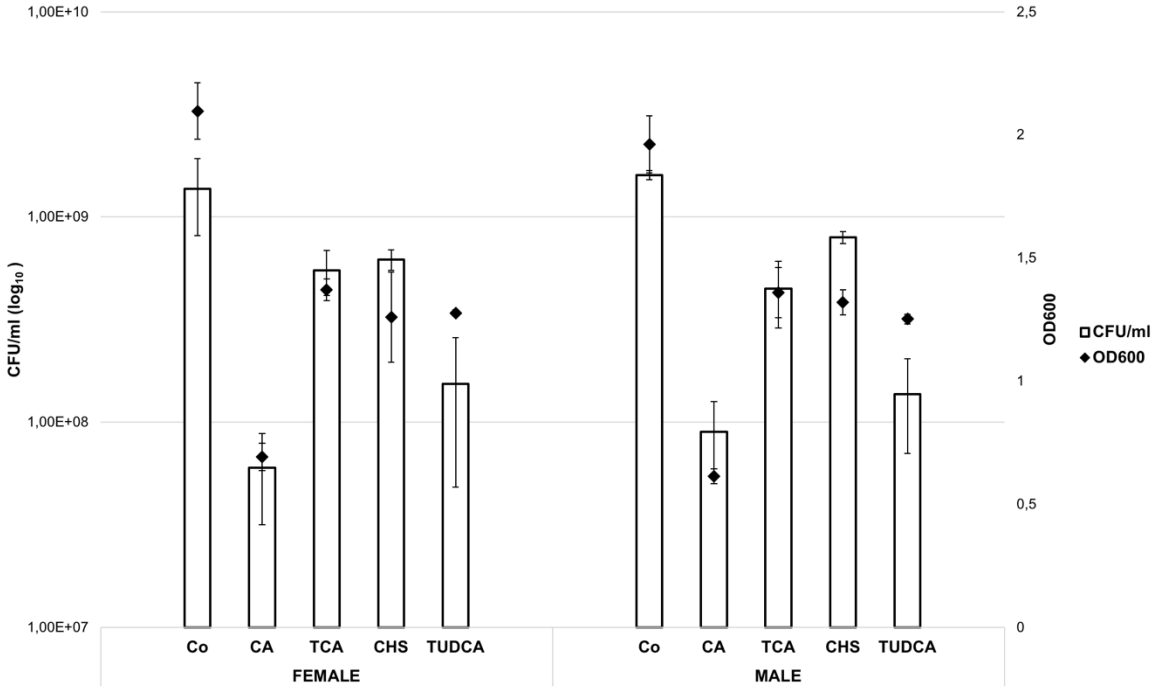

Figure 6. CFU/ml (log_{10}) and OD600 of samples from female and male mice. Aliquots of 24-hour cultures in bile acid selection LB were plated in volumes of 100µl on LB agar plates. Data are mean ± SD of 3 mice per group.

In both genders, bacterial growth in bile acid containing media showed the same tendencies. Determined by CFU/ml, CA suppressed bacterial growth the most, followed by TUDCA, TCA and CHS. Within the female group, CA suppressed bacterial growth by 96%, TUDCA by 89%, TCA by 60% and CHS by 55% when compared to control. Within the male group, CA suppressed bacterial growth by 95%, TUDCA by 91%, TCA by 72% and CHS by 50% when compared to control. When comparing bacteria obtained from female vs. male mice, bacterial growth was greater in male controls, male CA samples, and male CHS samples (+17%,

+50%, +28%). Male TCA and TUDCA samples showed weaker bacterial growth (-19%, -11%) than their counterparts obtained from female mice.

OD600 values in both groups showed corresponding tendencies. A remarkable decrease in OD600 compared to control, was observed with CA treatment. OD600 values of TCA, CHS and TUDCA treatment were lower in both groups, when compared to control. The observed suppression of bacterial growth determined by CFU/ml in TUDCA (vs. TCA and CHS) was not mirrored in the corresponding OD600 values. In general, OD600 values of the female and male group were quite similar in controls and the different treatments. CFU/ml were therefore diverging from OD600 values.

GI Medium

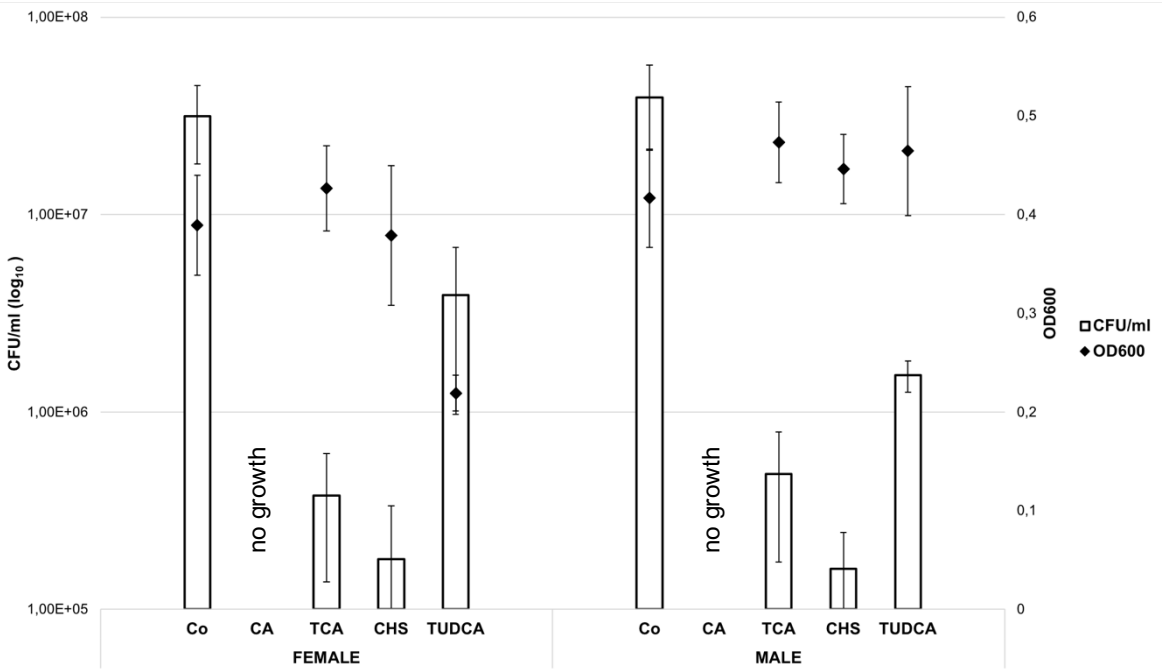

Figure 7. CFU/ml (log_{10}) and OD600 of samples from female and male mice. Aliquots of 24-hour cultures in bile acid selection GIM were plated in volumes of 100µl on GIM agar plates. No CFU/ml was observed with CA treatment. No OD600 value was observed with CA treatment. Data are mean ± SD of 3 mice per group.

In both genders, bacterial growth in bile acid selection media showed the same tendencies. Determined by CFU/ml, CHS suppressed bacterial growth the most, followed by TCA and TUDCA. No growth was observed with CA treatment. This is in contrast to bacterial growth in LB medium, where the ranking order of growth suppression was CA, TUDCA TCA and CHS. Presumably, CA did suppress bacterial growth in GIM to the point where no growth at all was observed.

Within the female group, CHS suppressed bacterial growth by 99,4%, TCA by 98,8% and TUDCA by 87,6%, when compared to control. No growth was observed with CA treatment. Within the male group, CHS suppressed bacterial growth by 99,6%, TCA by 98,8% and TUDCA by 96,1%, when compared to control. When comparing the female to the male group, bacterial growth was stronger in male controls and male TCA samples (+24%, +28%). Male CHS and TUDCA samples showed lower CFU/ml (-11%, -61%) than their female counterparts.

In the female group, only CA and TUDCA OD600 values were in analogy with bacterial growth suppression determined by CFU/ml. The remaining OD600 values showed no correspondence to bacterial growth determined by CFU/ml. Similar OD600 values were observed for control, TCA and CHS samples in both groups, and for the TUDCA sample only in the male group. The observed differences and trends in bacterial growth determined by CFU/ml were therefore mostly not reflected by OD600 measurements.

Additionally, as observed in 2.1.4., CFU/ml and OD600 values of GIM cultures are generally lower than those of LB cultures, reflecting the selective properties of GIM on specific gut microbiota that are more sensitive to bile acids. Highest values of CFU/ml in GIM are roughly two orders of magnitude less than the highest values of CFU/ml in LB and highest OD600 values in GIM are roughly a fourth of the highest OD600 values in LB.

In general, bile acid treatments in GIM had stronger effects on cultivable bacteria than bile acid treatments in LB. CA showed no growth in GIM, suggesting antimicrobial effects of CA to be very specific on microbiota grown in GIM. Also, CHS showed a remarkably greater suppression of microbial growth in GIM compared to LB. Interestingly, when comparing TCA and TUDCA between the two media, the effects of TCA and TUDCA were different (suppression of TUDCA > TCA in LB medium, suppression of TCA > TUDCA in GIM medium).

4.1.6. Bacterial growth of SHAM vs. BDL mice in BA media

Bile duct ligation is a common procedure in rodents to induce hepatic cholestasis and to inhibit bile flow to the intestines. Complete depletion of bile acids in the intestines is the result. We aimed to assess differences in bacterial growth between BDL and sham-operated animals in bile acid selection media (LB and GIM).

Faeces from three female and four male mice with different BDL status was diluted in NaCl relative to the weight to ensure equal dilution ratios (1:2 or 1:4). Details are listed in table 5.

Mouse No.	Sex	BDL Status	Stool weight (mg)	NaCl dilution volume (µl)	Inoculation volume (µl)
9	w	SHAM	380	760	25
10	w	7d	90	360	50
11	w	7d	167	668	50
12	m	SHAM	302	604	25
13	m	SHAM	320	640	25
14	m	7d	172	688	50
15	m	7d	220	880	50

Table 5. Details of three female and four male mice

Mice who underwent bile duct ligation had less stool than their sham-operated counterparts. After vortexing and centrifuging (1000rpm^{-1} x 60s) the samples, 25µl or 50µl (according to the dilution ratio) of the suspension were inoculated in bile acid selection LB and GIM plus controls (no bile acids) and incubated for 24 hours at 37°C x 180rpm^{-1}. Aliquots of the control and bile acid selection tubes were taken, diluted in series and plated on standard LB and GIM agar plates in volumes of 100µl. Details are shown in figures 8 – 11.

LB Medium

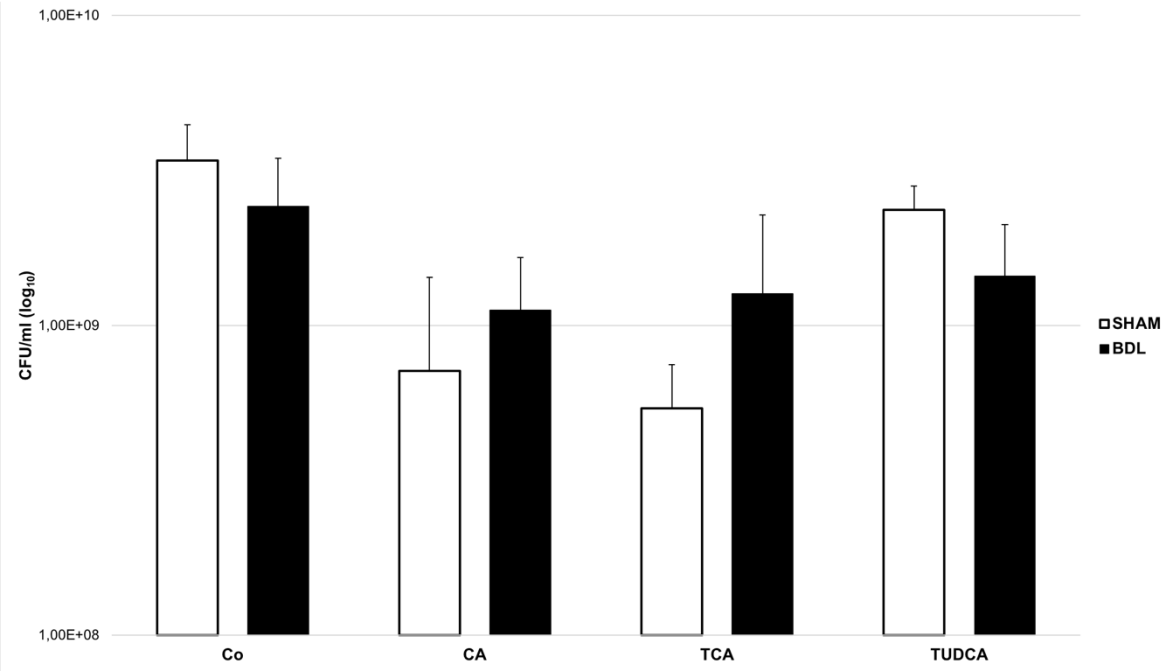

Figure 8. CFU/ml (log_{10}) of samples from SHAM vs. BDL mice in LB. Aliquots of 24-hour cultures in bile acid selection LB, were plated in volumes of 100µl on LB agar plates. Data are mean ± SD of 3 SHAM and 4 BDL mice.

In general, treatment with bile acids suppressed bacterial growth determined by CFU/ml in both, the SHAM and the BDL group, when compared to controls. Within the controls, CFU/ml of the SHAM group was higher than in the BDL group. In the SHAM group, growth suppression was strongest in TCA (-84%), followed by CA (-79%) and TUDCA (-31%) when compared to control. In the BDL group, growth suppression was strongest in CA (-54%), followed by TCA (-48%) and TUDCA (-40%), when compared to control. Conclusively, suppression of bacterial growth was weaker in the BDL group under CA (54% vs. 79%) and TCA (48% vs. 84%), but not under TUDCA (40% vs. 31%) treatment.

LB Medium

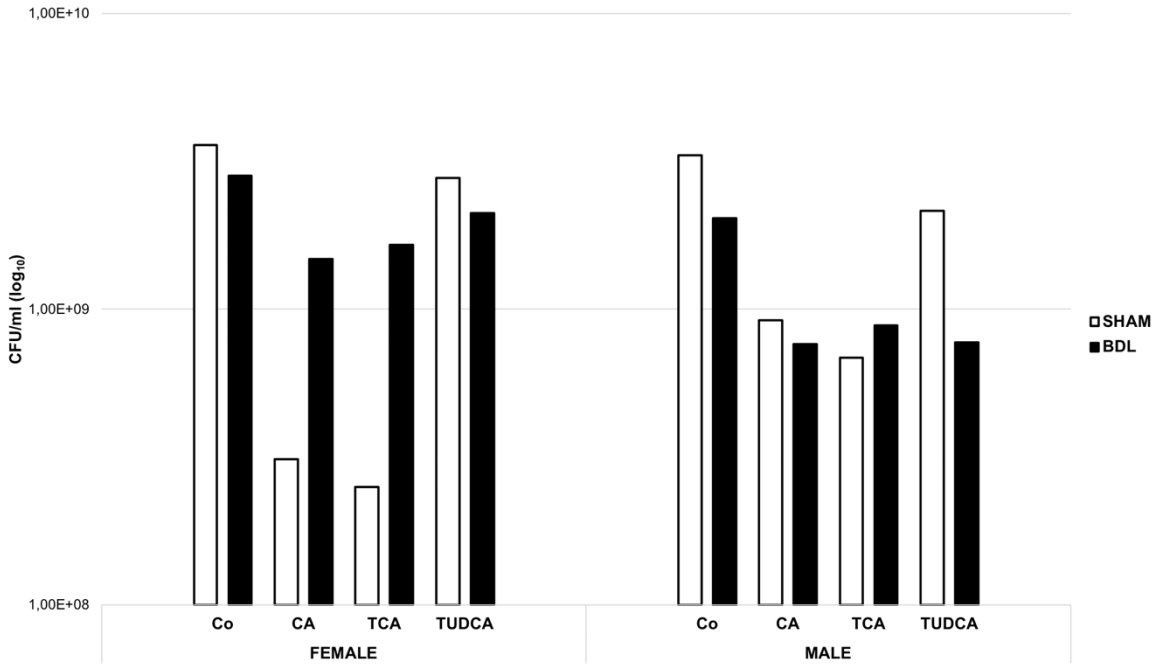

Figure 9. CFU/ml (log_{10}) of samples from SHAM vs. BDL mice in LB, separated by sex. Aliquots of 24-hour cultures in bile acid selection LB, were plated in volumes of 100µl on LB agar plates.

When separated by sex, controls and the TUDCA samples showed similar tendencies in both, the SHAM and BDL group respectively. Bacterial growth, determined by CFU/ml was stronger in the SHAM group than in the BDL group in these treatments. The differences observed in figure 8, between the SHAM and BDL group in treatments CA and TCA, seem to be mainly linked to a remarkable suppression of bacterial growth in the female SHAM group under these treatments vs. the female BDL group, when compared to untreated controls (-91%, -93% in the female SHAM group vs. -48%, -42% in the female BDL group). Suppression rates within the male group were more coherent. In the male SHAM group, CA and TCA treatment suppressed bacterial growth by 72% and 79% respectively, whereas in the male BDL group, CA and TCA treatment suppressed bacterial growth by 62% and 57% respectively. Conclusively, absolute suppression rates were higher in the female SHAM group and lower in the female BDL group when compared to the corresponding male groups.

Furthermore, a remarkable suppression under bile acid treatment was observed in the male BDL group. In contrast, effects of bile acids in the female BDL group were weaker.

This observation does not apply to the SHAM group, where CA and TCA, but not TUDCA treatment showed a remarkable suppression of bacterial growth especially in the female SHAM group. In the male SHAM group, a similar effect was observed, but here suppression in CA and TCA treatment was weaker overall.

GI Medium (GIM)

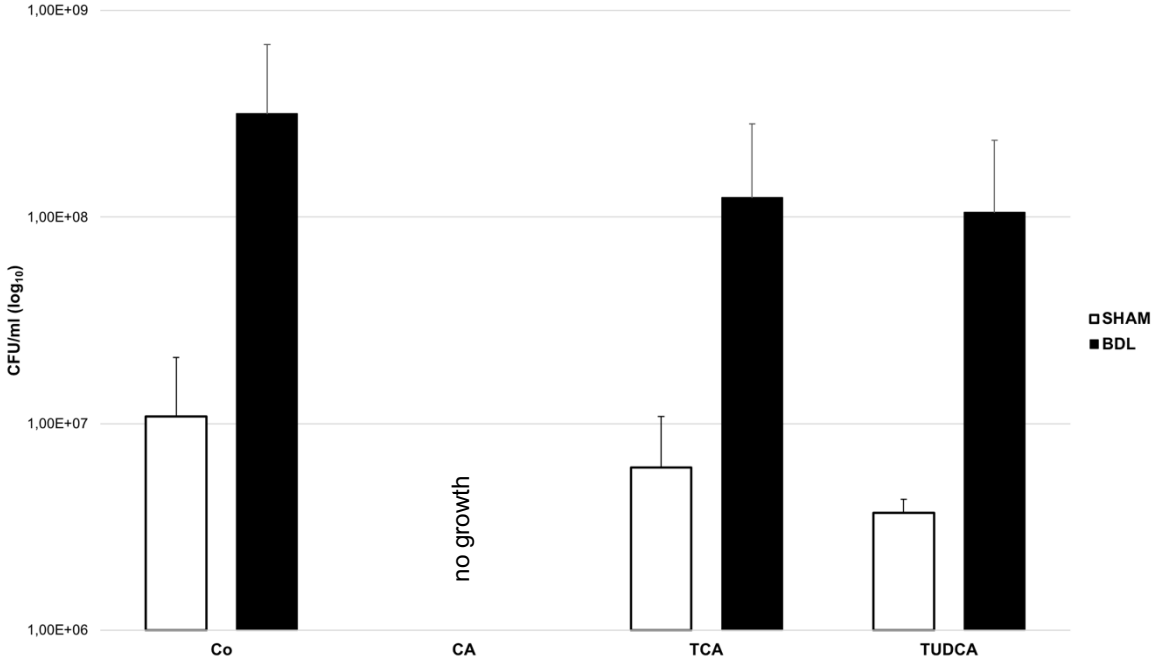

Figure 10. CFU/ml (log_{10}) of samples from SHAM vs. BDL mice in GIM. Aliquots of 24-hour cultures in bile acid selection GIM, were plated in volumes of 100µl on GIM agar plates. No growth was observed under CA treatment. Data are mean ± SD of 3 SHAM and 4 BDL mice.

Consistent with the findings in LB medium, treatment with bile acids suppressed bacterial growth, in both, the SHAM and the BDL group, when compared to controls. Under CA treatment, no growth was observed. In the SHAM group, we observed a suppression of 44% under TCA treatment and of 66% under TUDCA treatment, when compared to control. In the BDL group, similar tendencies were observed. TCA suppressed bacterial growth by 61% and TUDCA by 67% when compared to control. Interestingly, treatment with TUDCA suppressed bacterial

growth the most in GIM, which contrasts the findings in LB medium, where TCA (SHAM) or CA (BDL) showed the most suppression (see figure 8).
In general, bacterial growth, determined by CFU/ml was stronger in the BDL group, which stands in contrast to the findings in LB medium, where this only applied to treatment with TCA (and CA, but no comparison can be made with GIM in this case). Another conspicuous difference to the findings in LB medium is that growth between SHAM and BDL differs remarkably more within all treatments.

GI Medium (GIM)

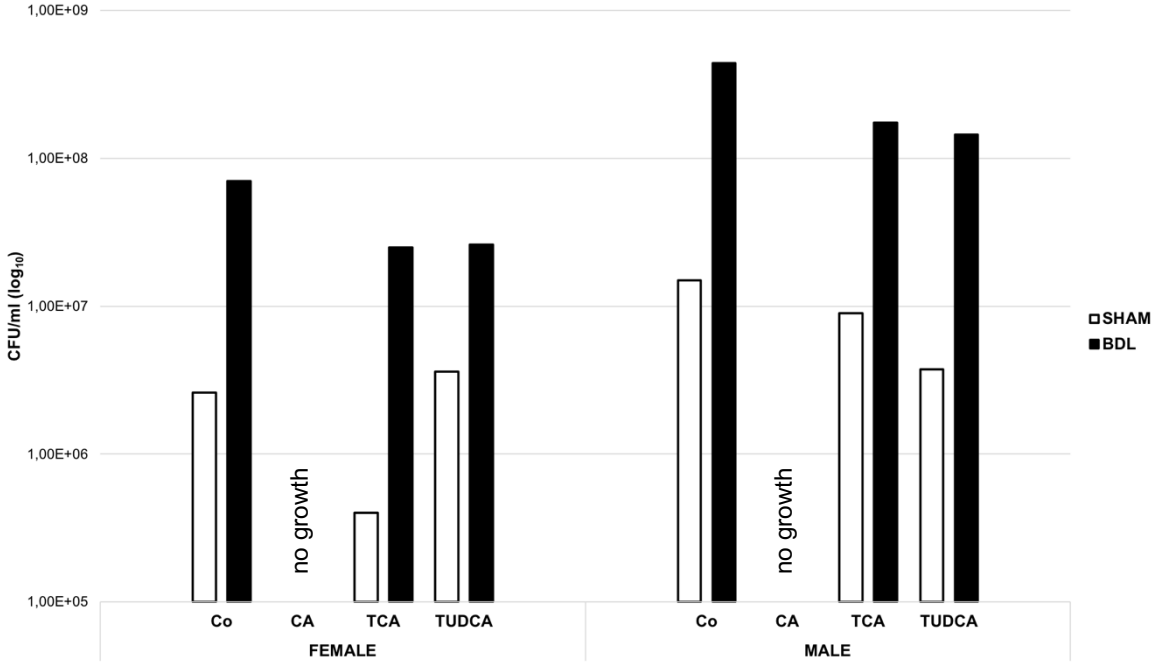

Figure 11. CFU/ml (log_{10}) of samples from SHAM vs. BDL mice in GIM, separated by sex. Aliquots of 24-hour cultures in bile acid selection GIM, were plated in volumes of 100µl on GIM agar plates. No growth was observed under CA treatment.

When separated by sex, controls and TCA samples showed the same tendencies in both, the SHAM und BDL group. In the SHAM group TCA suppressed bacterial growth by 85% (female) and 40% (male) vs. suppression by 64% (female) and 60% (male) in the BDL group. Treatment with TUDCA showed a paradox result in the female group: In the SHAM sample, bacterial growth was induced by 38%, whereas in the BDL sample, it was reduced by 63%. In the male group, the results were coherent: Bacterial growth was suppressed under treatment with TUDCA by 75% in the SHAM samples and 67% in the BDL samples. Overall, in the BDL

group, suppression rates of the different treatments in the female and in the male group were similar. In contrast to the findings in LB medium, bacterial growth was stronger in the BDL group than in the SHAM group.

4.2. Biotransformation of bile acids *in vitro*

To investigate the activity of the major bacterial enzymes BSH and 7α-dehydroxylase *in vitro*, faecal bacteria were incubated with certain conjugated and unconjugated bile acids and supernatants of 24-hour cultures were analysed with regard to bile acid metabolites using liquid chromatography high resolution mass spectrometry (LC-MS) as described previously (105).

4.2.1. Aerobic cultivation

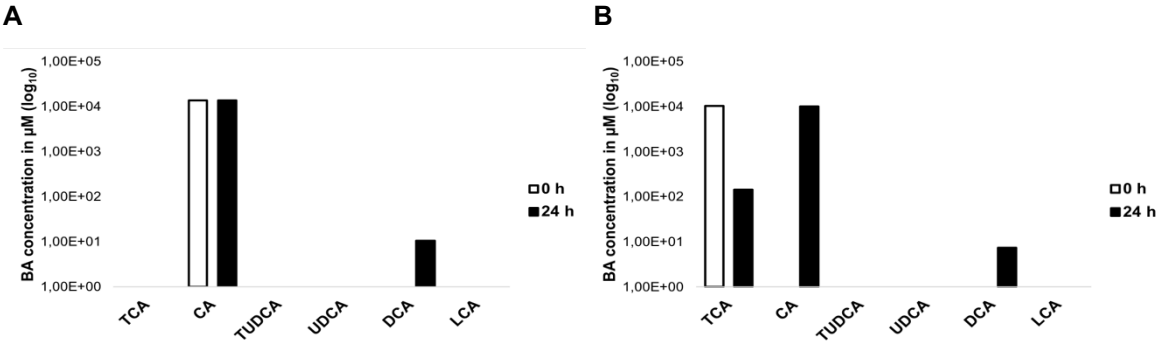

Figure 12. Modification of CA (A) and TCA (B). Concentrations in µM (log_{10}) in bile acid selection LB medium after 24-hour cultures of faecal samples in aerobic conditions.

In the CA treated bacterial culture, only 0.08% of CA was converted to DCA. Bacterial culture treated with TCA showed sufficient deconjugation of 98.43% of TCA into CA. Again, only 0.07% of CA was then converted to DCA, indicating that the second step of BA biotransformation is not executed in these bacterial samples under aerobic conditions.

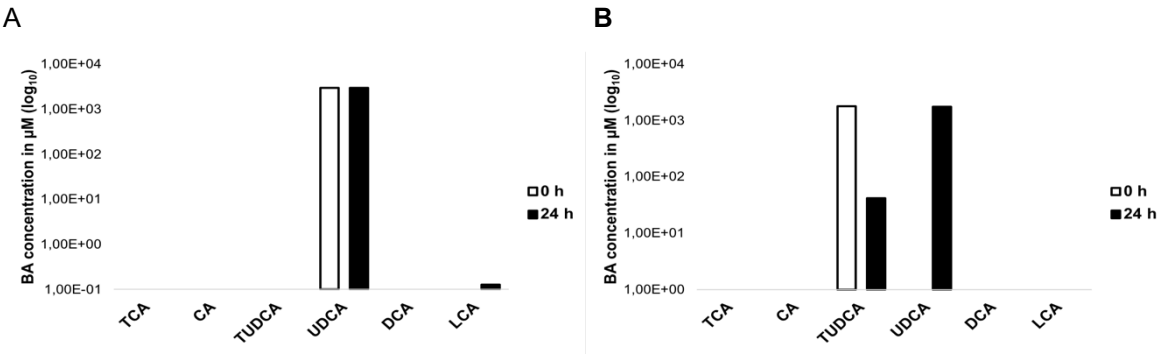

Figure 13. Modification of UDCA (A) and TUDCA (B). Concentrations in µM (log_{10}) in bile acid selection LB medium after 24-hour cultures of faecal samples in aerobic conditions.

Bacterial culture treated with UDCA yielded only a trace amount of the secondary bile acid LCA (<0.01% of UDCA was converted into LCA). On the contrary, bacterial culture treated with TUDCA showed sufficient deconjugation of 97.03% of TUDCA into UDCA. Again, consequent LCA generation was not observed.

Taken together, BSH activity (deconjugation) of BAs by faecal bacteria under aerobic *in vitro* culture conditions is highly effective. In contrast, the consecutive step, 7-alpha hydroxylation, is not performed.

4.2.2. Anaerobic cultivation

To further examine the activity of the two main bacterial enzymes in bile acid cultivation, we conducted the same experiment in anaerobic conditions and again analysed specific bile acid metabolites by LC-MS (105). It is known that the ability of 7α-dehydroxylation is reserved to just two gut microbial genera, *Eubacterium* and *Clostridium*, which are both obligate anaerobes (67,83).

After we observed no sufficient 7α-dehydroxylation in aerobic conditions, we hypothesized that the responsible genera may grow under anaerobic conditions and show active 7α-dehydroxylation resulting in the formation of the secondary bile acids DCA and LCA.

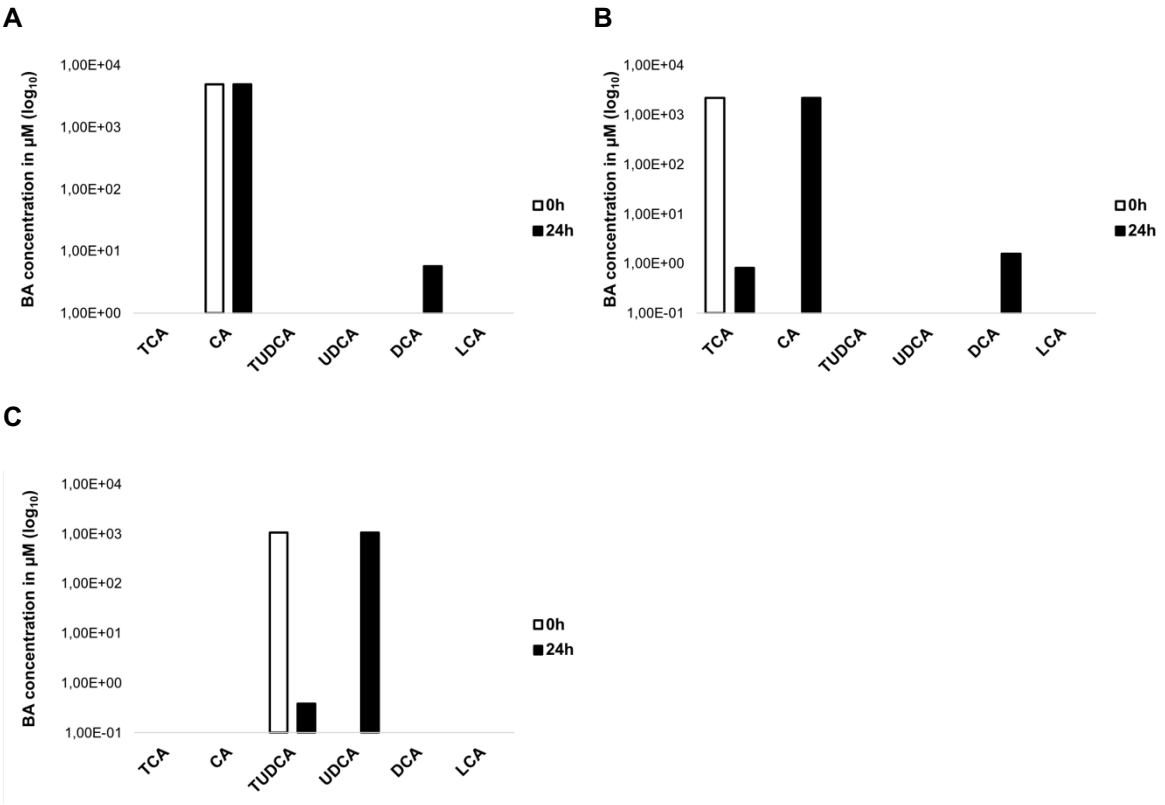

Figure 14. Modification of CA (A), TCA (B) and TUDCA (C). Concentrations in µM (log_{10}) in bile acid selection LB medium after 24-hour cultures of faecal samples in anaerobic conditions. Data are mean of two samples.

In the CA treated cultures, only 0.12% of CA was biotransformed into DCA. Deconjugation of TCA was in turn sufficient as 99.84% of it was biotransformed into CA. Again, only 0.07% of generated CA was then dehydroxylised to DCA.

In the TUDCA treated cultures, 98.72% of TUDCA was biotransformed into UDCA, showing sufficient deconjugation. The following step, generation of LCA, was not observed.

Conclusively, no 7α-dehydroxylation was observed under anaerobic conditions, which is consistent with previous findings from Wahlström et al., who were investigating 7α-dehydroxylation under anaerobic conditions *in vitro* with human faecal samples (106).

4.3. Gene expression analysis

Previous studies revealed that CA and UDCA repress hepatic gene expression of Cyp7a1 *in vivo* in both Fxr +/+ and Fxr -/- mice (107). It is known that CDCA is a potent FXR agonist, and therefore a modulator of BA-associated gene expression *in vitro*. However, other bile acids (including UDCA and CA) do not activate FXR *in vitro* (108).

Co-culturing of HepG2 cells with CDCA, CA, TCA and UDCA was done and relative mRNA levels of Cyp7a1 and Shp were measured. Details are displayed in figure 15.

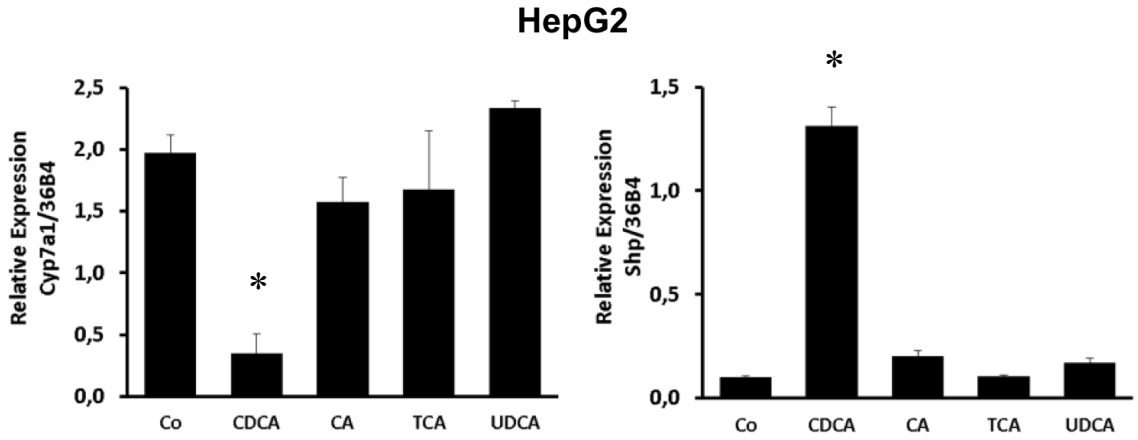

*Figure 15. Relative mRNA expressions of Cyp7a1 and Shp in HepG2 cells co-cultured with different bile acids. CDCA 100µM; other bile acids 250µM. Data are mean ± SD of triplicates for each sample. * P < 0.05 vs. control.*

Cyp7a1 expression was significantly repressed by CDCA, but not by CA, TCA and UDCA *in vitro*. Shp expression concordantly was significantly induced by CDCA, but not by CA, TCA and UDCA. These findings align with the above presented data from the Makishima et al. study which investigated FXR activation *in vitro* by different bile acids (108).

In previous studies, an effect of CA, TCA and UDCA on hepatic gene expression was demonstrated *in vivo* (109,110). We hypothesized that bioactive bacterial metabolites of modified BAs lead to differences in hepatic expression of BA-associated genes *in vivo*. To investigate if bacteria impact on hepatic gene expression, we treated HepG2 cells with supernatants of bacterial cultures in bile

acid selection media. Again, mRNA expression levels of Cyp7a1 and Shp were measured. Details are displayed in figure 16 and 17.

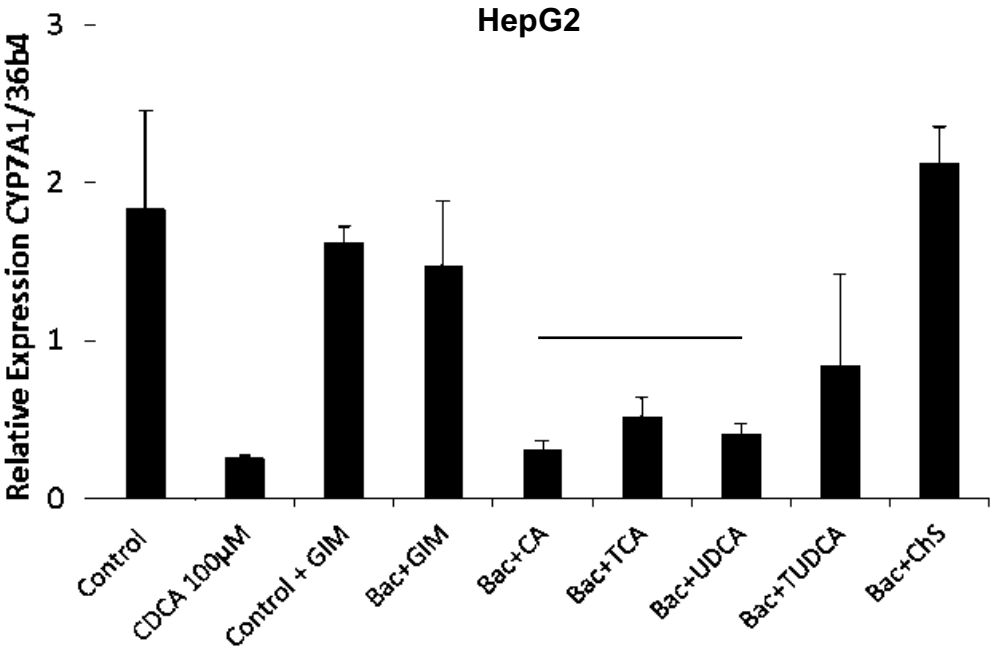

Figure 16. Relative mRNA expressions of Cyp7a1. HepG2 cells co-cultured with supernatants of bile acid selection GIM. CDCA 100µM; other bile acids 250µM. Data are mean ± SD of triplicates for each sample. – P < 0.05 vs. Bac+GIM.

The hepatic expression of Cyp7a1 was downregulated by CDCA which served as a positive control (as described above). Co-culturing of HepG2 cells with either GIM medium alone or bacterial culture supernatant without BAs (Control+GIM, Bac+GIM), had no effect on hepatic Cyp7a1 expression. In contrast, co-culturing with supernatants of different bile acid selection GIM cultures, revealed downregulation of Cyp7a1 expression. Bac+CA, Bac+TCA, and Bac+UDCA significantly repressed Cyp7a1 expression by roughly the same extent as CDCA. Bac+TUDCA showed insignificant repression of Cyp7a1, when compared to CDCA. Bac+ChS (cholylsarcosine – the cholic acid derivative that is resistant to deconjugation and cannot be metabolized by bacteria) showed no effect.

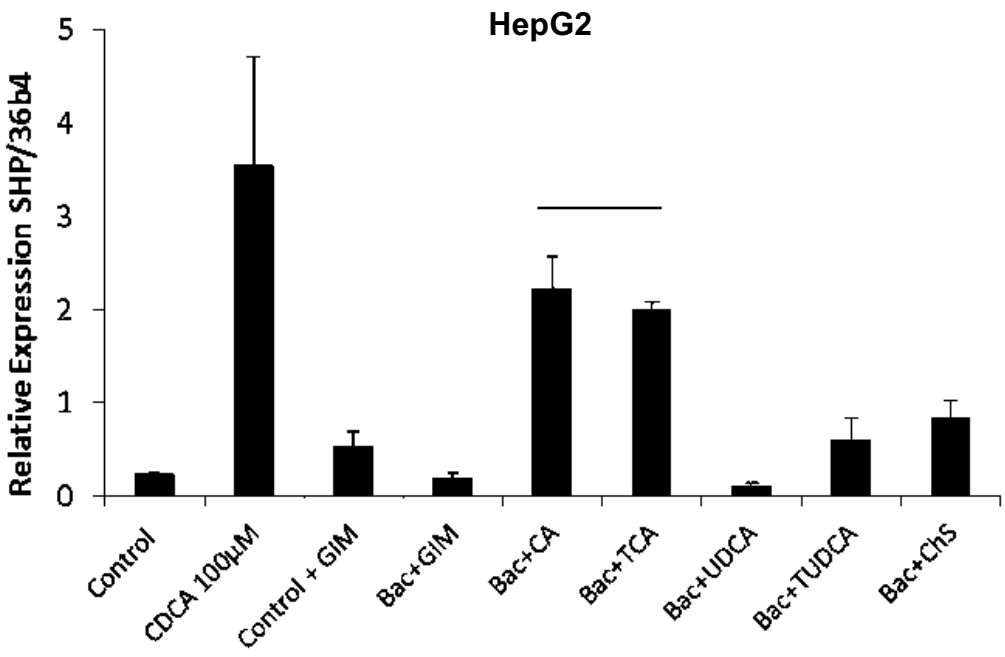

Figure 17. Relative mRNA expressions of Shp. HepG2 cells co-cultured with supernatants of bile acid selection GIM. CDCA 100µM; other bile acids 250µM. Data are mean ± SD of triplicates for each sample. – P < 0.05 vs. Bac+GIM.

Moreover, the hepatic expression of Shp was upregulated by CDCA which served as a positive control (as described above). Co-culturing of HepG2 cells with a bacterial culture supernatant (Controls, Bac+GIM), had no effect on hepatic Shp expression. In line with the results observed for Cyp7a1, co-culturing with supernatants of GIM bacterial cultures and bile acids, revealed substantial upregulation of Shp expression. In particular, Bac+CA and Bac+TCA significantly induced Shp expression. In contrast, Bac+TUDCA, Bac+UDCA and Bac+ChS had no effect on Shp, when compared to controls and Bac+GIM. These data is in line with *in vivo* results of mice treated with UDCA and TUDCA, showing a lack of induction of Shp (111,112).

Conclusively, bacterial cultures of faecal samples in GIM either produce certain metabolites or modify BAs, which then are potent to modulate hepatic expression of Cyp7a1 and Shp *in vitro*. Using aliquots of CA and TCA cultures showed concordant effects on both, Cyp7a1 (repression) and Shp (induction). Aliquots of cultures of UDCA treated bacteria only had an effect on Cyp7a1 expression, but not on Shp expression. The impact of aliquots of TUDCA cultures was weaker and showed only tendencies of modulatory effects on Cyp7a1 and Shp expression.

5. Discussion

5.1. Summary

The present study aimed to investigate the interaction between bile acids and gut microbiota in an *in vitro* experimental design. We investigated the effects of free and taurine-conjugated bile acids on gut microbiota growth in different cultivation settings. Furthermore, the bile acid biotransformation potential of gut microbiota grown in aerobic and anaerobic conditions and the modulatory effects of metabolites of bacterial cultures in bile acid selection media on hepatic expression of genes associated with bile acid metabolism were investigated.

Bacterial growth of mouse gut microbiota in different bile acid supplemented media was remarkably distinct. Gut microbiota showed attenuated growth when co-cultured with bile acids. In most of the experiments this effect was independent of growth medium, composition or sex. Furthermore, BDL – which depletes BAs from the gut *in vivo* – did desensitize these bacteria later on to BA-induced growth suppression *in vitro*, as observed in stronger bacterial growth of faecal samples from BDL vs. SHAM mice.

CA suppressed bacterial growth the most, as determined by CFU/ml and OD600. We assume that the lack of growth under CA in GIM, is either a direct consequence of the toxic properties of CA, or due to a selection of certain bacteria in this medium. The suppression of bacterial growth under CA was therefore robust between the different media used, the BDL status of the mice and the sex. Suppression rates in cultures with TCA, TUDCA and CHS showed diverging results. TCA also suppressed bacterial growth, but not as consistent as CA with regard to culture medium and BDL status. Suppression under TUDCA was second strongest in LB with faecal samples of non-operated mice. However, suppression of bacterial growth of faecal samples from SHAM and BDL mice (i.e. operated mice) under TUDCA was weakest when compared to the other bile acids. In GIM, suppression under TUDCA was weakest with samples of non-operated mice, when compared to the other bile acids. Samples of operated mice (SHAM and BDL) showed diverging results.

CHS showed weak suppressive effects in LB medium, but visible effects in GIM medium, where suppression was second strongest after CA. Further experiments are needed to elucidate the effects of TCA, CHS and TUDCA in the different media and with different faecal samples more precisely.

Moreover, the activity of the two most important representatives of gut bacterial enzymes, bile salt hydrolase (BSH) and 7α-dehydroxylase was observed *in vitro* under aerobic and anaerobic conditions. Deconjugation of taurine-conjugated bile acids into free primary bile acids in both, aerobic and anaerobic bacterial cultures was efficiently performed, whereas the cultured gut bacteria showed reduced ability to convert the free primary bile acids into their secondary counterparts in both environments. The absence of bacteria with 7α-dehydroxylase activity (e.g. *Eubacterium* and *Clostridium*) in our cultures is presumably the reason for this result.

Additionally, we were able to show that metabolites of gut microbiota cultures in bile acid selection media are potent modulators of hepatic expression of Cyp7a1 and Shp. Modulation of hepatic gene expression by bile acids has been demonstrated recently in *in vivo* experiments (107). However, the precise pathways and mechanisms of these effects remain unclear. This study transfers these findings to an *in vitro* experimental setting and shows that gut microbiota are able to modulate hepatic gene expression through biotransforming or metabolizing bile acids or the release of metabolically active components which impact on gene transcription in the host. These data is consistent with a recent study from Zhang et al. which had a similar approach (113).

5.2. Bacterial growth in standard and BA supplemented media

As early as in the beginning of the 20th century, a differential medium containing bile acids was invented by Alfred MacConkey (114). Since then MacConkey agar has become a standard culture medium specifically designed to harbour enteric gram-negative bacteria. As supplementation, normally ox gall is used. MacConkey agar might be suitable to culture and select enteric bacteria, but not to examine

and characterize the effects of different kinds of bile acids alone on bacterial growth. We therefore designed culture media containing one single kind of bile acid.

Bacterial cultures in cholic acid (CA) selection media yielded the most consistent results. Unsurprisingly, of the tested bile acids, CA, a trihidroxy-unconjugated BA, is the most hydrophobic. Hydrophobicity has been found to be the most important determinant of toxicity of bile acids in previous studies (69,70). However, keeping in mind that gut microbiota are exposed heavily to CA, the extent of suppression (up to 96%) is still impressive and may have physiologic implications in the inhibition of bacterial overgrowth and the homeostasis of certain intestinal communities.

Our findings are consistent with results from Islam et al., who reported significant lower bacterial counts in an *in vivo* setup with CA-fed rats (115). Moreover, our findings align with various *in vitro* studies, which frequently reported the antimicrobial activity of BAs in bacterial cultures (67,116,117).

The strong suppression of bacterial growth under CA treatment might also be due to generation of DCA in the culture. DCA which is highly hydrophobic and thus highly toxic, may therefore be partly involved in the effect observed under CA in both, LB and GIM medium, where no growth under CA challenging was observed (115). As GIM favours the growth of intestinal bacteria especially, 7α-dehydroxylation, might have been more present in those cultures, resulting in a pronounced biotransformation of CA into DCA. To prove this hypothesis, other free bile acids (e.g. CDCA) should be included in further experiments, and possible biotransformation and generation of secondary BAs within the culture should be investigated.

Interestingly though, we did not observe sufficient 7α-dehydroxylation determined by the generation of secondary bile acids, when analysing supernatants of centrifuged bile acid bacterial cultures via LC-MS. However, the missing secondary bile acids might have been in the bacterial pellet.

Several other possibilities for the absence of bacterial growth under CA in GIM are conceivable. Bacterial growth in GIM determined by CFU/ml was decreased in general when compared to LB which may result in just-non-detectable CFU/ml in the GIM cultures. Also, one needs to keep in mind that bacterial strains, grown in

the two media, may be completely different. It is possible that CA impacts the bacteria grown in GIM to a greater extent than the bacteria grown in LB.

Also, due to the differential characteristics of GIM (e.g. special composition of SCFAs and other compounds) (100), only bacterial strains may have grown, which are highly sensitive to CA. Furthermore, the dilution of the faecal sample might have been too high, not allowing sufficient bacterial growth of certain bacterial communities. Moreover, complexation of CA to other components of GIM, resulting in increased antimicrobial properties of the medium, might be an explanation for the non-detectable growth under CA in GIM.

Suppression of bacterial growth in TCA and CHS was not as consistent as in CA. CHS (i.e. Cholylsarcosine), a semi-synthetic bile acid analogue, which is resistant to intestinal degradation and biotransformation, has the same structure as CA. Instead of conjugation with taurine or glycine at the C24 position, sarcosine is linked to the molecule (101). Interestingly, in LB medium, the suppression rates of TCA and CHS were similar, whereas in GIM, CHS showed stronger suppression than TCA. This again, is most possibly based on differences between LB and GIM as described above and may indicate that CHS is more toxic on gut microbiota as GIM selects especially enteric strains. This may have clinical relevance as CHS is used as bile acid replacement therapy and to treat disorders regarding lipid malabsorption (101). Interestingly, we observed no effect of CHS on hepatic gene expression in the following experiments (see chapter 4.3.), indicating that the modulation of hepatic gene expression relies on bile acid related metabolites, rather than bile acids itself.

TUDCA did also suppress bacterial growth in the cultures. In TUDCA LB, CFU/ml of bacteria obtained from non-operated mice, was lower than TCA and CHS, rather mimicking the effects of CA. This stands in contrast to the results in GIM, where CFU/ml under TUDCA challenging showed the weakest suppression of bacterial growth when compared to TCA and CHS. Strains grown in GIM medium therefore seem to be more resistant to TUDCA as compared with TCA and CHS, than strains grown in LB medium. This observation though could not be made with samples obtained from SHAM and BDL mice.

In general, the antimicrobial effects of the conjugated bile acids TCA and TUDCA (conjugation decreases hydrophobicity which in turn determines cytotoxicity) are weaker than the effects of CA, but not UDCA (data not shown), which is consistent

with previous findings (67,118). Moreover, TUDCA and its unconjugated derivative UDCA have been previously found to attenuate cytotoxic effects of hydrophobic bile acids such as DCA and CDCA (69,70). These protective effects might also have repercussions on bacterial growth and may result in weaker suppression. A follow-up study may therefore be investigating the effects of bile acid combinations (e.g. TUDCA and CA) on bacterial growth and if addition of TUDCA or UDCA is able to alleviate the antimicrobial effects of CA.

We observed remarkable differences in bacterial growth of faecal samples from SHAM vs. bile duct ligated animals. In GIM, CFU/ml of BDL mice were over one order of magnitude higher than those of SHAM individuals. Quite possibly an explanation for this finding may be higher bacterial counts in BDL mice due to the lack of BAs and their antimicrobial properties *in vivo* as reported by various other studies. Another conceivable factor is that bacterial cultures of SHAM mice consisted mostly of previously BA-sensitized gut microbiota, due to constant exposure to BAs, which may result in higher sensitivity to bile acids yielding lower CFU/ml in this group (119–121).

Additionally, we measured the optical density of the samples at a wavelength of 600nm (OD600). In LB, OD600 values of control, CA, TCA and CHS corresponded well with bacterial growth determined by CFU/ml. Only under TUDCA treatment, the OD600 value was false high when compared to the corresponding CFU/ml. In GIM, OD600 values did not correspond with the CFU/ml of the bile acid treatments. OD600 values need to be interpreted with care (see chapter 5.7), as the data may not allow inferences on living cell counts within the sample. Our findings highlight the importance of this problem.

5.3. Bile acid biotransformation in an aerobic and anaerobic environment

Bacterial biotransformation of bile acids is essential for enterohepatic cycling and bile acid metabolism. It is one of the key features of the gut microbiome that contributes to intestinal homeostasis (66,72).

Biotransformation potential of faecal bacteria cultured in bile acid supplemented LB medium was investigated via LC-MS. Faecal bacteria were co-cultured with free and conjugated bile acids and the metabolites of the two main bacterial enzymatic reactions (deconjugation, BSH activity: formation of free primary bile acids; 7α-dehydroxylation: formation of secondary BAs) were investigated.

The first step, enzymatic hydrolysis of the amide bond at C24, is performed sufficiently in both, aerobic and anaerobic conditions, with both taurine-conjugated bile acids, TCA and TUDCA. These findings align with previous findings from Wahlström et al. (122).

Consistent with this, previous studies observed BSH activity in a variety of mostly gram-positive bacterial strains like Lactobacillus, Bifidobacterium, Enterococcus, Clostridium and Bacteroides and ascribe bacterial BSH activity to be a conserved function of gut microbiota specifically (85,86,123).

Interestingly, BSHs were found to be more affine to glycine-conjugated BAs, but in the absence of glycine-conjugates, bacterial deconjugation seems to target taurine-conjugates sufficiently as well. Supposedly, gut microbiota from mice, where mostly taurine-conjugates occur, do have a higher potential to deconjugate taurine-BAs than gut microbiota from men. To investigate this hypothesis, further investigations of bacterial bile acid biotransformation processes, may include human stool (85).

The second step, 7α-dehydroxylation, was not performed sufficiently in aerobic conditions, albeit it is thought that BSH positive bacteria are associated with 7α-dehydroxylating bacteria. However, 7α-dehydroxylating activity has been found in certain members of just two bacterial genera: *Eubacterium* and *Clostridium*, both of which belong to class *Clostridia* and are obligate anaerobes (67,83). As oxygen is toxic to these genera, it is not surprising that sufficient 7α-dehydroxylation (determined by the formation of secondary bile acids DCA and LCA) was not observed in experiments conducted in aerobic conditions.

Strikingly, when conducting the same experiments in anaerobic conditions, still no 7α-dehydroxylation could be observed. The results of the anaerobic experiments therefore resemble those observed under aerobic conditions, indicating that sufficient 7α-dehydroxylation may not rely on the presence of certain genera in anaerobic conditions alone, but rather may require different stimuli, e.g. the interaction with intestinal mucosa or more time in cultivation. However, after co-

culturing faecal samples with Caco2-cells – a cell model for intestinal mucosal environment – and consequent analysation via LC-MS, no additional generation of secondary bile acids in the supernatant was observed (data not shown). Future experiments may try to mimic the intestinal environment more closely and investigate the formation of secondary bile acids in such environments.

Our findings align with data from Wahlström et al., who had a similar approach and also observed no 7α-dehydroxylation in anaerobic conditions with human faecal samples (122). Moreover, in this study, the authors observed a mismatch between supplemented TCA and its deconjugated counterpart CA after culturing. Despite TCA being almost fully deconjugated, the amount of CA only added up to roughly two thirds of the initial amount of TCA. This indicates that in addition to deconjugation, also other metabolites must have been generated which may have been missed by LC-MS (e.g. epimerized and isomerized BA derivatives). This also may be applied to our study. It may very well be plausible that a part of the initially used bile acids has been absorbed by the cultured bacteria (122). Also, secondary murine bile acids, may have been missed, as the murine products of 7α-dehydroxylation are hyodeoxycholic acid and murideoxycholic acid, which we did not include in our analysis (90,91). Further LC-MS analyses should include the investigation of the formation of the stated secondary murine bile acids and isomerized or epimerized BAs.

5.4. Transactivation of bile acid target genes by bacterial metabolites

Bile acids regulate their own synthesis via an elaborate feedback mechanism on hepatic bile acid-associated gene expression (66,72,93,97). Previous studies showed that bile acid feeding in mice, resulted in altered hepatic gene expression of Cyp7a1, Shp, Ost-a/Ost -β and Cyp8b1 (107,109,110,124).

Moreover, hepatic Cyp7a1 feedback mechanism was partly independent from FXR. Zollner et al. showed that Fxr -/- mice also exert negative feedback on Cyp7a1 expression upon CA and UDCA feeding suggesting these mechanisms to be in part independent from FXR activation (107).

We found that only CDCA, but not CA, TCA and UDCA are able to directly alter hepatic expression of Cyp7a1 and Shp in an *in vitro* design. This contrasts findings from Lew et al., who found CDCA, DCA, CA and UDCA to be able to directly modulate expression of Bsep in HepG2 cells. In their work CDCA and DCA increased Bsep mRNA transcription already at 75µM. However, the effect under CA and UDCA was only observed using very high doses of CA and UDCA (400-600µM) (125). We might have missed the effects of CA and UDCA due to the dose of 250µM per bile acid we used in these experiments.

However, provided that this effect is mediated by FXR as a central bile acid sensing element, our findings are in turn consistent with Lew et al., who reported that among the tested bile acids, only CDCA is a true agonist of FXR. CA and UDCA were reported to be only partial agonists, which may explain our finding that only CDCA was able to supress Cyp7a1 and induce Shp expression remarkably. Further experiments are needed, to examine the specific effects of various bile acids on hepatic gene expression (125).

In light of the findings of the above stated studies on bile acid feedback mechanisms, we hypothesized that bacterial metabolites or modifications of these bile acids, may be potent to (in)-directly alter hepatic expression of genes associated in bile acid metabolism. Zhang et al. were investigating if supernatants of bacterial cultures had an effect on Fxr expression and FXR target genes and demonstrated that supernatants from two distinct bacterial strains belonging to gut microbiota (*B. dorei* and *E. limosum*) were transactivating the FXR target genes Ibabp and Ost-α (113).

Our approach included bile acids in the bacterial cultures. We hypothesized that cultures under bile acid treatment had different impact on hepatic bile acid associated gene expression. In consistency with *in vivo* data from Zhang et al., remarkable suppression of Cyp7a1 expression *in vitro* was observed with CA, TCA and UDCA culture aliquots and a tendency of suppression was observed with TUDCA culture aliquots (109). Furthermore, this is consistent with the above stated studies on negative feedback mechanisms, bile acids exert on hepatic gene expression (66,72,93,97). Concordantly, Shp expression was induced with CA and TCA culture aliquots, whereas UDCA and TUDCA in bacterial supernatant aliquots had no effect on Shp expression. In line with our findings, Mueller et al. showed that Shp expression was not effected by UDCA in an *in vivo* study with human

participants (111), which is however antithetical to the data of Song et al. which showed that UDCA altered hepatic Shp expression in mice fed an UDCA supplemented diet, although FXR was not activated in these animals (110).
We therefore postulate that certain bacterial metabolites of modified bile acids are potent modulators of the expression of Cyp7a1 and Shp. Further experiments are needed, to examine the effects of bacterial bile acid metabolites on the expression of other genes like Bsep, Ost-a/Ost -β and Cyp8b1. Moreover, we propose analysis of these bacterial metabolites as the next logical step. The first data obtained by nuclear magnetic resonance spectroscopy (NMR-spectroscopy) already highlighted differences in the bacterial supernatants treated with different bile acids. However, a detailed investigation of these metabolites was beyond the scope of this work.

5.5. Conclusion

With this study, we gave an overview of the interaction between specific bile acids and gut microbiota. Growth of gut microbiota from female and male, BDL and sham-operated mice was investigated in bile acid culture media, as well as biotransformation of bile acids and modulation of hepatic gene expression. Gut microbiota growth shows substantial differences in various bile acid supplemented media and between LB and GIM medium. Furthermore, differences in gut microbiota growth between female and male mice were observed and between gut microbiota from BDL and sham-operated animals. Bacterial biotransformation is possible *in vitro*, but only bacterial bile salt hydrolysis (BSH) functioned sufficiently. In addition, cultures of gut microbiota in bile acid supplemented media yield metabolites which are potent to alter hepatic expression of genes of bile acid metabolism.

5.6. Limitations

Certain limitations need to be pointed out. For the experiments, only a small number of animals was used. Especially for the experiments with faeces from SHAM and BDL female and male mice, greater numbers of animals are needed to find statistically robust differences.

Also, measurements of OD600 values to examine bacterial growth in liquid cultures need to be interpreted with care. The photometric approach just yields the optical density of a sample, which may not reflect actual living cell counts inside the sample. Cell debris etc. may falsely result in high OD600 values.

Another limitation of this study is the aerobic character of most of the experiments. In the gut, mostly obligate anaerobic bacteria of phylum *Firmicutes* and *Bacteroidetes* are being found, many of which are susceptible to oxygen. Conclusively, we mainly observed bacterial growth of facultative anaerobes or aerobes, which in turn does not resemble the intestinal flora well. Further studies may perform such experiments strictly in anaerobic conditions.

Moreover, faecal samples obtained from mice caeca might not reflect actual gut microbiota composition, as many members of the intestinal community are mucosa adherent. This limitation might distort the results we observed in the different experiments.

Additionally, one needs to keep in mind that bile acid profiles of men and mice differ significantly and results, conducted in mice experiments, may not be transferred straightforwardly to human physiology.

5.7. Outlook

For our experiments, we chose a quantitative approach. Future experiments may assess qualitative differences in gut microbiota growth under different bile acid treatments. We observed differences in CFU/ml and OD600, but which bacterial species actually are responsible for growth has to be elucidated in further studies. Different bacterial strains may predominate in different bile acid treatments. We therefore propose bacterial 16s-RNA sequencing of the different bacterial cultures.

Moreover, only faecal content from mice was used. Future experiments may include stool samples from human donors. Samples from healthy donors and samples from individuals who suffer from diseases like IBDs or PSC may be included in such studies to further elucidate how gut microbiota of different habitats react to bile acid selection pressure.

Furthermore, a metabolomic approach may be useful to investigate the bacterial metabolites which arise from gut microbiota metabolism in different bile acid culture media. This may also reveal the metabolites responsible for the modulation of hepatic gene expression.

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