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Changes in the bile acid composition in response to caloric restriction in mice

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Abbreviation

Ad lib	Ad libitum
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AUC	Area under the curve
BA	Bile acid
BA supp	Taurine-conjugated BA supplementation
BAs	Bile acids
BAT	Brown adipose tissue
BSH	Bile salt hydrolase
BSHi	Bile salt hydrolase inhibitor
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CR	Caloric restriction
CYP	Cytochrome P450
DAMPs	Pathogen-associated molecular patterns
DCA	Deoxycholic acid
DHCA	Dehydrocholic acid
DMEM	Dulbecco's Modified Eagle Medium
EA	Ethacrynic acid
Eef1a1	Eukaryotic translation elongation factor 1 alpha 1
EtOH	Ethanol
eWAT	Epididymal white adipose tissue
FA	Formic acid
FT	Fecal transplant
Fxr	Farnesoid X receptor
GSH	Glutathione
GSTs	Glutathione S-transferases
HPLC	High-performance liquid chromatography
HSDH	Hydroxysteroid dehydrogenase
Ibat	Ileal apical sodium-dependent bile acid cotransporter
IL	Interleukin
IFN	Interferon
LCA	Lithocholic acid
LCMS	Liquid Chromatography Mass Spectrometry
LTD	Low taurine diet

MAPEG	membrane-associated proteins in eicosanoid and glutathione metabolism
MCA	Muricholic acid
MeOH	Methanol
Mgst1	Microsomal GSH S-transferase 1
MHC	Major histocompatibility complex
MHY1485	4,6-dimorpholino-N-(4-nitrophenyl)-1,3,5-triazin-2-amine
MM	Mastermix
Mrp1	Multidrug resistance protein 1
mTOR	Mammalian Target of Rapamycin
mTORC	Mammalian target of rapamycin complex
NB	No bedding
NTD	Normal taurine diet
Ostα	Organic Solute Transporter subunit alpha
Ostβ	Organic Solute Transporter subunit beta
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PPARγ	Peroxisome proliferator-activated receptor-γ
PXR/Pxr	Pregnane X Receptor
qPCR	Quantitative real-time PCR
Rt	Retention times
SCFA	Short-chain fatty acids
SI	Small intestine
SREBP	Sterol regulatory element-binding protein
Stat1	Signal transducer and activator of transcription 1
STD	Standard
sWAT	Subcutaneous white adipose tissue
Tau	Taurine diet 4%
TauT	Taurine transporter
TauTi	Taurine transporter inhibitor
TCA	Taurocholic acid
TDCA	Taurodeoxycholic acid
TIR	Toll interleukin-1 receptor
TLCA	Taurolithocholic acid
Tlr3	Toll-like receptor 3
TOR	Target of rapamycin
TRIF	TIR-domain-containing adaptor-inducing IFN-β

TUDCA	Tauroursodeoxycholic acid
UDCA	Ursodeoxycholic acid
WAT	White adipose tissue

TABLE OF CONTENT

Table of figures

List of tables

1 Abstract

Caloric restriction (CR) is well known for its effects on longevity and health promotion, but its influence on the composition and metabolism of bile acids (BAs) is not yet fully determined. This thesis aims to investigate the effects of CR on BA composition and metabolism in mice.

Liquid Chromatography Mass Spectrometry (LCMS) was used to measure conjugated and unconjugated BAs in ileum, liver, plasma, proximal colon and adipose tissue. Additionally, bile salt hydrolase (BSH) activity was assessed in feces. Quantitative real-time PCR (qPCR) was used to quantify gene expression of transporters, receptors, transcription factors and gut bacteria.

The results suggest a distinct CR phenotype characterized by an elevated BA pool size in the ileum, liver, plasma, and proximal colon. The thesis also explores the mechanisms underlying the observed increase in BA pool size during CR, indicating that changes in the expression and activity of transporters, receptors, and transcription factors involved in the synthesis and metabolism of BA may be responsible.

In conclusion, this thesis provides insight into the impact of CR on BA composition and metabolism in mice. The observed augmentation of the BA pool size during CR may be due to changes in the expression and functionality of transporters, receptors, and transcription factors that play key roles in BA synthesis and metabolism. Furthermore, this project also suggests that changes in gut microbiota may affect BA metabolism and vice versa.

Zusammenfassung

Kalorische Restriktion (CR) ist für ihre Auswirkungen auf Langlebigkeit und Gesundheitsförderung bekannt, aber ihr Einfluss auf die Zusammensetzung und den Stoffwechsel von Gallensäuren (BA) ist noch nicht vollständig geklärt. Ziel dieser Arbeit ist es, die Auswirkungen von CR auf die Zusammensetzung und den Stoffwechsel von Gallensäuren bei Mäusen zu untersuchen.

Mit Hilfe der Flüssigchromatographie-Massenspektrometrie (LCMS) wurden konjugierte und unkonjugierte Gallensäuren im Ileum, in der Leber, im Plasma, im proximalen Kolon und im Fettgewebe gemessen. Zusätzlich wurde die Aktivität der Gallensalzhydrolase (BSH) in den Fäkalien bestimmt. Mittels quantitativer Echtzeit-PCR (qPCR) wurde die Genexpression von Transportern, Rezeptoren, Transkriptionsfaktoren und Darmbakterien quantifiziert.

Die Ergebnisse deuten auf einen ausgeprägten CR-Phänotyp hin, der durch eine erhöhte BA-Poolgröße im Ileum, in der Leber, im Plasma und im proximalen Dickdarm gekennzeichnet ist. Die Arbeit untersucht auch die Mechanismen, die dem beobachteten Anstieg der BA-Poolgröße während der CR zugrunde liegen, und weist darauf hin, dass Veränderungen in der Expression und Aktivität von Transportern, Rezeptoren und Transkriptionsfaktoren, die an der Synthese und dem Metabolismus von BA beteiligt sind, dafür verantwortlich sein könnten.

Zusammenfassend lässt sich sagen, dass diese Arbeit einen Einblick in die Auswirkungen von CR auf die BA-Zusammensetzung und den BA-Stoffwechsel bei Mäusen gibt. Die beobachtete Vergrößerung des BA-Pools während der CR könnte auf Veränderungen in der Expression und Funktionalität von Transportern, Rezeptoren und Transkriptionsfaktoren zurückzuführen sein, die eine Schlüsselrolle bei der BA-Synthese und dem BA-Stoffwechsel spielen. Außerdem deutet dieses Projekt darauf hin, dass Veränderungen in der Darmmikrobiota den BA-Stoffwechsel beeinflussen können und umgekehrt.

2 Introduction

2.1 Caloric restriction

Reducing calorie intake is a common way to lose weight and improve health, and it may also increase lifespan. Luigi Cornaro wrote about the positive effects of reducing calorie intake in his work “Discorsa della vita sobria” during the 1500s. The first experimental studies of the effects of food restriction in animals started in the early 1900s. In 1917 Osbourne and his colleagues showed that food restriction has positive effects on the lifespan of rats. It also affected their reproductive performance in later life [1].

2.1.1 Definition

CR protocols applied in various studies vary in their degree of restriction, ranging from 10% to 50% reduction in daily caloric intake. The duration of CR can also vary, from short-term interventions lasting a few weeks to lifelong treatment. Some CR protocols restrict all nutrients, while others selectively restrict macronutrients and supplement micronutrients to study the specific effects of CR and prevent malnutrition [2].

2.1.2 Effects

In 1935, McCay et al. showed that the implementation of a 40% CR regimen in rats, initiated at the weaning stage and continued throughout their life, exhibited an extension in lifespan, approaching a nearly two-fold increase [3]. Many followed this early work and it was shown that CR is associated with positive effects on the mean and maximum lifespan in a wide range of multiple species, regardless of sex, ranging from various rat and mouse strains to yeasts, invertebrate animals, fish, hamsters and dogs. [4] [5]. In rodents, implementing CR throughout their entire lives has the potential to extend lifespan by as much as 50%. However, the impact of this restriction diminishes gradually when it is initiated later in life. [6]. This effect is associated with impact on age-related illnesses, including a reduced risk of cancer, neurodegenerative conditions, autoimmune disorders, cardiovascular diseases, and type 2 diabetes mellitus [5]. CR is linked to a complex network of metabolic pathways. These pathways involve insulin-like growth factor 1, sirtuins, adenosine monophosphate (AMP)-

activated protein kinase (AMPK), and target of rapamycin (TOR). The sympathetic and neuroendocrine systems, thyroid hormones, adipokines, and ghrelin have also been related with the positive effects of CR [5]. This collection of processes associated with CR influences the entire body, as indicated by decreases in inflammation, body fat mass, resting metabolic rate, and body temperature. Moreover, CR is also associated with enhanced insulin sensitivity [7]. Due to the varied outcomes associated with CR and the intricate nature of the involved pathways, the precise mechanisms behind the health benefits of CR are not yet fully comprehended.

2.2 Bile acid metabolism

2.2.1 Definition

Bile acids (BAs) play a crucial role in the physiological processes of intestinal nutrient absorption and the biliary secretion of lipids, harmful metabolites, and xenobiotics [8]. BAs are amphiphile molecules that are produced from cholesterol in the liver and stored in the gallbladder [9]. Hepatic BA synthesis is responsible for most of the daily cholesterol turnover in humans [8]. In bile, BAs form micelles with phospholipids and cholesterol. These micelles are released from the gallbladder into the small intestine (SI) in response to food ingestion. BAs play a crucial role in facilitating the digestion and absorption of dietary lipids, steroids, drugs, and lipophilic vitamins in the intestine. The majority of BAs are efficiently absorbed in the ileum, and transported back to the liver through the portal blood circulation and subsequently resecreted into the gallbladder [10] [11]. Due to the high reabsorption rate of BAs in the SI (approx. 95 %), the BA pool is mostly preserved. The human BA pool typically contains approximately 2-4 g of BAs. Each day, hepatocytes synthesize approximately 0.2-0.6 g of BAs to compensate for the loss of BAs through fecal excretion [12]. There is evidence indicating that BA play a noteworthy role as signaling molecules in various physiological processes. These include the regulation of lipid, glucose, and energy metabolism, drug metabolism, as well as modulation of immune response [11].

2.2.2 Primary BAs

BAs produced from cholesterol in hepatocytes are called primary BAs. They are synthesized through two main biosynthetic pathways known as the classical pathway and the alternative pathway. The specific contributions of these pathways to the production of hepatic BAs vary among different species. In adult humans, the classical pathway for BA synthesis is the primary contributor, accounting for approximately 90% of total BA production in hepatocytes. On the other hand, the alternative pathway plays a minor role, contributing less than 10% to the overall hepatic BA production. In rodents, both pathways make comparable contributions to the production of BAs. Once synthesized, the side chain of BAs is highly efficiently conjugated to glycine or taurine. Conjugated BAs exhibit solubility, impermeability to cell membranes, and resistance to precipitation under physiological pH conditions. The amide bond formed during conjugation resists cleavage by pancreatic carboxypeptidases, resulting in the high stability of conjugated BAs within the intestinal lumen [12]. In mice, 95% of BAs are conjugated to taurine rather than glycine [8], while the proportion of glycine to taurine conjugation in humans is about 3:1. [13].

2.2.3 Secondary BAs

When bile enters the SI, some of the BAs are further modified by bacterial bile salt hydrolases (BSH) in the ileum and colon to produce secondary BAs. During this modification, the taurine-, and glycine-conjugated BAs are deconjugated [14]. Unconjugated primary and secondary BAs are hydrophobic and passively absorbed in the ileum and colon [10]. The human BA pool includes the primary BAs, cholic acid (CA), and chenodeoxycholic acid (CDCA), as well as secondary BAs, such as deoxycholic acid (DCA) and a small quantity of lithocholic acids (LCA) [15]. Primary BAs are produced in hepatocytes, while secondary BAs are created from primary BAs by bacterial enzymes in the small and large intestine. The human BA pool is made up of around 80% CDCA and CA, 20% DCA, small amounts of LCA and UDCA, and other secondary BAs in lesser proportions. CDCA and CA are typically found in nearly equal quantities in humans, but this can vary between individuals. In mice,

taurocholic acid (TCA), tauro α- muricholic acid and tauro β- muricholic acid are the most common BAs [12].

2.2.3.1 Microbiota

Diets rich in fat, disturbances in circadian rhythms, variations in feeding schedules and fasting, the use of medications, consumption of alcohol, and hormonal changes have the potential to impact the composition of the intestinal bacteria, leading to modifications in BA metabolism, homeostasis, and the metabolism of the host organism [16]. The biotransformation of primary BAs into secondary BAs takes place in the SI and colon. The SI has a low bacterial population but is the main site for the reabsorption of BAs and the digestion and absorption of nutrients. In comparison, the large intestine has a very high bacterial population [12]. In the colon, bacterial 7α-dehydroxylases transform CA to DCA, and CDCA to LCA [17]. DCA possesses strong antimicrobial properties, which help regulate bacterial overgrowth and support the integrity of the intestinal barrier. The activities of BSH and 7α-hydroxysteroid dehydrogenase (HSDH) influence the level of secondary BA synthesis, the specific types of BAs present, and the hydrophobic nature of BAs within the enterohepatic circulation. Mice treated with antibiotics or raised in a germ-free environment demonstrate an elevated level of BA synthesis and an increased BA pool size of approximately 30% compared to mice raised in a conventional setting [18]. Therefore, the gut bacteria can influence the overall size of the BA pool in the liver, gallbladder, bile, and intestine. Further, intestinal microbiota produces short-chain fatty acids (SCFA) like acetate, butyrate, and propionate through the utilization of polysaccharides, cellulose, and starch. The Gram-positive bacteria *Clostridium*, *Enterococcus*, *Bifidobacterium*, and *Lactobacillus*, as well as the Gram-negative genus *Bacteroides*, exhibit increased BSH activity in the SI and colon. These bacterial genera possess elevated levels of HSDH activity and play a role in various stages of 7α-dehydroxylation, which is essential for the synthesis of secondary BAs by intestinal microbiota [14]. Studies of the gut microbiome of humans and mice have revealed a link between certain diseases and gut microbiota composition. Conditions like non-alcoholic fatty liver disease, type 2 diabetes mellitus, and hepatocellular

carcinoma have been associated with specific microbial profiles [12]. When examining human feces, it has been observed that around 90% of the bacteria present belong to the two phyla *Firmicutes* and *Bacteroidetes*. The proportions of *Firmicutes* and *Bacteroidetes* in the gut have been linked to obesity in both mouse models and human participants [19].

2.2.3.2 Taurine

Taurine is a semi-essential amino acid that is not utilized in protein synthesis. It is ubiquitous in mammalian tissue and the most prevalent free amino acids in heart, retina, skeletal muscle, brain, and leukocytes [20]. In previous studies, it was shown that taurine has anti-inflammatory, antioxidative [21] and osmoprotective [22] effects. Further taurine stimulates the expression of anti-inflammatory factors in human intestinal epithelial Caco-2 cells [23]. It additionally reduces the proliferation of harmful bacteria and enhances the synthesis of SCFA [24]. Numerous physiological impacts of taurine rely on its intracellular levels, primarily regulated by the taurine-producing enzymes cysteine dioxygenase and cysteine sulfinate decarboxylase, as well as the taurine transporter (*TauT*) [25]. Therefore, taurine can also be produced endogenously, primarily in the liver [26]. Yet, the primary source of taurine is dietary protein [27]. CR is linked to elevated levels of taurine-conjugated BAs such as TCA and tauroursodeoxycholic acid (TUDCA) in the intestinal contents of mice undergoing CR compared to those fed *ad libitum* [28]. In a recent study conducted by Gregor A. et al., the researchers explored the effects of increased taurine-conjugated BA levels in the gut due to CR. They hypothesized that the rise in taurine-conjugated BAs associated with CR would coincide with an increase in free taurine concentrations resulting from deconjugation in the gut [29].

2.2.4 Receptors and transporters involved in BA metabolism & inflammation

2.2.4.1 Taurine Transporter (TauT)

Most biological functions of taurine rely upon the intracellular concentration of taurine, which is determined for a given cell by its capacity to transport taurine from the extracellular medium and its ability to synthesize taurine. The contribution of taurine

transport depends on the expression level and regulation of *TauT*, but also on the concentration of taurine in the extracellular medium, which is related to the taurine concentration in the blood. *TauT* is a sodium- and chloride-dependent transporter with high affinity and low capacity. Taurine transport requires at least two natrium ions and one chloride ion to transport a taurine molecule across the cell membrane. *TauT* show a narrow specificity for β-amino acids like taurine, hypotaurine, and β-alanine [25].

2.2.4.2 Ileal apical sodium-dependent bile acid cotransporter (Ibat)

The ileal apical sodium-dependent bile acid cotransporter (*Ibat*) is a glycoprotein that plays a crucial role as a BA transporter. The highest expression levels are detected in the ileum. *Ibat* is situated in the apical brush border membrane of enterocytes in the ileum and facilitates the initial absorption of BAs. Its expression is also observed in tissues involved in the enterohepatic circulation of BAs. Additionally, *Ibat* is expressed in renal proximal tubule cells, biliary epithelium, and kidneys. In both the kidney and ileum, the majority of the available BAs in the adjacent lumen are absorbed. *Ibat* recognizes various physiological substrates, including primary unconjugated BAs such as CA, DCA, CDCA, and UDCA, as well as their taurine-conjugates. [30].

2.2.4.3 Multidrug resistance-associated protein 1 (Mrp1)

Initially recognized for its ability to confer multidrug resistance in lung cancer cells, the human ATP-binding cassette transporter multidrug resistance protein 1 (*Mrp1*) has subsequently been found to have additional functions. *Mrp1* is involved in the protection of certain tissues against xenobiotics and facilitating the cellular elimination of the proinflammatory compound cysteinyl leukotriene C4, as well as a wide range of other organic anions derived from both endogenous and exogenous sources. Many of these compounds are conjugates of glutathione (GSH) or glucuronide, which are products of phase II drug metabolism. Furthermore, *Mrp1* contributes to the cellular efflux of both reduced and oxidized forms of GSH, thereby influencing numerous physiological and pathological processes, including oxidative stress [31].

2.2.4.4 Microsomal GSH S-transferase 1 (Mgst1)

Glutathione S-transferases (GSTs) are a group of enzymes that catalyze the conjugation of various compounds, such as carcinogens, mutagens, toxins, and pharmacologically active substances, with glutathione (GSH) [32]. Their primary role is to attach electrophiles to GSH, effectively neutralizing them and protecting the cells. Microsomal glutathione S-transferase 1 (*Mgst1*) is an enzyme bound to the cell membrane, predominantly found in the liver. It is present in the endoplasmic reticulum as well as the inner and outer mitochondrial membranes. Smaller amounts of *Mgst1* are also found in other tissues. This particular enzyme becomes active when the cells experience oxidative stress [33]. *Mgst1* belongs to the MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) superfamily, which includes enzymes that participate in detoxification processes and provide protection against lipid peroxidation [34].

2.2.4.5 Signal transducer and activator of transcription 1 (Stat1)

Signal transducers and activators of transcription (STATs) are latent transcription factors located in the cytoplasm. They play a crucial role in facilitating numerous biological reactions such as cell growth, viability, programmed cell death, and cellular differentiation. [35]. Interferons (IFNs) are the primary stimulants for *Stat1* activation. In addition, other cytokines such as Interleukin-2 (IL), IL-6, platelet-derived growth factor, epidermal growth factor, hepatocyte growth factor, tumor necrosis factor, and angiotensin II can also trigger *Stat1* activation [36]. *Stat1* has various biological functions, including the regulation of cell growth through the control of cell cycle-related gene expression. It promotes the expression of Cyclin-dependent kinase inhibitors P21 and P27, while also suppressing the expression of cyclin to further regulate cell growth [37]. Moreover, *Stat1* plays a role in controlling cell differentiation. By undergoing phosphorylation, *Stat1* can govern the differentiation process of human granulocytes and osteoblasts [38] [39]. Additionally, *Stat1* actively contributes to cell apoptosis. Its primary mechanism for promoting apoptosis involves stimulating the production of various apoptosis-related proteins, including precursor forms of caspase 1, 3, and 11. Furthermore, *Stat1* interacts with the tumor protein p53, further

influencing the apoptotic pathways [40] [41]. Moreover, *Stat1* plays a role in immune system regulation by actively participating in various antigen presentation processes that are dependent on the major histocompatibility complex (MHC) [42]. Additionally, it is involved in the initial development of B-cells [43]. A study conducted by Dupuis S. et al. demonstrated that the lack of *Stat1* or a mutation in the *Stat1* gene significantly heightens the body's vulnerability to parasites, bacteria, viruses, and other pathogens [44].

2.2.4.6 Organic Solute Transporter subunit alpha (Ostα)

Ostα serves as another BA transporter responsible for facilitating the export of BAs across the basolateral membrane [30] It functions as the primary efflux transporter for BAs in the intestine, playing a crucial role in excreting BAs into the portal circulation [8]. *Ostα* is predominantly located on the lateral and basolateral plasma membrane of ileal enterocytes and exhibits high expression levels in hepatocytes, cholangiocytes, and renal proximal tubule cells [30]. Additionally, *Ostα* plays a significant role in promoting the efflux of secondary BAs through the sinusoidal membrane [8]. Studies conducted by Dawson PA. revealed that humans exhibit higher gene expression levels of *Ostα* in the liver, whereas rodents have nearly undetectable levels of *Ostα/β* expression in the liver. However, in the case of cholestasis, both humans and rodents experience a dramatic increase in *Ostα/β* gene expression. Inactivation of the *Ostα* gene leads to alterations in BA metabolism and impairs BA absorption. Furthermore, experiments conducted on *Ostα* knockout mice demonstrated that the efflux of BAs is impaired, resulting in the accumulation of BAs in ileal enterocytes [30].

2.2.4.7 Toll-like receptor 3 (Tlr3)

Toll-like receptor 3 *(Tlr3)*, a key component of the innate immune receptor family, plays a crucial role in regulating immune responses. It facilitates the maturation and differentiation of immune cells and is involved in responding to pro-inflammatory factors. Activation of *Tlr3* occurs through recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), which are central to the pathophysiology

of numerous inflammation-associated diseases [45]. *Tlr3*, as a member of the TLR family, triggers the transcriptional induction of type I IFNs, proinflammatory cytokines, and chemokines to produce an antiviral response in the host. Research has demonstrated that *Tlr3* is unique among TLR family members in that it is the only RNA sensor entirely dependent on Toll interleukin-1 receptor (TIR)-domain-containing adaptor-inducing IFN-β (TRIF) [46]. TLRs are primarily found on endosome and lysosome membranes or on the surfaces of macrophages, dendritic cells, and other cell types. They play a role in detecting a variety of PAMPs associated with microbial cell walls and endosomal nucleic acids. [47] [48].

*2.2.4.8 Pregnane X Receptor (*PXR/*Pxr)*

Pregnane X Receptor (PXR) is a nuclear receptor known for its pivotal role in enhancing the body's defense against xenobiotics [49]. PXR is primarily activated by BAs, sterols, and various pharmaceutical agents. Additionally, it can stimulate the expression of drug-metabolizing cytochrome P450 (CYP) enzymes such as CYP3A, CYP2B, and CYP2C [50]. PXR also regulates many genes involved in BA metabolism, including CYP7A1, the rate-limiting enzyme in BA synthesis. *Pxr* knockout mice have been shown to have dysregulated CYP7A1 levels [49]. Apart from BAs, oxysterols and cholesterol also possess the ability to activate PXR. Li T. et al. conducted a study where *Pxr* knockout mice, subjected to a high-cholesterol diet, manifested hepatorenal failure. This suggests that PXR might be essential for the detoxification of cholesterol metabolites. These findings provide support for the potential involvement of PXR in the activation of CYP27A1, which is part of the alternative BA pathway. This activation could serve as an adaptive response to effectively eliminate excess cholesterol within the intestine [51].

2.3 mTOR

2.3.1 Definition

Mammalian Target of Rapamycin (mTOR) is a protein kinase that regulates cellular growth and metabolic processes in reaction to changes in nutrient availability, the presence of

growth factors, and the cellular energy status. Its dysregulation is a common occurrence in various conditions such as cancer and metabolic disorders [52]. mTOR, specifically mTORC1 (mTOR complex 1), acts as a sensor for nutrients and energy at both the cellular and systemic levels. The signaling responses of mTORC1 in crucial metabolic organs like the liver, adipose tissue, muscle, and hypothalamus regulate the overall metabolism of the whole body during periods of feeding and fasting. In a healthy state, the balance of glucose in the body is maintained by storing glucose as glycogen when nutrient levels are high. Conversely, when blood glucose levels decrease, glycogen is broken down into glucose, ensuring glucose homeostasis. These processes are tightly regulated by insulin and its downstream signaling to mTORC2 and mTORC1. Additionally, excessive nutrients can be stored as triglycerides in white adipose tissue (WAT) [52]. Activation of mTORC1 has been associated with the promotion of obesity and the accumulation of lipids, potentially through the upregulation of transcription factors such as peroxisome proliferator-activated receptor-γ (PPARγ) and sterol regulatory element-binding protein 1 (SREBP1) and SREBP2 [53] [54]. Consistently consuming an excessive amount of food results in persistent high mTOR signaling and disrupts the body's metabolic regulation. This can ultimately cause obesity, and type 2 diabetes. The development of diabetes and cancer can be attributed to dysregulated metabolic homeostasis and disrupted growth control mechanisms, respectively. Therefore, it is unsurprising that mTOR is involved in the underlying causes of these conditions [52]. Another aspect of TOR worth considering is its involvement in extending lifespan. Prolonged inhibition of mTOR signaling, emulates the effects of dietary restriction, a well-documented method of prolonging lifespan [55] [56].

2.3.1.1 Rapamycin

Rapamycin works as an allosteric mTOR inhibitor. Additionally, mTORC1 is acutely sensitive to inhibition by rapamycin [52]. Harrison et al. conducted a study demonstrating that adult mice treated with rapamycin exhibited a notable increase in lifespan and improved health indicators compared to a control group that did not receive treatment [57]. The extension of lifespan through TOR inhibition has been observed in various organisms, including yeast,

flies, and worms. Moreover, mTOR inhibition has shown considerable clinical advantages in managing cancer, diabetes, and their associated complications, as well as alleviating age-related disorders [58] [59] [52]. Li J. et al. showed in their review that the inhibition of the mTOR pathway by rapamycin has been found to have both beneficial and detrimental effects on the metabolism [60].

2.3.1.2 MHY1485

4,6-dimorpholino-N-(4-nitrophenyl)-1,3,5-triazin-2-amine (MHY1485) is a potent cell-permeable mTOR activator, and also potently inhibits autophagy. MHY1485, a compound with the chemical name 4,6-dimorpholino-N-(4-nitrophenyl)-1,3,5-triazin-2-amine, acts as a potent activator of mTOR and exhibits high cell permeability. Additionally, it demonstrates strong inhibitory effects on autophagy. Choi Y.J. et al. investigated the impact of MHY1485 on autophagic process interference by hindering the fusion of lysosomes in Ac2F cells derived from rat hepatocytes. The morpholino triazine structure of MHY1485 is recognized for its ability to bind to mTOR. The findings revealed that MHY1485 triggered mTOR activity, an additional modulator of autophagy. Based on these results, the authors highly propose that MHY1485 has promising potential as a novel autophagy inhibitor [61].

3 Aims

The primary objective of this master thesis is to investigate the effects of CR on taurine-conjugated and unconjugated BAs in mice. Moreover, the expression levels of several transporters, receptors, and transcriptional factors associated with BA metabolism were evaluated with the aim of understanding the underlying mechanisms. The effects of cage bedding, fecal microbiota transplants, and alterations in the gut bacteria were also investigated to provide insights into the broader connection. Additionally, the BSH activity was measured to assess the ability to deconjugate BAs through microbiota in feces. Furthermore, diets containing different amounts of taurine were administered to mice to assess the impact of dietary taurine on the composition of BAs. Lastly, the CR phenotype was challenged through pharmacological agents that modulate the mTOR pathway. This thesis contributes to a better understanding of the complex relationship between CR and BAs.

4 Methods

4.1 Materials & equipment

4.1.1 Liquid Chromatography Mass Spectrometry (LCMS)

LCMS Shimadzu 8040:

- Autosampler SIL-20AC HT
- Liquid Chromatograph LC-20 AD
- Degassing Unit DGU-20A5
- Atlantis T3 3 µm column (2.1 × 150 mm, Waters, Milford, MA, USA)
- LabSolutions (PC software)
- High Pressure Switching Valve FCV-20AH2o UV/VIS Detector SPD-20A
- Column Oven CTO-20AC

High-performance liquid chromatography (HPLC) vials: 2 ml; Sigma-Aldrich Chemie GmbH

Precellys tubes: Fisherbrand™ Free-Standing Microcentrifuge Tubes with Screw Caps

4.1.2 Quantitative real-time PCR (qPCR)

Dispenser: 0.5 - 10 µl, 5 - 100 µl, Eppendorf Xplorer®; Eppendorf

qPCR plate: Frame Star® 384 Well Skirted PCR Plate, Roche Style; 4titude® via Brooks Life Sciences

qPCR seal: 4titude® via Brooks Life Sciences

Nanodrop: NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer; ThermoFisher Scientific

qPCR: applied biosystems®, Quant studioTM 6 Flex Real-time PCR System, OptiFlexTM Optics System; Life Technologies Holding (Singapore)

DNA Kit: AllPrep® DNA/RNA Micro Kit; QIAGEN GmbH

4.1.3 BAs

- **Bile Salts;** MDL: MFCD00130648; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Chenodeoxycholic acid** (CDCA) ≥96% (C24H40O4); CAS: 474-25-9; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Cholic acid**, sodium salt (CA) (C24H39O5Na); CAS: 361-09-1; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Deoxycholic acid**, sodium salt (DCA) (C24H39NaO4); CAS: 302-95-4; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Dehydrocholic acid** (DHCA) ≥99% (C24H34O5); CAS: 81-23-2; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Sodium Taurolithocholate** (TLCA) (C26H44NO5SNa); CAS: 6042-32-6; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Taurocholic acid**, sodium salt (TCA) (C26H44NO7SNa); CAS: 145-42-6; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Taurodeoxycholic acid**, sodium salt (TDCA) (C26H44NO6SNa); CAS: 1180-95-6; Merck KGaA (Darmstadt, Germany)
- **Tauroursodeoxycholic acid**, sodium salt (TUDCA) (C26H44NNaO6S); Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Ursodeoxycholic acid** (UDCA) ≥99% (C24H40O4); CAS: 128-13-2; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)

4.1.4 General usage

Microcentrifuge tubes: 1.5ml and 2ml; Eppendorf Austria GmbH (Vienna, Austria)

Pipette: 2.5, 10, 100, 200, 1000 µl; Eppendorf Reference® 2; Eppendorf

Tips: 0.1 - 20 µL, 10 - 100 µl, 100 -1 000 µl, epT.I.P.S.® 384; Eppendorf

4.1.5 Further equipment

- **Vortexer:** Reax 2000; Heidolph
- **Scale:** Sartorius Laborwaage Entris Model ENTRIS224-1S

- **Centrifuge big:** Thermo Scientific™ Megafuge™ 8; fisher scientific
- **Centrifuge small:** Microcentrifuges, ventilated/refrigerated, Micro Star 17 / 17R, VWR
- **Concentrator:** Concentrator plus/Vacufuge® plus, Eppendorf
- **Rocking shaker:** Fisherbrand™ Sea Star orbital shaker package; fisher scientific
- **Hot water bath tub:** GFL 1002; Müller-Scherr; Linz
- **Precellys shaker:** Homogenisator, Precellys® 24; VWR

4.2 Reagents and Chemicals

4.2.1 Solvents for LCMS

The mobile phase A consisted of water and eluent B was acetonitrile/methanol (MeOH) (3/1, v/v), both containing 0.1% formic acid (FA) and a concentration of 20 mM ammonium acetate.

Solvent A: Water + 0.01% FA + 20 mM Ammoniumacetate (1.54 g/l)

Solvent B: Acetonitrile 3:1 MeOH + 0.01% FA + 20 mM Ammoniumacetate (1.54 g/l)

4.2.2 Reagents

- **Ethanol:** absolute EMPLURA®; Merck
- **Methanol:** ROTISOLV® HPLC Gradient Grade; ROTH
- **Acetonitrile:** ROTISOLV® HPLC Gradient Grade; ROTH
- **Formic acid:** ROTIPURAN® ≥98%, p.a., ACS; ROTH
- **HPLC water:** ROTISOLV® HPLC Gradient Grade, ROTH
- **Ammoniumacetate:** ≥97%, p.a., ACS, ROTH
- **Dulbecco´s Modified Eagle Medium:** ROTI®Cell DMEM: ROTH
- **2-Mercaptoethanol**: ≥99%, p.a., 14.3M, ROTH

4.3 Sample preparation

4.3.1 LCMS

4.3.1.1 Tissue (liver, small intestine, colon, adipose tissues)

Samples were prepared according to the protocol of Gregor et al. Tissue was weighted in Precellys homogenizing tubes with 1.4 mm ceramic beads, and nine times the volume of MeOH absolute at −20 °C was added. Samples were homogenized in the Precellys 24 Tissue Homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) twice for 30 s at 4000 rpm, and centrifuged for 5 min at 3500 x g at 4°C. The supernatants were transferred to new 1.5 ml Eppendorf tubes, and, to remove the remaining debris, the centrifugation step was repeated this time at 13000 x g. Supernatants were transferred into new Eppendorf tubes and after the second centrifugation, supernatants were directly transferred into HPLC vials [29] [62].

4.3.1.2 Plasma

To achieve proper homogenization, 50 µl of plasma sample were added with 150 µl of MeOH, followed by vigorous vortexing and shaking for a duration of 10 minutes. After shaking, the mixture was centrifuged for 10 minutes at 4 °C with 12000 x g to remove any particulate matter and obtain a clear supernatant. 100 µl of the supernatant were then evaporated at 60 °C for 25 minutes to remove the solvent and concentrate the sample. The sample was redissolved in 50 µl of MeOH and transferred into HPLC vials.

4.3.1.3 BSH assay

Fresh feces were collected at the dissection and immediately incubated with Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) (240 µl/10 mg) and a taurine-conjugated-BA mixture (10 µl/10 mg) containing TCA, TLCA, TUDCA, and TDCA (all Sigma-Aldrich, St. Louis, MO, USA). The mixture was incubated in a 37 °C water bath. Medium samples were collected over 30 min; evaporated and frozen at -20 °C for measurement of BSH activity. For the measurement, 60µl of MeOH was added to the

sample, shaken for 10 seconds, and then centrifuged for 5 minutes at 4 °C at maximum speed. The supernatant was transferred to HPLC vials.

4.3.1.4 Preparation of Standards

In order to calculate a standard curve for the concentration of an analyte in samples, at the beginning of a measurement, a standard mix of all BAs was prepared and measured. Those standards were prepared by a dilution series. First, stock solutions of all BAs (TCA, LCA, CDCA, UDCA, TLCA, TDCA, DHCA, TUDCA, and bile salts (CA & DCA)) with a concentration of 1 mg/1 ml MeOH were prepared. Standard 1 (STD 1) was prepared by mixing 100 µl of stock solution from TCA, LCA, bile salts, CDCA, DHCA, TUDCA, UDCA, and 20 µl of TLCA and TDCA and filled up with 260 µl MeOH to 1 ml. The next steps follow a typical dilution series, by taking 500 µl of STD 1 and pipetting it into STD 2, and adding 500 µl MeOH. After vortexing again, 500 µl of STD 2 was pipetted into STD 3 with 500 µl MeOH again. And so forth, except STD 6, which is prepared from STD 4 (Figure 1).

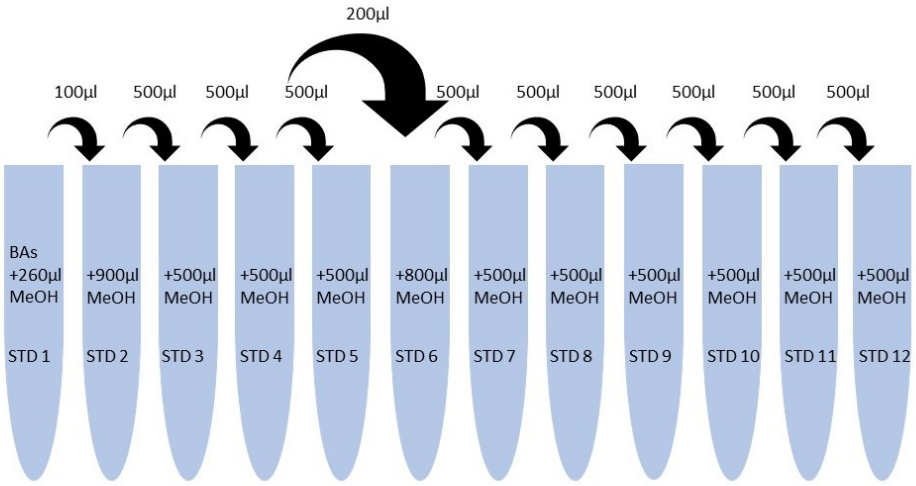


Figure 1 Dilution series

The standards that had been prepared were transferred to HPLC vials to measure them using LCMS. By utilizing the known concentrations of the standards and determining the

area under the curve (AUC) from the measurement, a standard curve was created for each BA through the use of Microsoft® Excel.

4.3.2 qPCR

4.3.2.1 DNA extraction

For DNA extraction the AllPrep® DNA/RNA Micro Kit from QIAGEN GmbH was used. For the DNA extraction around 10 mg of tissue was weighed in and put into Eppendorf tubes. At the beginning of the extraction process, the required buffers from the AllPrep® DNA/RNA Micro Kit were prepared by adding 25 ml ethanol (96-100%) to the bottle containing 19 ml buffer AW1 concentrate, then 30 ml ethanol (96-100%) was added to the bottle containing 13 ml buffer AW2 concentrate, and 10 µl b-mercaptoethanol per 1 ml buffer was added to buffer RLT Plus before use. The first step was to disrupt the tissue and homogenize in 350 µl Buffer RLT Plus with a pipet tip until it was fully homogenized. After briefly centrifuging, the supernatant was taken and transferred to an AllPrep DNA Mini spin column placed in a supplied 2 ml collection tube. The next step was to centrifuge the lysate for 30 s at 20,000 x g. The AllPrep DNA Mini spin column was placed in a new 2ml collection tube and then 350 µl of Buffer AW1 was added and again centrifuged for 30 s at 20,000 x g. After discarding the flow-through, 20 µl of Proteinase K was added to 60 µl Buffer AW1 and applied to the AllPrep DNA Mini spin column membrane, and incubated for 5 minutes. In the next step, 350µl Buffer AW1 was added to the spin column and centrifuged as before. Then 500 µl Buffer AW2 was added to the AllPrep DNA Mini spin column and centrifuged for 2 min at 20,000 x g to wash and dry the column. After carefully removing the spin tube from the collection tube, it was placed in a new 1.5 ml collection tube and 100 µl Buffer EB was added directly to the spin column membrane. After 1 min incubation, another centrifugation step followed for 1 min at 8000 x g to elute the DNA. Samples were frozen for further analysis. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA. A ratio of ~1.8 was generally accepted. To further prepare the samples, the DNA was diluted to the

same concentration calculated with the dilution formula (c1 x V1 = c2 x V2) with the NanoDrop™ using nuclease-free water.

4.4 Model organism

4.4.1 Mice C57BL/6

For the experiments, male C57BL/6NRj were used and purchased from Janvier Inc. Labs (Le Genest, France). The mice were divided according to their diet groups and kept in a standard housing cage with a 12h light/dark cycle. At the age of 10 weeks, the treatment started and lasted for 2 weeks. The mice were fed with standard chow (V153x R/M-H auto diet from SSNIFF-Spezialdiäten GmbH, Soest, Germany) unless otherwise specified. The groups had no significant differences in body weight at the beginning of the experiments. The mice treated with CR were submitted to 2 weeks of CR with a reduction of 25% of daily food intake. At the end of the treatment, the mice were dissected after euthanasia via isoflurane overdose.

4.4.2 Bedding

As cage bedding, Lignocel select, poplar, cubic; from J. Rettenmaier & Söhne GmbH + Co KG (Vienna, Austria) was used. Additionally, mice were housed in cages without any bedding, which was wiped daily.

4.5 Experiments

In this thesis, experiments were carried out with different diets and supplementations to analyze the influence of CR conditions on the BA metabolism of mice. The mice were divided into groups of 3-8. Mice usually consume about 4 g of food per day when fed *ad libitum*. The standard chow diet contained a mixture of grains and grain by-products, oilseed products, minerals, vegetable oils, vitamins, and trace elements.

4.5.1 Experiment I

In the first experiment, mice were divided into two groups to confirm the differences between Ad lib and CR in different tissues. Ileum, liver, plasma, and proximal colon samples

were analyzed and compared. During a period of two weeks, the mice subjected to CR were administered 75% of their daily caloric intake.

4.5.2 Experiment II

In this experiment, the groups consisted of Ad lib and CR mice with and without bedding (Ad lib NB & CR NB), as mice tend to eat more bedding during CR than Ad lib fed mice [63]. Additionally, CR mice with a fecal transplant (FT) from Ad lib or CR-treated mice (CR NB FT Ad lib & CR NB FT CR) were also compared. Ileum, liver, and three different adipose tissues (epididymal white adipose tissue (eWAT), subcutaneous white adipose tissue (sWAT) & brown adipose tissue (BAT)) were analyzed and compared. The cages without bedding were wiped daily. Additionally, a BSH assay was performed to determine differences in the deconjugation capacity of BAs in feces through the impact of bedding and fecal transplant. Furthermore, qPCR was performed for specific gut bacteria (*Bacteroides fragilis, Bifidobacterium, Akkermansia muciniphila, Lactobacillus, Clostridium leptum*) and BSH in the cecum. The fecal transplant groups were gavaged twice within 14 days with 200µl of an antibiotic mixture containing vancomycin 0.5g/l, neomycin 1g/l, ampicillin 1g/l, and metronidazole 1g/l. For the fecal transplant, fresh feces were mixed with PBS (phosphate-buffered saline), vortexed, and centrifuged for 3 min at 1000 x g. Then the mice were gavaged with the supernatant.

4.5.3 Experiment III

For the third experiment, the mice received the usual Ad lib vs CR treatment and three taurine supplemented diets; taurine (Tau), low taurine diet (LTD), and normal taurine diet (NTD). The taurine diet groups were also split into Ad lib and CR. The “Tau” groups had 4% taurine added to their drinking water and were fed standard chow. In contrast, LTD did not contain any taurine, while NTD had 0.5 % taurine added (Table 1). Notably, the standard chow contains small amounts of methionine and cysteine which are absent in LTD and NTD, respectively. In this experiment, jejunum and liver samples were analyzed and compared to observe the influence of dietary taurine on the BA composition.

Table 1 Composition of LTD and NTD

	Low-taurine diet (LTD)	**Normal-taurine diet (NTD)**
Casein	10	10
Cornstarch	53,3	51,8
Sucrose	10	10
Maltodextrin	12,5	12,5
Soybean oil	5	5
Fiber mix	3	3
Vitamin mix w/o choline	1	1
Mineral mix	6	6
Choline	0,2	0,2
Taurine	**0**	**0,5**
L-cysteine	0	0

4.5.4 Experiment IV

The fourth experiment was conducted to challenge the BAs phenotype in CR mice. For this purpose, the animals were divided into four treatment groups, each divided into Ad lib and CR:

- **BSHi:** BSH inhibitor (CAPE with 6mg/ml concentration and 30mg/kg, Sigma-Aldrich Chemie GmbH Steinheim, Germany)
- **BA** supp: BA supplementation (with taurine-conjugated BA by gavage; 50mg/kg of TUDCA, TDCA, TLCA, TCA)
- **Fluvastatin:** statin treatment (20mg/kg by gavage, Sigma-Aldrich Chemie GmbH Steinheim, Germany)
- **Vehicle:** vehicle control with ethanol (EtOH) gavaged

Plasma samples were analyzed and measured, additionally, qPCR was performed to evaluate the expression of genes related to the BA metabolism in cecum samples, including *TauT, Ibat, Mrp1, Mgst1, Stat1, Ostα, Tlr3*, and *Pxr*.

4.5.5 Experiment V

For the fifth experiment, the extent of deconjugation of BAs over time in feces was estimated in three BSH assays. The mice were divided into seven groups with different treatments (Table 2), which were further divided into Ad lib and CR, respectively.

Table 2 Experimental groups BSH assays

	Group treatment	**Group name**
BSH Assay 1	BA supplementation	BA supp
	Fluvastatin	Fluvastatin
	BSH inhibitor	BSHi
BSH Assay 2	Rapamycin	Rapamycin
	MHY1485	MHY1485
BSH Assay 3	TauT inhibitor	TauTi
	Ethacrynic acid	EA

Mice were treated with MHY1485 (1mg/kg/day, ca. 25µg/mouse/day, Sigma-Aldrich Chemie GmbH Steinheim, Germany) and rapamycin (3mg/kg/day, ca. 75µg/mouse/day, Sigma-Aldrich Chemie GmbH Steinheim, Germany) as mTOR activator and inhibitor. Further, ethacrynic acid (EA) (6mg/kg by gavage, Sigma-Aldrich Chemie GmbH Steinheim, Germany) and TauT inhibitor (imidazole-4-acetate 1mM in drinking water, Sigma-Aldrich Chemie GmbH Steinheim, Germany) was assessed.

4.6 Measurement of the samples

4.6.1 LCMS

For the measurement, samples were analyzed using an LCMS-8040 Liquid Chromatograph Mass Spectrometer (Shimadzu Corporation, Kyoto, Japan) with an Atlantis T3 3µm column (2.1 ×150mm, Waters, Milford, MA, USA). The column temperature was at 30°C. The gradient was maintained from an initial 30% solvent B for 5 min, to 100% solvent B at 25 min, which was held constant for 20 min. Afterwards, the composition was reversed to the initial 30% solvent B ratio within 2 min, followed by a 10 min re-equilibration. For data analysis, the expected retention times (Rt) for each BA were used to define the right peaks (Table 3). After collecting the AUCs for the BAs and standards, the concentration of samples was calculated using the linear equitation y= kx+d with Microsoft® Excel.

Table 3 Detection of BAs

BA	m/z	Rt
LCA	375 SIM Mode	32,7
UDCA	410,20>357,2	29,7
DCA	410,35>357,3	29,7
CDCA	410,35>374,4	29,7
DHCA	420,30>385,2	21,9
CA	426,35>373,2	25,8
TLCA	501,35>466,2	25,8
TUDCA	517,32>464,2	18,6
TDCA	517,32>500,2	23,3
TCA	533,35>516,4	19,5

4.6.2 qPCR

qPCR reactions were carried out using the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). For pipetting the samples onto the qPCR plates, the plates were put on ice, and a pipetting scheme was followed. The wells were filled with 2 µl of DNA and 8 µl mastermix (MM) (Table 4). The SYBR Green MM was mixed with RNAse-free water, forward, and reverse primer. 8 µl of MM was required per well. By multiplying the number of wells, the total amount of MM could be calculated.

Table 4 Mastermix preparation

SYBR Green	5 µl
Primer forward	0,3 µl
Primer reverse	0,3 µl
RNAse free water	2,4 µl
multiply by the number of wells	8 µl

After adding the MM, the plates were centrifuged at 1000 x g for 1 min and sealed for the qPCR measurement. The Ct values for each tested gene were normalized to Ct values from the reference gene *Eef1a1* (Eukaryotic translation elongation factor 1 alpha 1). For analysis, the ΔΔCt method was applied.

5 Statistics

For statistical tests, a paired sample t-test in Microsoft® Excel was carried out. A p-value lower than 0.05 was considered statistically significant.

6 Results

6.1 Experiment 1 Confirmation of differences between Ad lib and CR

6.1.1 Ileum

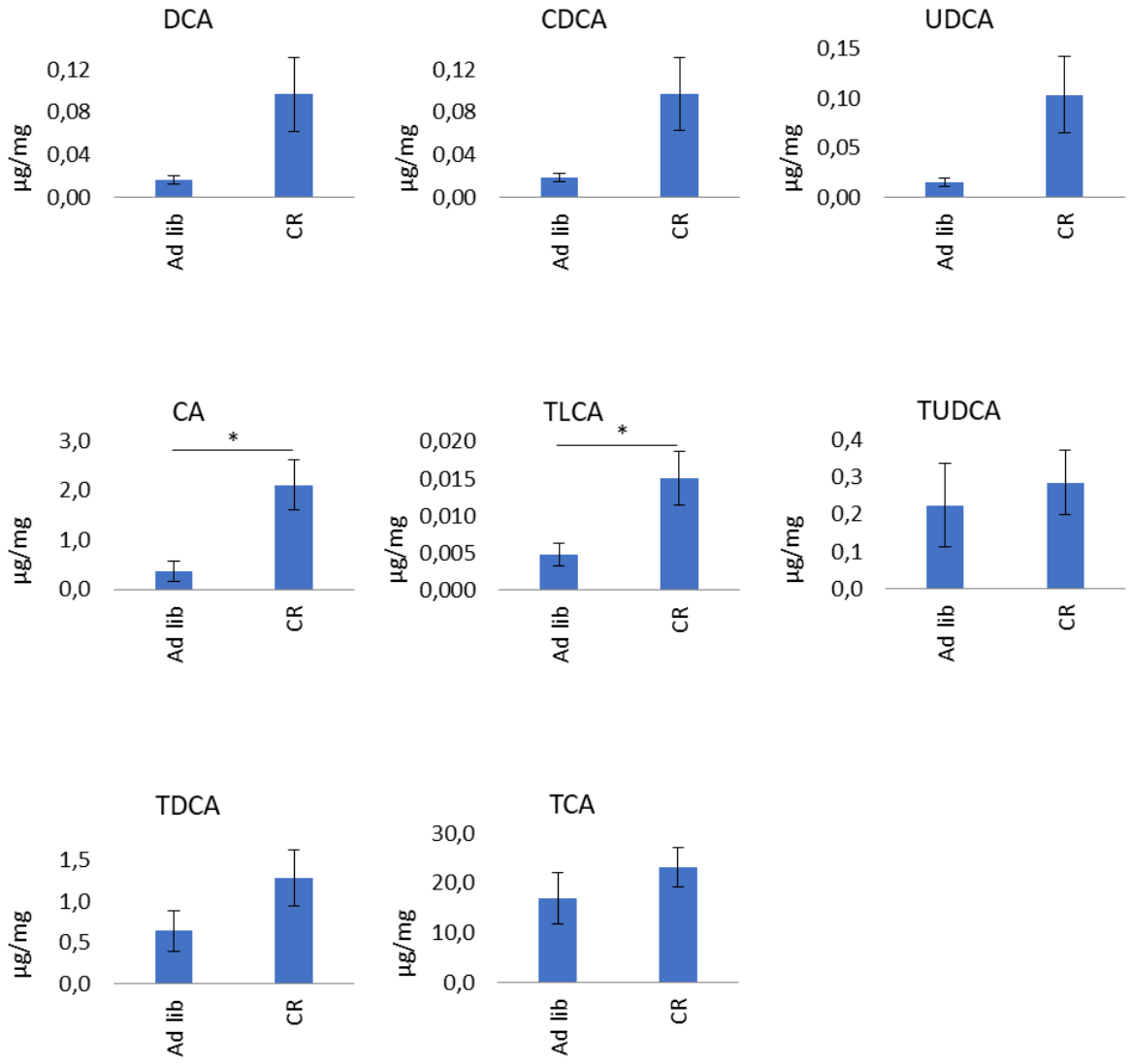


*Figure 2 BAs measured in ileum; Ad lib vs CR. Statistical significances are marked with * indicating p<0.05. Ad lib = ad libitum; CR = caloric restriction.*

LCMS was used to measure the composition of BAs in the ileum, liver, plasma, and proximal colon of *Ad lib and CR* mice. In the ileum, CR mice had statistically significantly more CA and TLCA. For the unconjugated BAs DCA, CDCA, and UDCA, there were also higher amounts in the CR group, but not statistically significant. The taurine-conjugated BAs showed a similar trend, but the differences between groups were smaller (Figure 2).

6.1.2 Liver

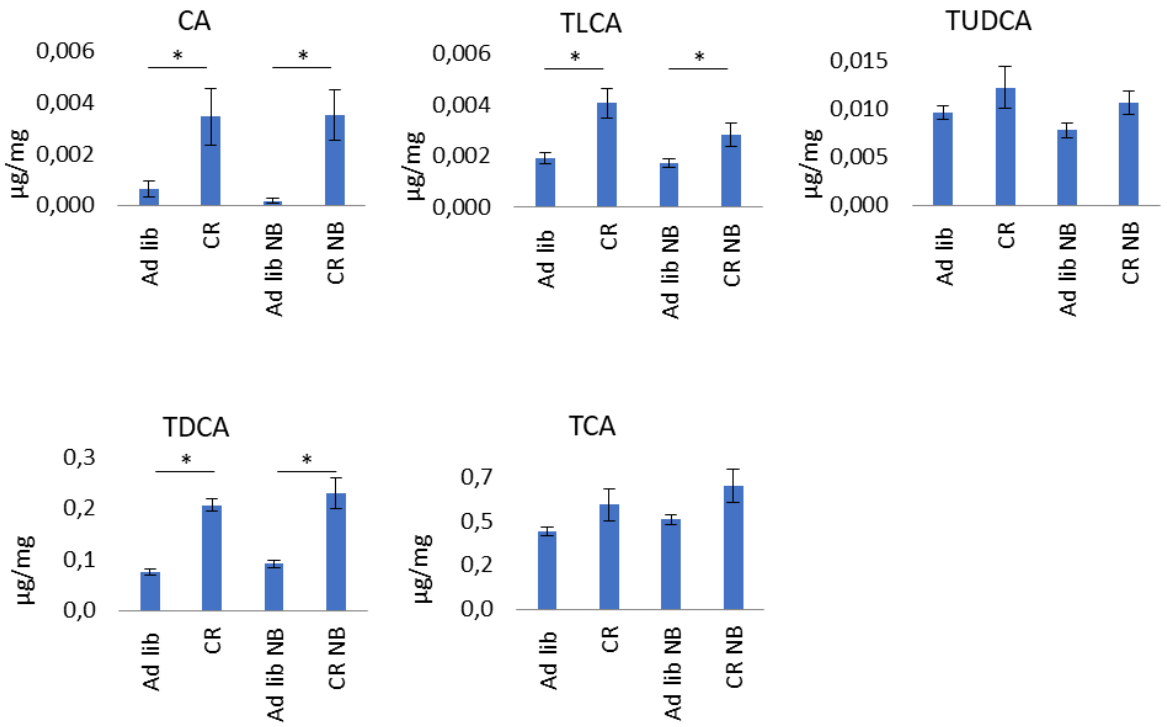


*Figure 3 BAs measured in liver; Ad lib vs CR. Statistical significances are marked with * for p<0.05. NB = no bedding.*

The results from the liver measurement showed statistically significant higher amounts of CA, TLCA, and TDCA in the CR groups, regardless of the presence of bedding. A similar trend was observed for TUDCA and TCA (Figure 3).

6.1.3 Plasma

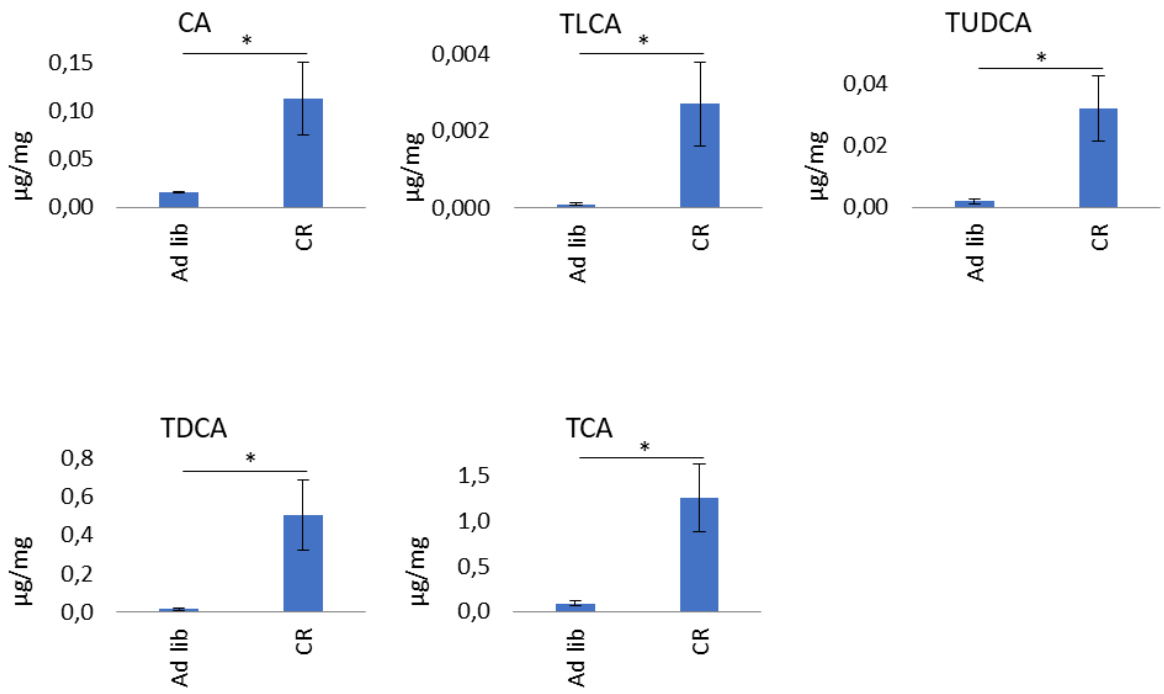


*Figure 4 BAs measured in plasma; Ad lib vs CR. Statistical significances are marked with * for p<0.05.*

In plasma, the results showed a statistically significant higher amount of all measured BAs in the CR group compared to the Ad lib group (Figure 4).

6.1.4 Proximal colon

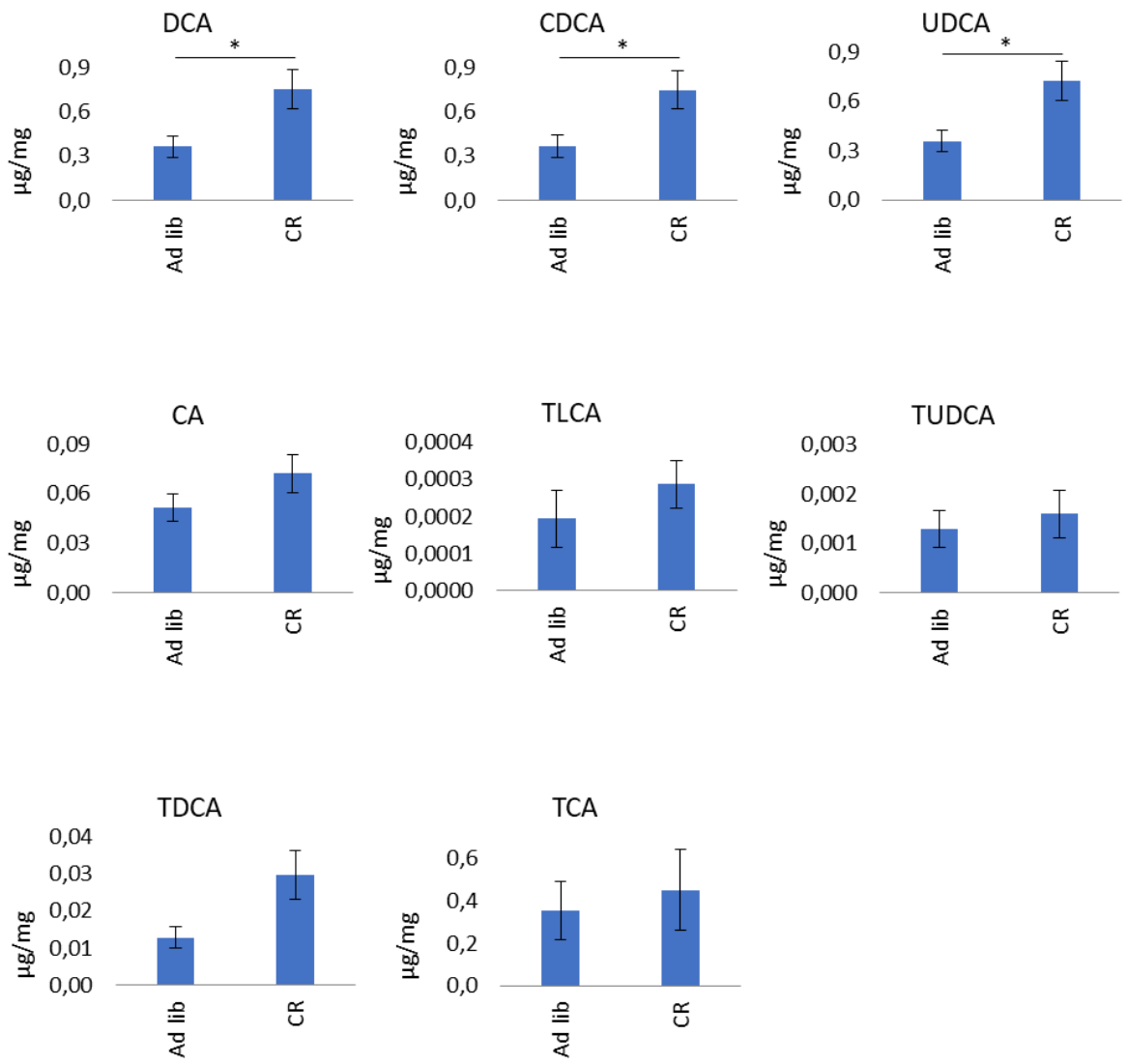


*Figure 5 BAs measured in proximal colon; Ad lib vs CR. Statistical significances are marked with * indicating p<0.05.*

For the proximal colon, the CR mice had higher amounts of BAs in general. For DCA, CDCA, and UDCA there was a statistically significant difference between the two groups. For the taurine-conjugated BAs, the difference was not as pronounced (Figure 5).

6.2 Experiment 2 Impact of bedding and fecal transplant

6.2.1 Ileum

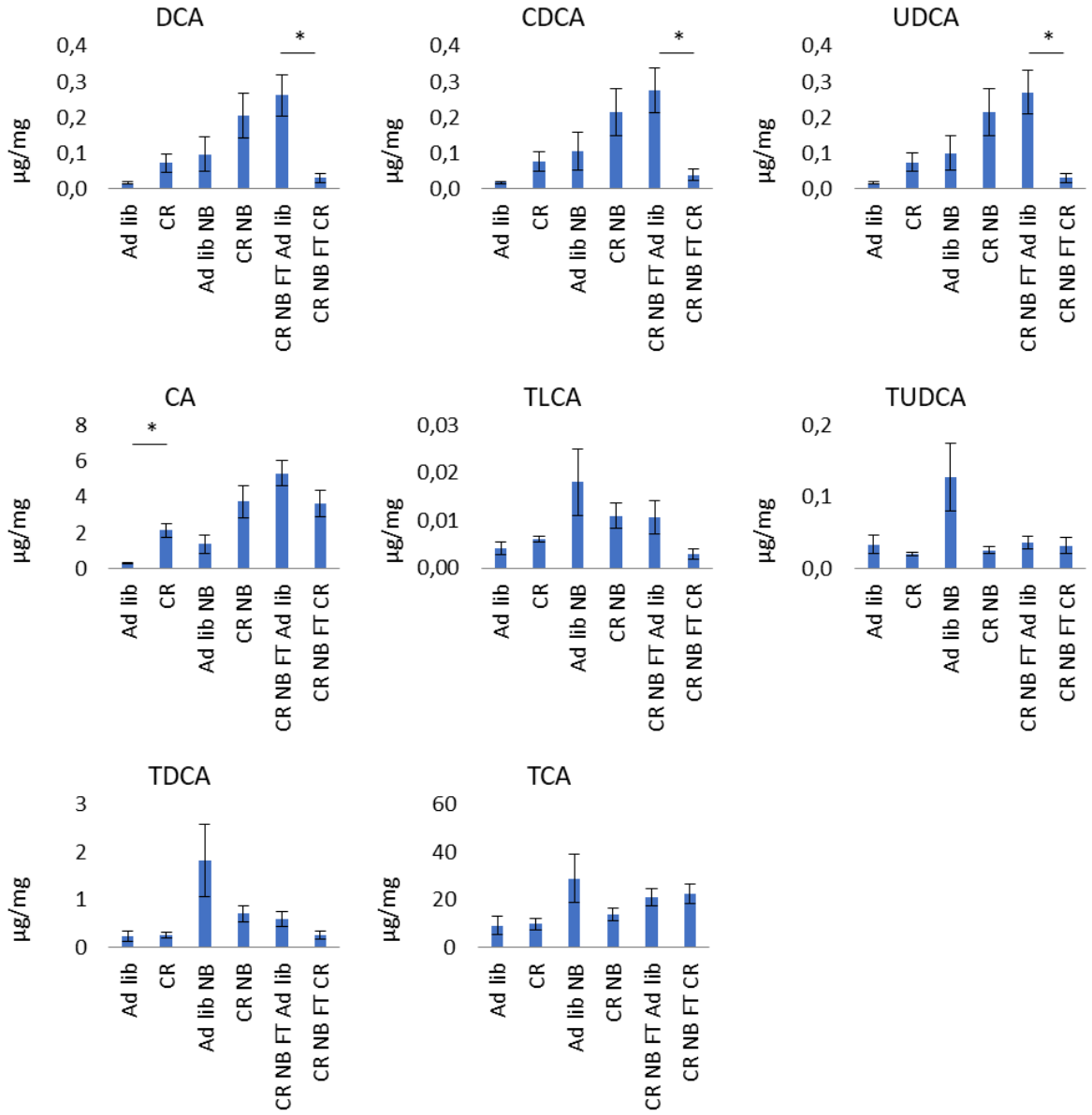


*Figure 6 BAs measured in ileum; beddings and FT. Statistical significances are marked with * for p<0.05. FT = fecal transplant.*

LCMS was used to measure the composition of BAs in the ileum, liver, and adipose tissue of *Ad lib and CR* mice with and without bedding and also CR mice with a fecal transplant from Ad lib or CR-treated mice. In addition, a BSH assay was performed to determine the

differences in the deconjugation capacity of BAs in feces. In the ileum, both CR groups generally had more unconjugated BAs than the Ad lib groups, except for the CR mice that received the CR fecal transplant. Those mice had less unconjugated BAs than the groups which received the fecal transplant from Ad lib mice. The taurine-conjugated BAs were present in the highest concentrations in the Ad lib NB group. There were also minor differences between TDCA and TLCA in the FT (fecal transplant) groups (Figure 6).

6.2.2 Liver

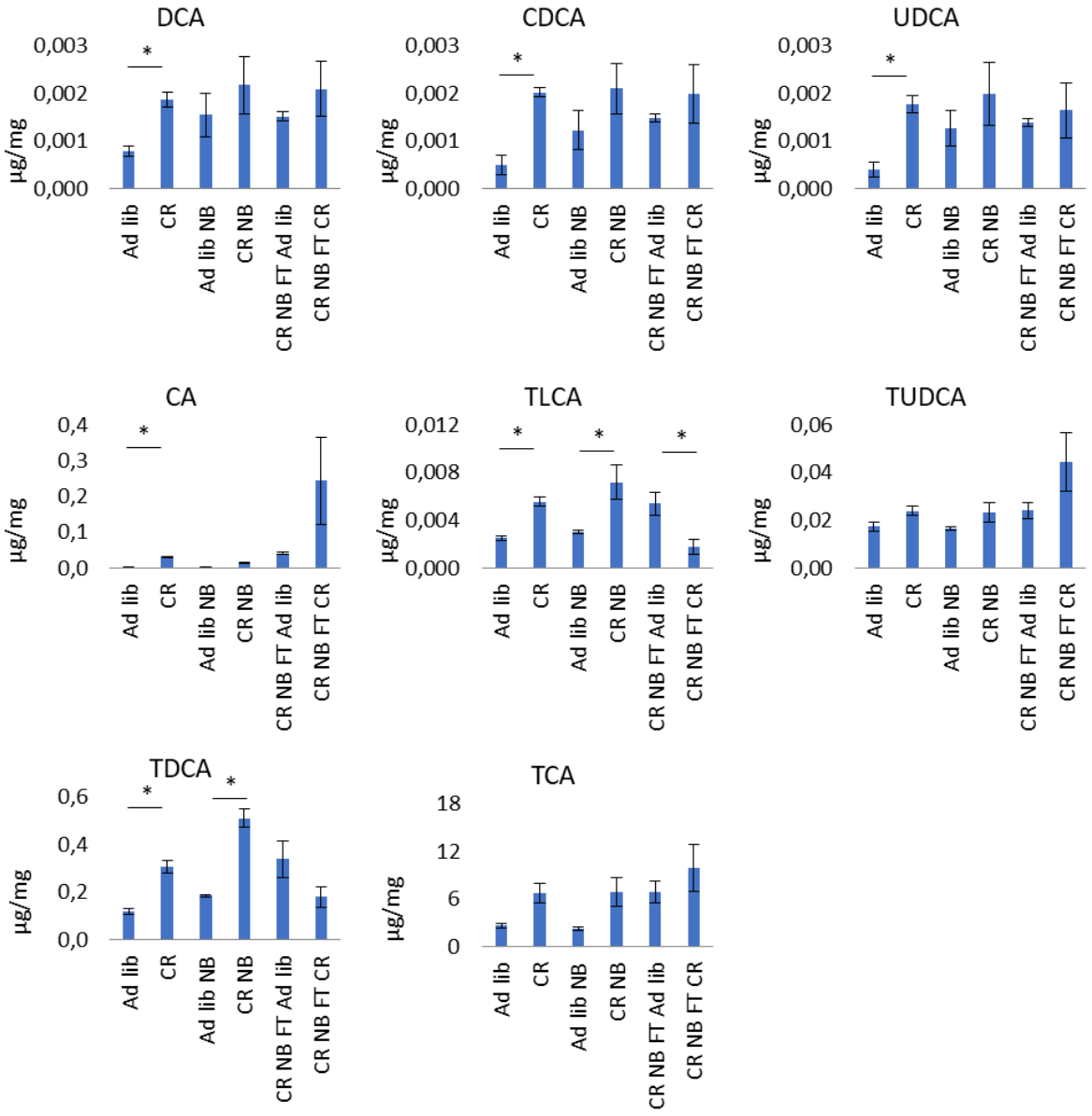


*Figure 7 BAs measured in liver; beddings and FT. Statistical significances are marked with * for p<0.05.*

The results from the liver tissue showed that CR mice had in general higher amounts of unconjugated and conjugated BAs in comparison to their Ad lib controls, but for the NB groups, the difference was not statistically significant in the unconjugated BAs. The same was the case for the FT groups. For the taurine-conjugated BAs, the mice receiving CR

transplant had higher amounts of TUDCA and TCA, but less TLCA and TDCA in the liver. For the NB groups, there were also differences in the amount of taurine-conjugated BAs (Figure 7).

6.2.3 Adipose tissue

6.2.3.1 Epididymal white adipose tissue (eWAT)

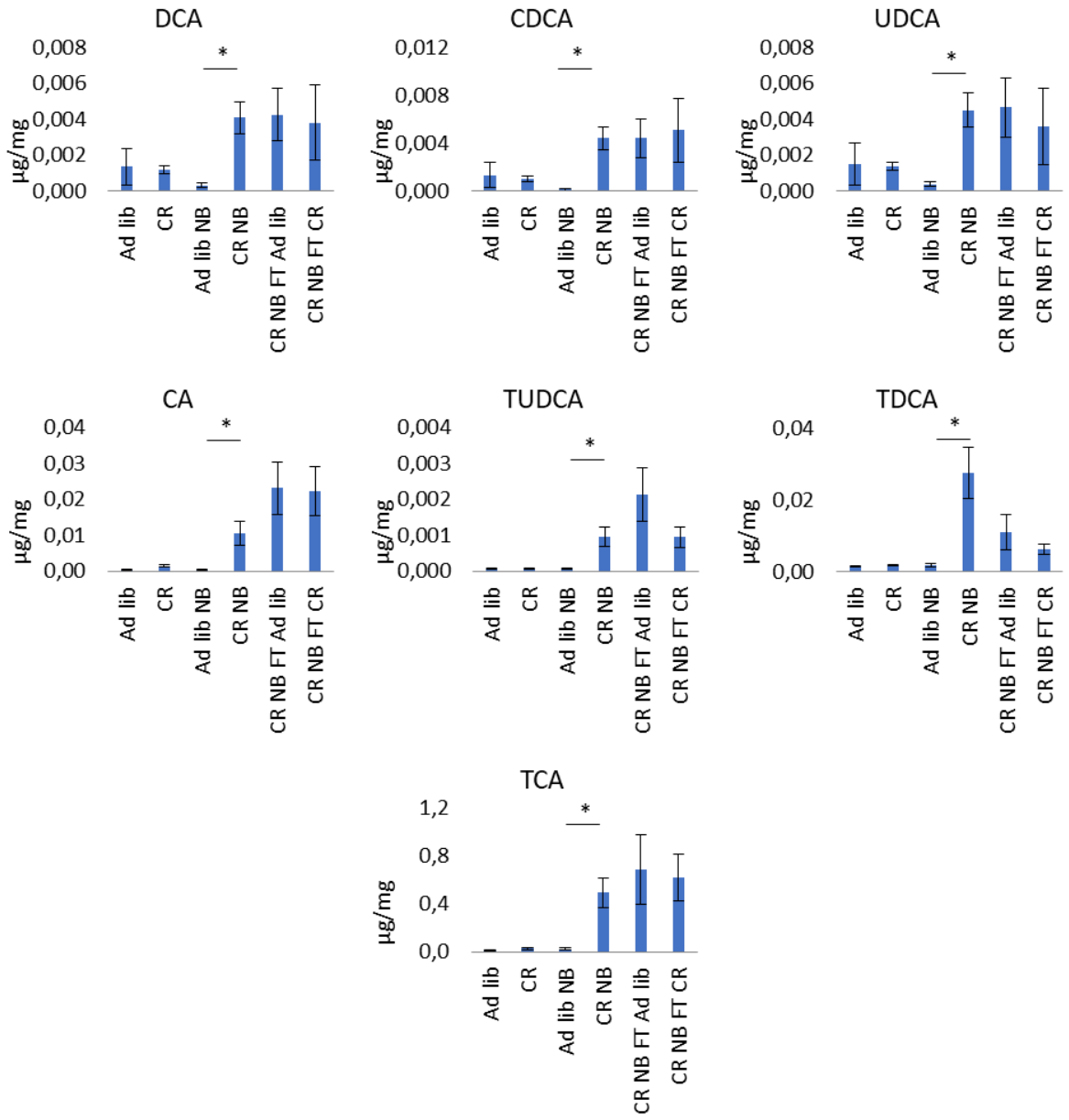


*Figure 8 BAs measured in eWAT; beddings and FT. Statistical significances are marked with * indicating p<0.05. eWAT = epididymal white adipose tissue.*

For the eWAT, a statistically significant difference between the Ad lib NB and CR NB groups for all BAs could be seen. For the other groups, there were no statistically significant

differences. In eWAT, there was no difference between the Ad lib and CR groups for unconjugated BAs (Figure 8).

6.2.3.2 Subcutaneous white adipose tissue (sWAT)

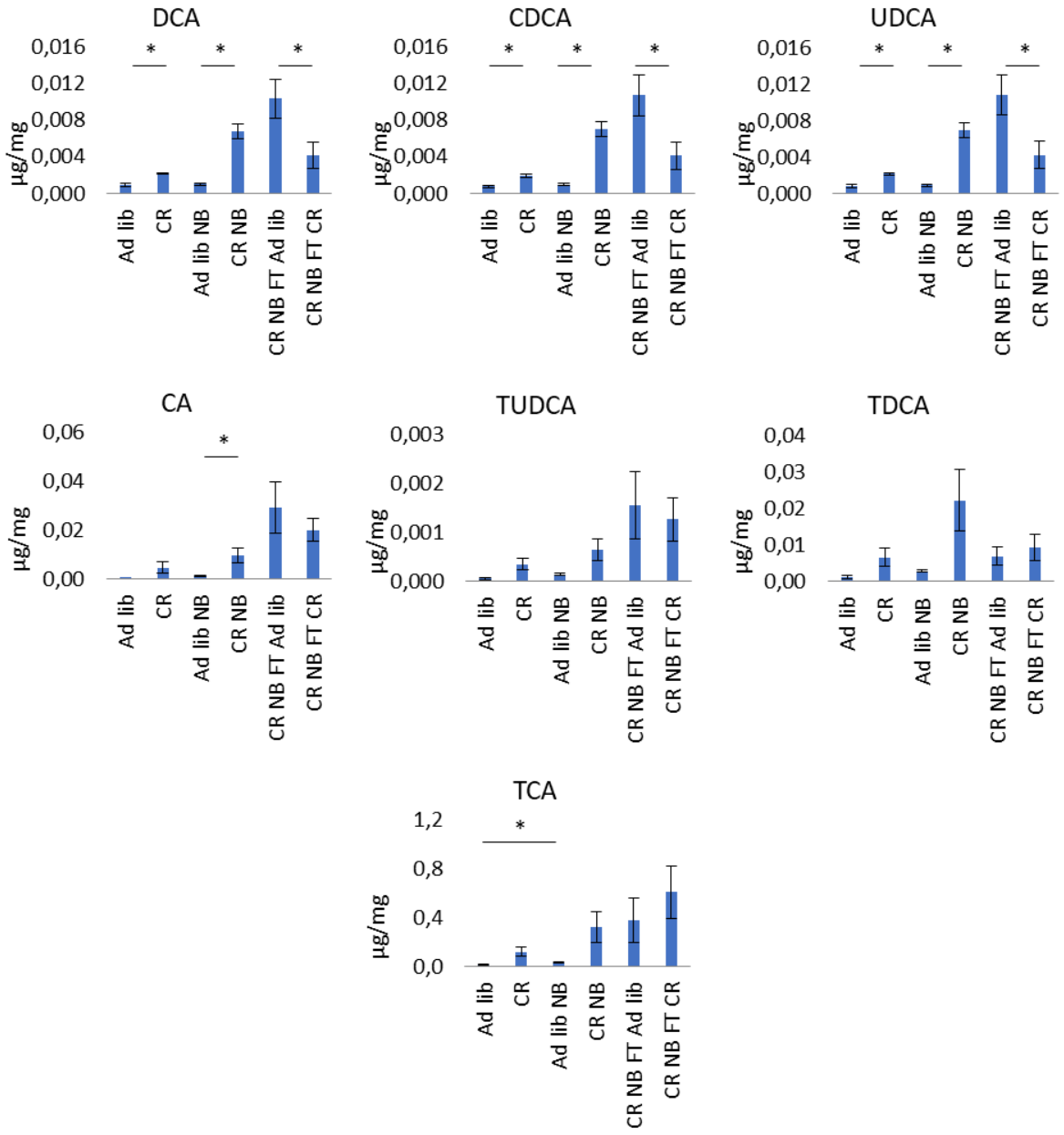


*Figure 9 BAs measured in sWAT; beddings and FT. Statistical significances are marked with * for p<0.05. sWAT = subcutaneous white adipose tissue.*

In sWAT, the results showed a statistically significant difference for DCA, CDCA, and UDCA for all Ad lib and CR groups. For CA, there was only for the Ad lib NB and CR NB groups a statistically significant difference. There was also a statistically significant difference between the Ad lib and Ad lib NB groups for TCA. In general, the CR groups had higher amounts of taurine-conjugated BAs than the Ad lib groups, except for the FT groups (Figure 9).

6.2.3.3 Brown adipose tissue (BAT)

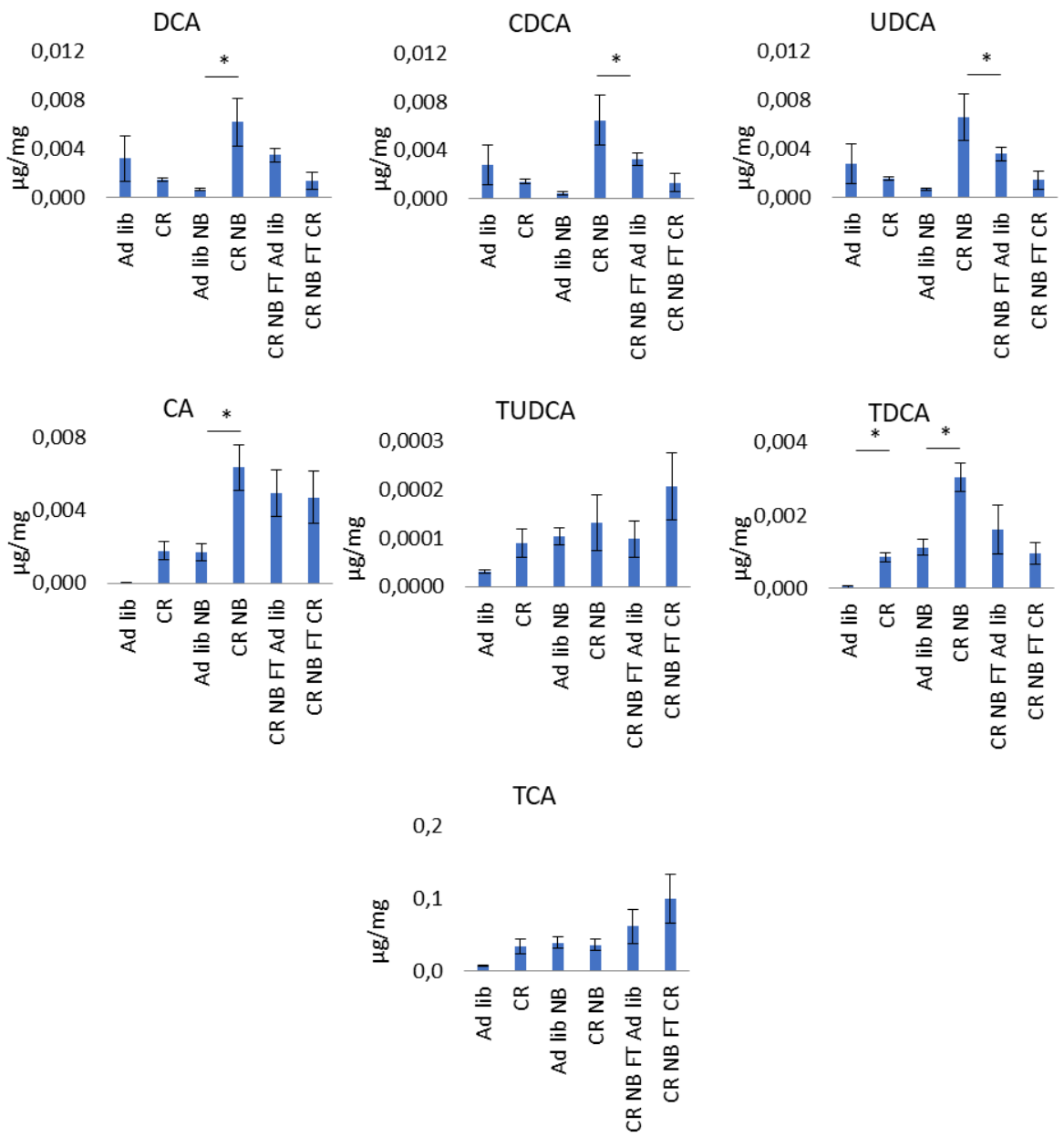
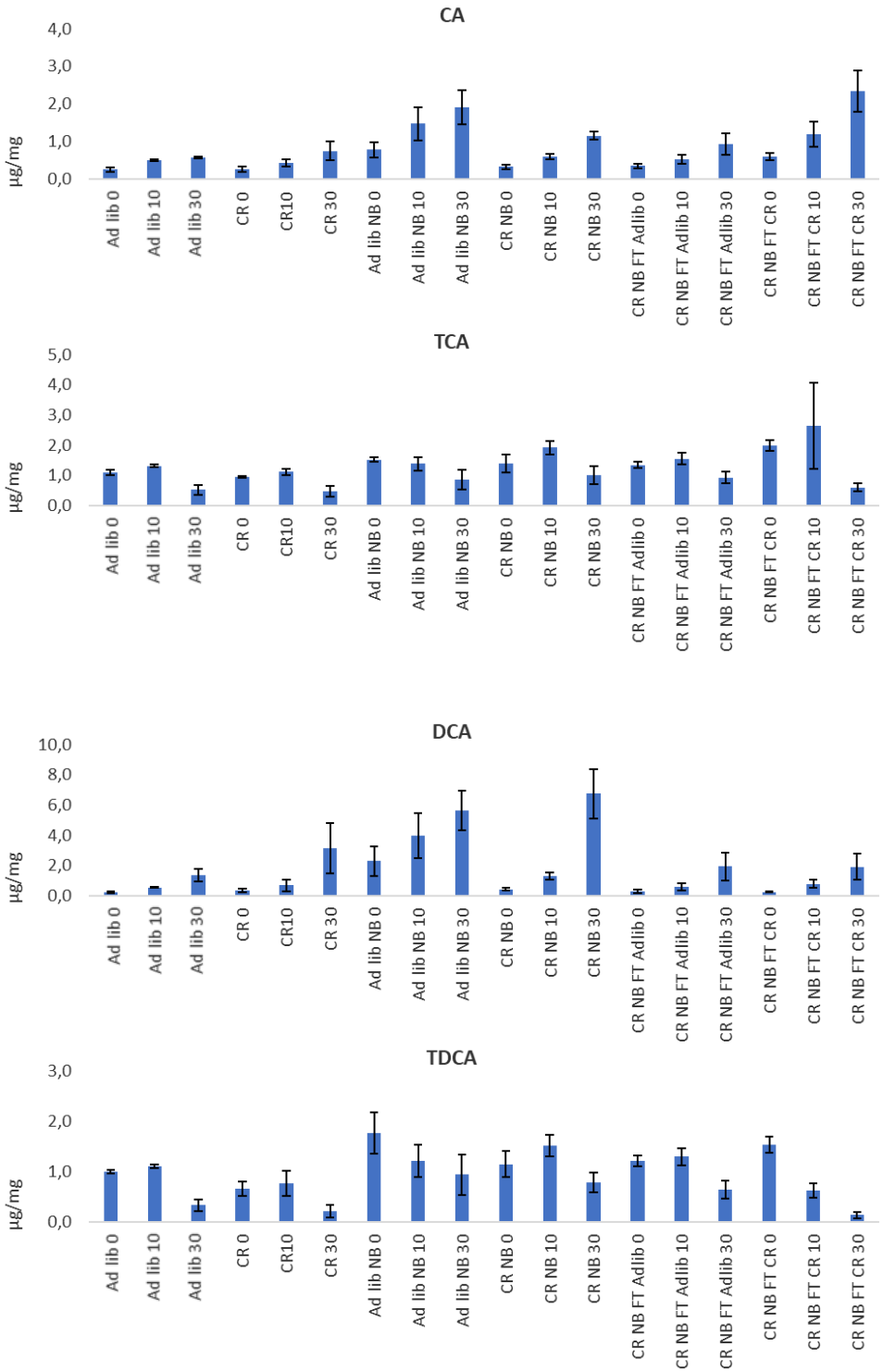


*Figure 10 BAs measured in BAT; beddings and FT. Statistical significances are marked with * for p<0.05. BAT = brown adipose tissue.*

For BAT, a statistically significant difference between the Ad lib NB and CR NB groups for DCA, CDCA, UDCA, and CA could be seen. Between the Ad lib and CR control groups, there was a smaller amount of DCA, UDCA, and CDCA in the CR mice. The same was observed for

the mice of the FT group. For the taurine-conjugated BAs, there was a statistically significant difference for Ad lib vs. CR with and without bedding only for TDCA. For TCA there were higher amounts in the CR groups compared to Ad lib, except in the groups without bedding. For the FT group, the results showed higher amounts of taurine-conjugated BAs in the CR transplanted group, with TDCA as an exception (Figure 10).

6.2.4 BSH feces



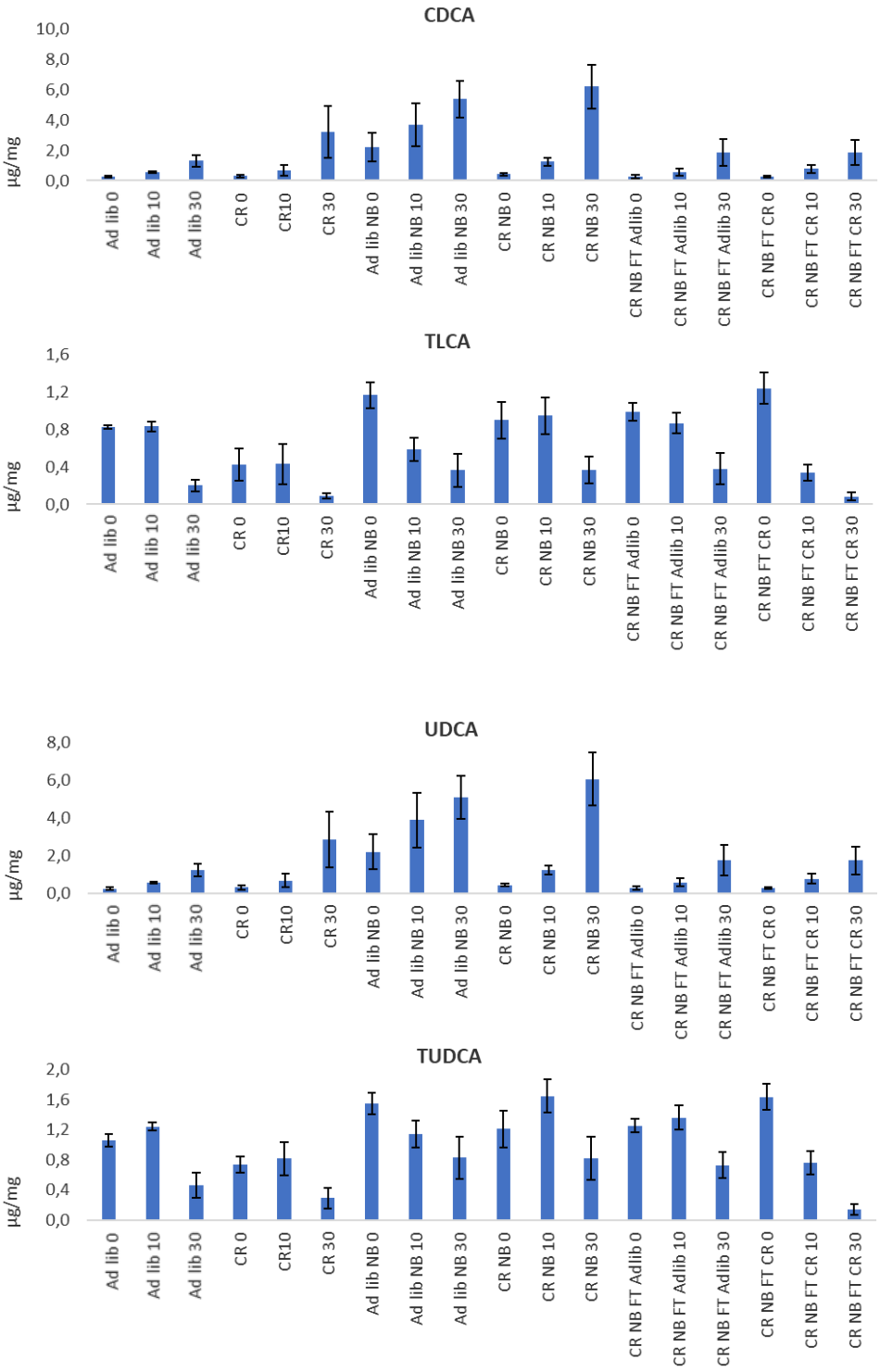


Figure 11 BAs measured over three time points to observe BSH activity in feces; bedding and FT. 0 = sample taken immediately; 10 = sample taken after 10 minutes; 30 = sample taken after 30 minutes.

Samples were taken at three time points to observe deconjugation of taurine-conjugated BAs over time. The results from the BSH assay showed an increase in all unconjugated BAs and a decrease over time for all taurine-conjugated BAs, which indicated deconjugation in all groups. There was a slight increase in taurine-conjugated BAs from the first to the second time point in the CR NB group and the CR NB FT CR group, but there was a greater decrease at the third time point (Figure 11).

6.2.5 qPCR microbiota & BSH in the cecum

6.2.5.1 qPCR cecum Ad lib vs. CR with/without bedding

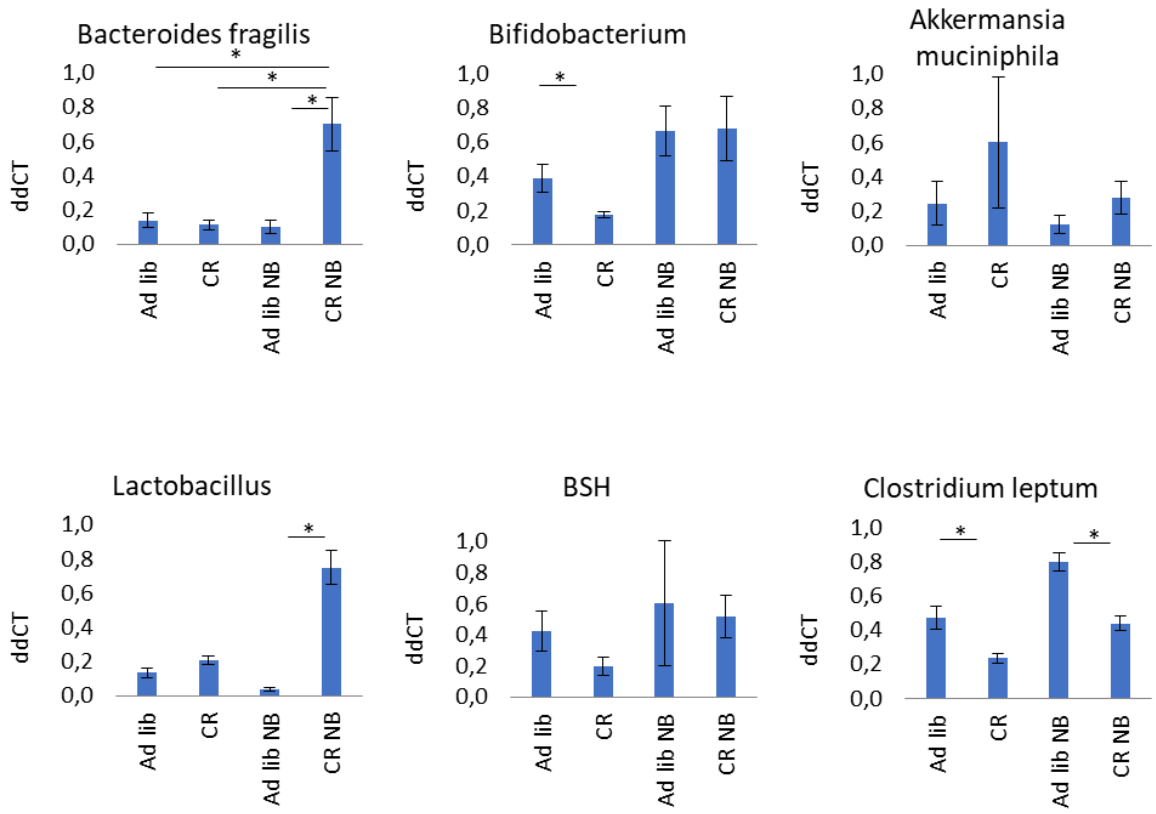


*Figure 12 qPCR results for microbiota and BSH in cecum; beddings. Statistical significances are marked with * for p<0.05. BSH = bile salt hydrolase.*

To further investigate the BAs deconjugation process and the influence of bedding, qPCR was performed for specific bacteria and BSH gene in the content of cecum. *Bacteroides fragilis* showed higher relative abundance in the cecum of the CR NB group. The levels in the other groups were comparable. There was a statistically significant difference between Ad lib and CR for *Bifidobacterium* abundance. For the NB groups, there was no difference. For *Akkermansia muciniphila* and *Lactobacillus*, there were higher amounts in the CR groups compared to the Ad lib groups. The difference was statistically significant in the case of the NB groups in *Lactobacillus*. *Clostridium leptum* showed statistically significantly decreased

levels for the CR groups, regardless of the presence of bedding. The results for BSH expression showed a decreased relative amount for the CR group compared to Ad lib. The NB groups looked more alike (Figure 12).

6.2.5.2 qPCR cecum Ad lib vs. CR with a fecal transplant from Ad lib and CR

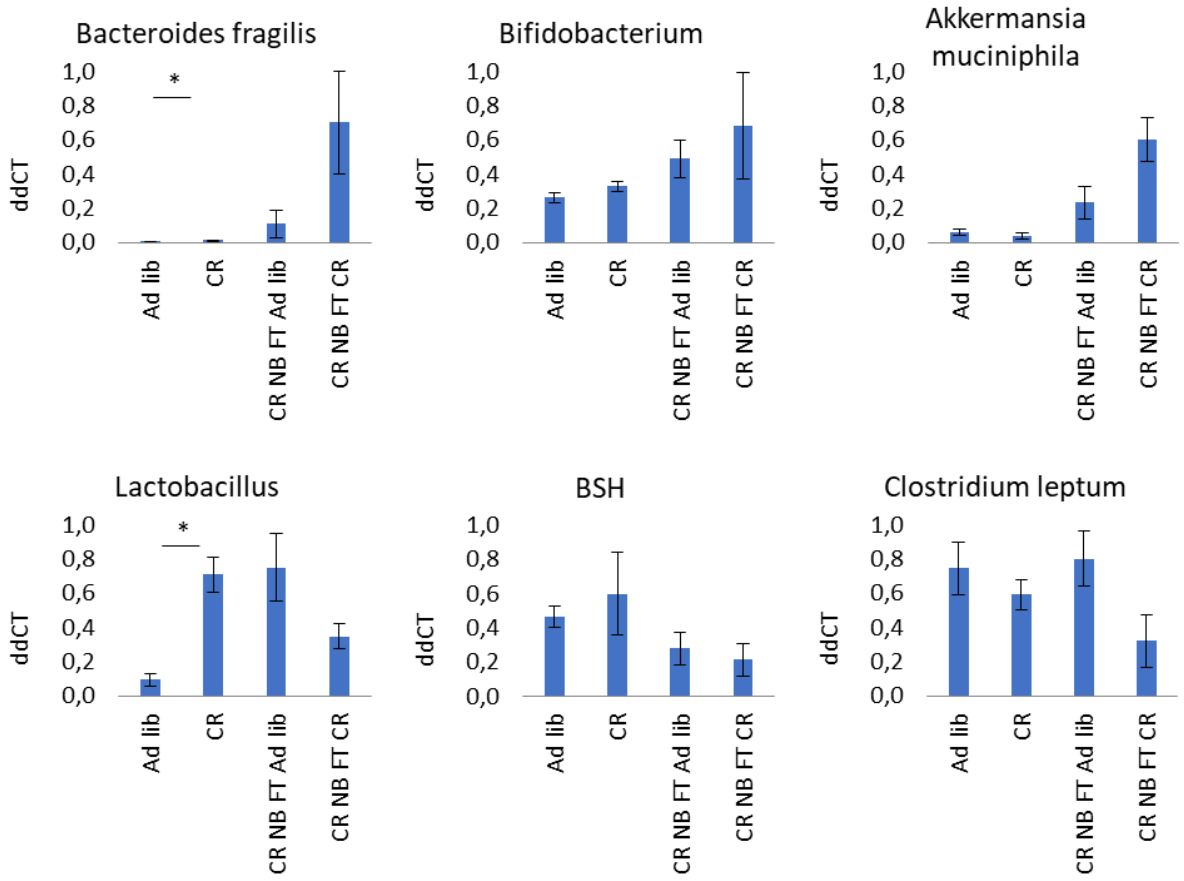


*Figure 13 qPCR results for microbiota and BSH in cecum; FT. Statistical significances are marked with * for p<0.05.*

Bacteroides fragilis was more abundant in the CR NB FT CR mice compared to the CR NB FT Ad lib group. There was a statistically significant difference between the Ad lib and CR groups. In the case of *Bifidobacterium*, no statistically significant differences were measured. *Akkermansia muciniphila* was also more abundant in the cecum of the CR NB FT CR group; however, not statistically significant. *Lactobacillus* levels were statistically significantly different between the Ad lib and CR groups. In the case of the FT groups, there

was a visible difference, but not statistically significant. The results for *Clostridium leptum* showed decreased amounts in the CR groups compared to Ad lib. BSH is higher expressed in the CR group, but not statistically significant. No differences were observed for the FT groups (Figure 13).

6.3 Experiment 3 Impact of taurine diets

6.3.1 Jejunum

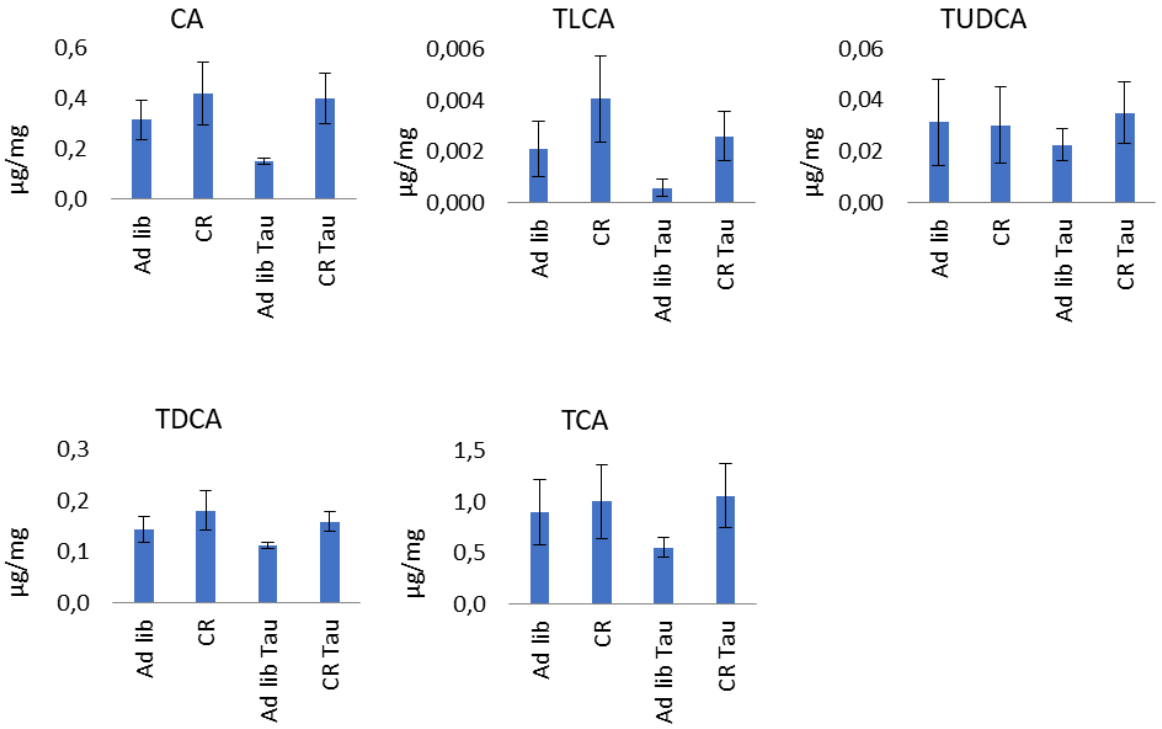


*Figure 14 BAs measured in jejunum; taurine diets. Statistical significances are marked with * for p<0.05. Tau= taurine diet (4%).*

To observe the effects of dietary taurine on BA composition, jejunum and liver samples of mice treated with varying quantities of taurine were analyzed. No statistically significant differences were detected in the jejunum samples; however, a noticeable trend was observed where the CR groups tended to have higher BA concentrations compared to their Ad lib counterparts (Figure 14).

6.3.2 Liver

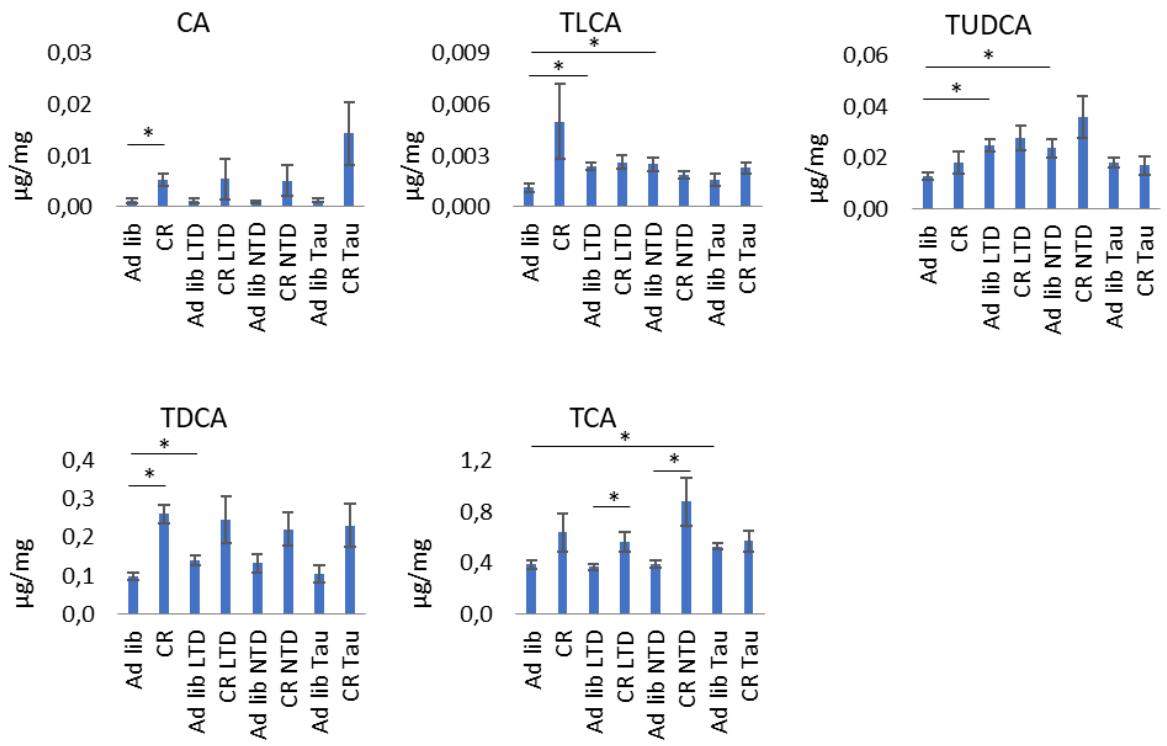


*Figure 15 BAs measured in liver; taurine diets. Statistical significances are marked with * for p<0.05. LTD= low taurine diet (0%); NTD= normal taurine diet (0.5%).*

For CA, the results showed higher amounts in all CR groups compared to their Ad lib counterparts, but it was only statistically significant for the Ad lib vs CR control. Within the taurine-conjugated BAs, there were higher amounts in the CR control compared to the Ad lib control, but only with a statistically significant difference for TDCA. Within the LTD groups, there were higher amounts for TDCA and TCA in the CR groups, but for TLCA and TUDCA, there were no differences. Also, the Ad lib LTD group showed statistically significant higher amounts in TLCA, TUDCA, and TDCA compared to the Ad lib control. The NTD groups showed higher values in TUDCA, TDCA, and TCA in the CR group. For TLCA, the CR NTD group had a slightly lower amount compared to the Ad lib NTD group. Ad lib Tau compared to the CR Tau group, showed higher amounts of TDCA for the CR group, but for TLCA, TUDCA, and TCA the Ad lib and CR groups had comparable amounts in their liver tissue (Figure 15).

6.4 Experiment 4 Impact of BSH inhibitor, bile acid supplementation, and fluvastatin

6.4.1 Plasma

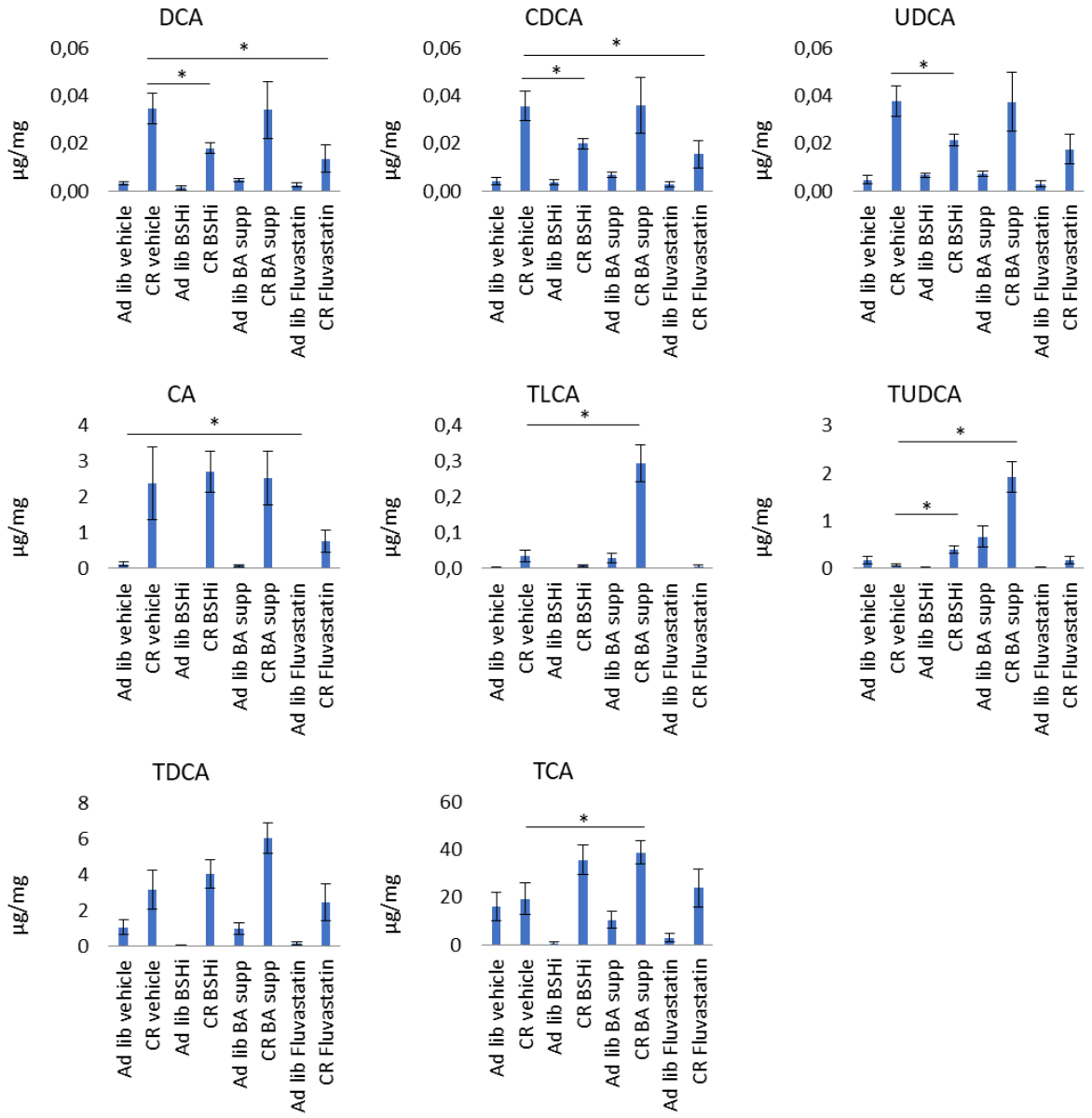


*Figure 16 BAs measured in plasma; BSHi, BA supp, and Fluvastatin. Statistical significances are marked with * for p<0.05. Ad lib vehicle= ad libitum vehicle (EtOH) control; CR vehicle= caloric restriction vehicle (EtOH) control; BSHi= bile salt hydrolase inhibitor; BA supp= taurine-conjugated bile acid supplementation; EtOH= ethanol.*

To challenge BAs phenotype in CR animals, mice were administered BSHi, BA supp, and fluvastatin. Plasma samples of CR groups had higher levels of unconjugated BAs DCA, CDCA, UDCA, and CA. Also, fluvastatin treatment reduced the levels of unconjugated BAs in the CR group compared to the control CR. For the BSH inhibitor groups, there were lower levels of BAs in the CR BSHi groups, compared to the CR vehicle control, except for CA. For the taurine-conjugated BAs, the results showed that the BSH inhibitor CR groups, had higher amounts of TUDCA, TDCA, and TCA compared to the CR vehicle control, but only statistically significant for TUDCA. The BA supp groups and the Ad lib & CR vehicle groups had comparable levels of unconjugated BAs. For taurine-conjugated BAs, the CR BA supp group had statically significantly more TLCA, TUDCA, and TCA compared to the CR vehicle control. The Ad lib fluvastatin group had fewer BAs than the Ad lib vehicle control group. But it was only for CA statistically significant. The CR groups showed statistically significantly lower amounts of DCA and CDCA in the fluvastatin-treated mice compared to the CR control. No statistically significant differences were measured in taurine-conjugated BAs between the fluvastatin and vehicle control groups (Figure 16).

6.4.2 qPCR gene assay

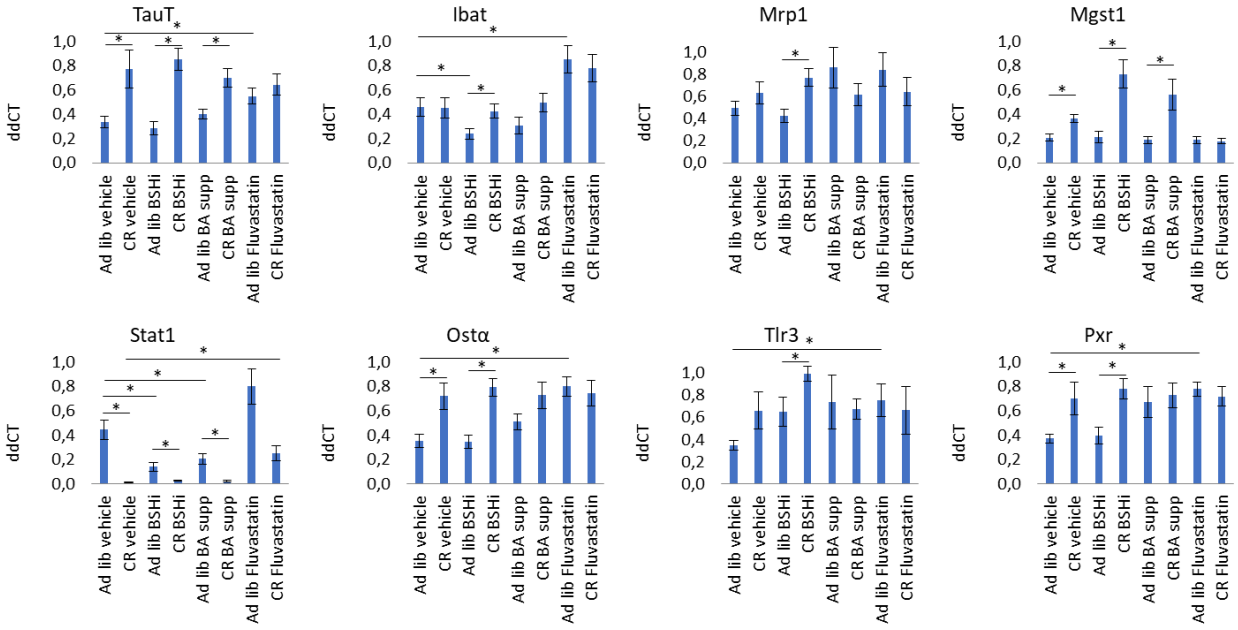


*Figure 17 qPCR results for TauT, Ibat, Mrp1, Mgst1, Stat1, Ostα, Tlr3, and Pxr in cecum; BSHi, BA supp, and Fluvastatin. Statistical significances are marked with * for p<0.05. As reference gene for data normalization, Eef1a1 was used.*

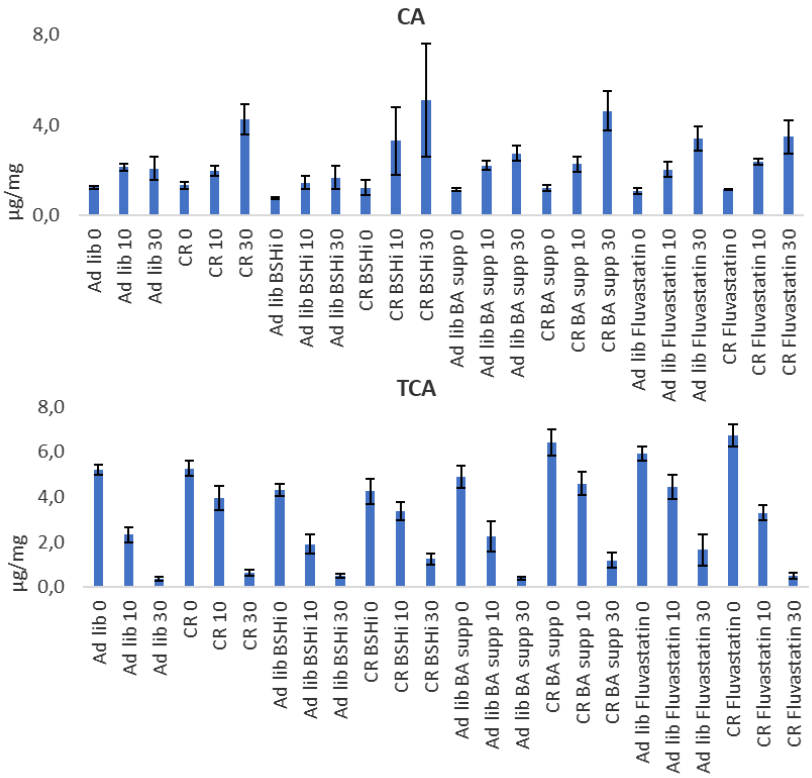
To evaluate the expression levels of genes related to BA metabolism, qPCR was performed. The results showed that the expression of *TauT* was higher in the CR groups compared with the Ad lib groups in all interventions, with statistically significant differences observed between the vehicle control group, BA supp groups, and BSHi groups. Additionally, the Ad lib fluvastatin group showed a statistically significant increase in *TauT* expression compared with the Ad lib vehicle control group. The analysis of *Ibat* expression showed no statistically significant differences between the Ad lib and CR vehicle control groups. However, a statistically significant difference was observed between the BSHi groups. The highest expression of *Ibat* was observed in the fluvastatin groups. The expression of *Mrp1* was analyzed and higher levels were observed in the CR vehicle control and BSHi groups compared to their Ad lib counterparts. The results showed that the BS supp and fluvastatin groups had a lower expression of *Mrp1* in their CR groups compared to their Ad lib counterparts, with only the difference between the BSHi groups being statistically

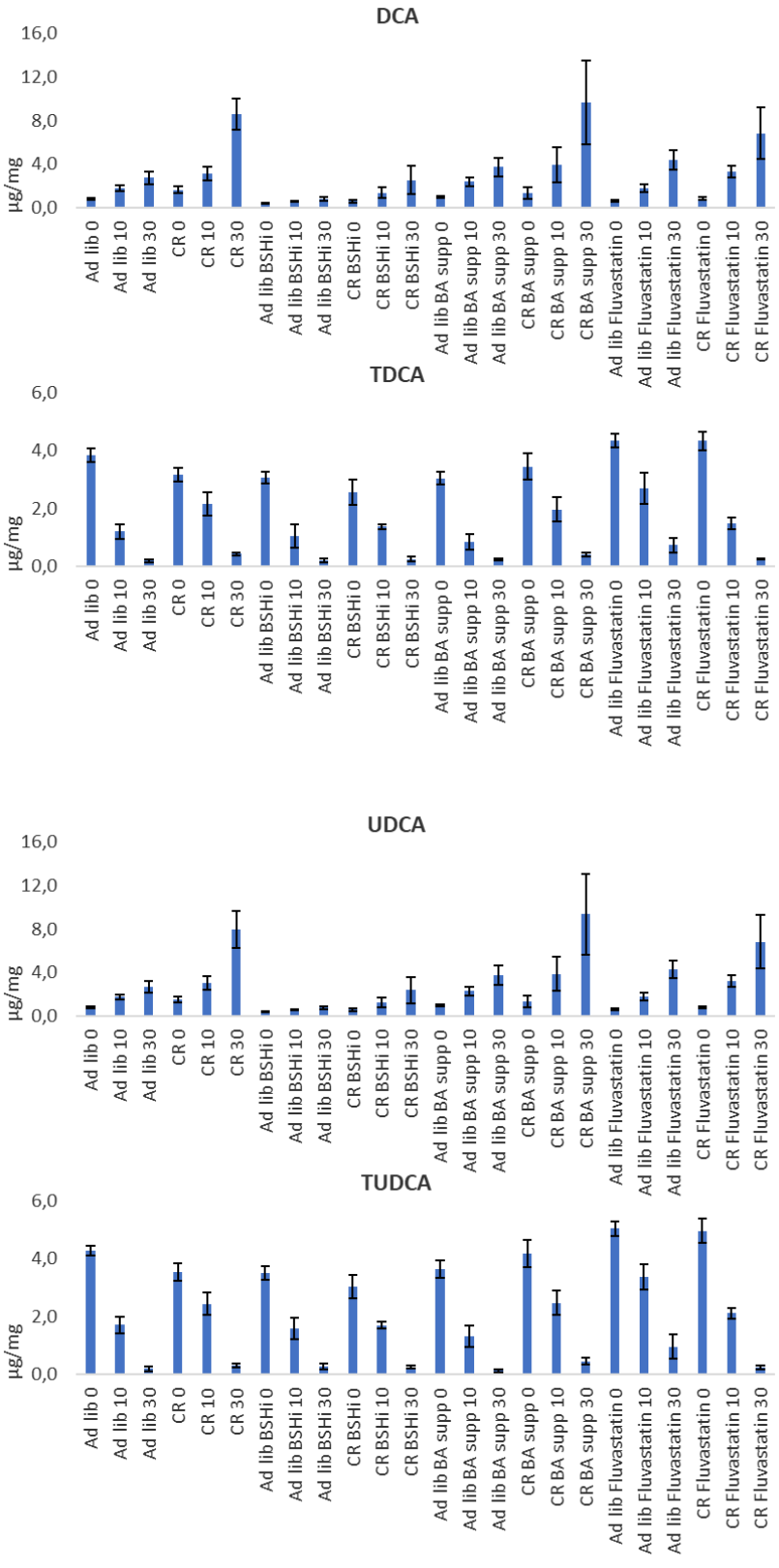
significant. For *Mgst1*, a statistically significant higher expression was noted in all CR groups compared to their Ad lib counterparts, with the exception of the fluvastatin intervention group. The levels of *Stat1* gene expression were decreased in all CR groups in comparison to their Ad lib counterparts. The Ad lib treatment with BSHi and BA supp showed statistically significant lower levels of *Stat1*, compared to the Ad lib control group. In contrast, the fluvastatin intervention group under CR conditions showed a statistically significant increase in *Stat1* expression when compared to the CR vehicle control group. The expression of the *Ostα* gene was found to be statistically significantly elevated in the CR vehicle control group and the CR BSHi group, in comparison to their respective Ad lib groups. Although a trend towards elevated expression was observed in the CR BA supp group, this difference was not statistically significant. The expression of the *Ostα* gene in the fluvastatin-treated groups exhibits minimal differences, however, the fluvastatin Ad lib group exhibited a statistically significant increase in *Ostα* expression compared to the Ad lib vehicle control group. For *Tlr3* and *Pxr*, the gene expression was found to be elevated in the CR vehicle control group and the CR BSHi group, when compared to their respective Ad lib groups. The CR BA supp and fluvastatin groups showed no statistically significant differences in expression. However, a statistically significant increase was observed in the fluvastatin Ad lib group, compared to the Ad lib vehicle control group. The results from the fluvastatin groups showed no statistically significant difference in the expression of the measured genes between the Ad lib and CR groups, with the exception of a decreased expression of *Stat1* in the CR group (Figure 17).

6.5 Experiment 5 Impact of BSH inhibitor; BA supplementation; mTOR activator/inhibitor; TauT-inhibitor, ethacrynic acid, and fluvastatin on BSH activity

6.5.1 BSH Feces

6.5.1.1 Ad lib vs CR plus BAs, fluvastatin & BSH inhibitor




DCA
16,0
12,0
8,0
4,0
0,0
µg/mg
Ad lib 0
Ad lib 10
Ad lib 30
CR 0
CR 10
CR 30
Ad lib BSHi 0
Ad lib BSHi 10
Ad lib BSHi 30
CR BSHi 0
CR BSHi 10
CR BSHi 30
Ad lib BA supp 0
Ad lib BA supp 10
Ad lib BA supp 30
CR BA supp 0
CR BA supp 10
CR BA supp 30
Ad lib Fluvastatin 0
Ad lib Fluvastatin 10
Ad lib Fluvastatin 30
CR Fluvastatin 0
CR Fluvastatin 10
CR Fluvastatin 30
TDCA
6,0
4,0
2,0
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µg/mg
UDCA
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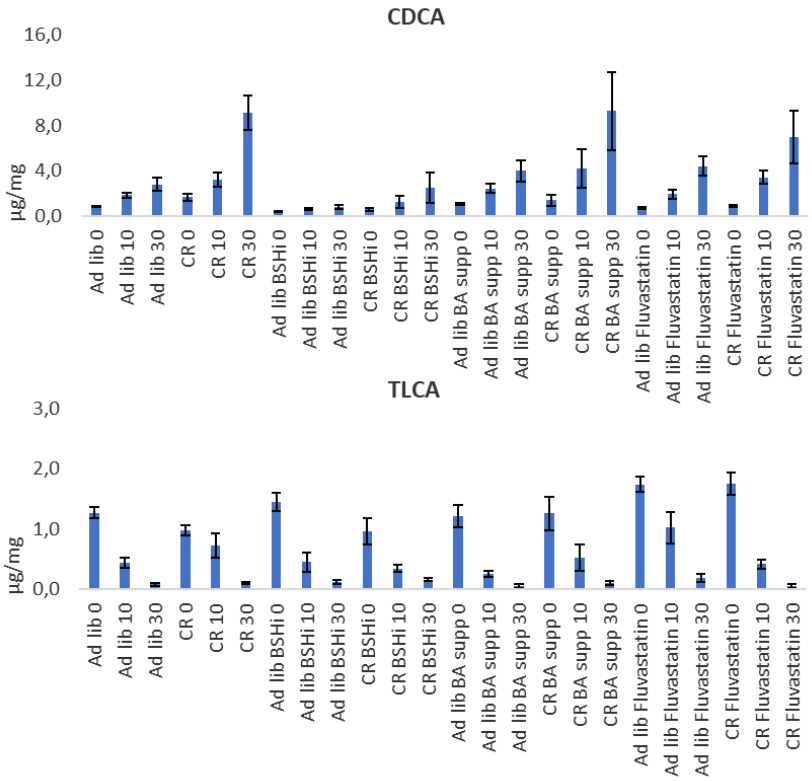
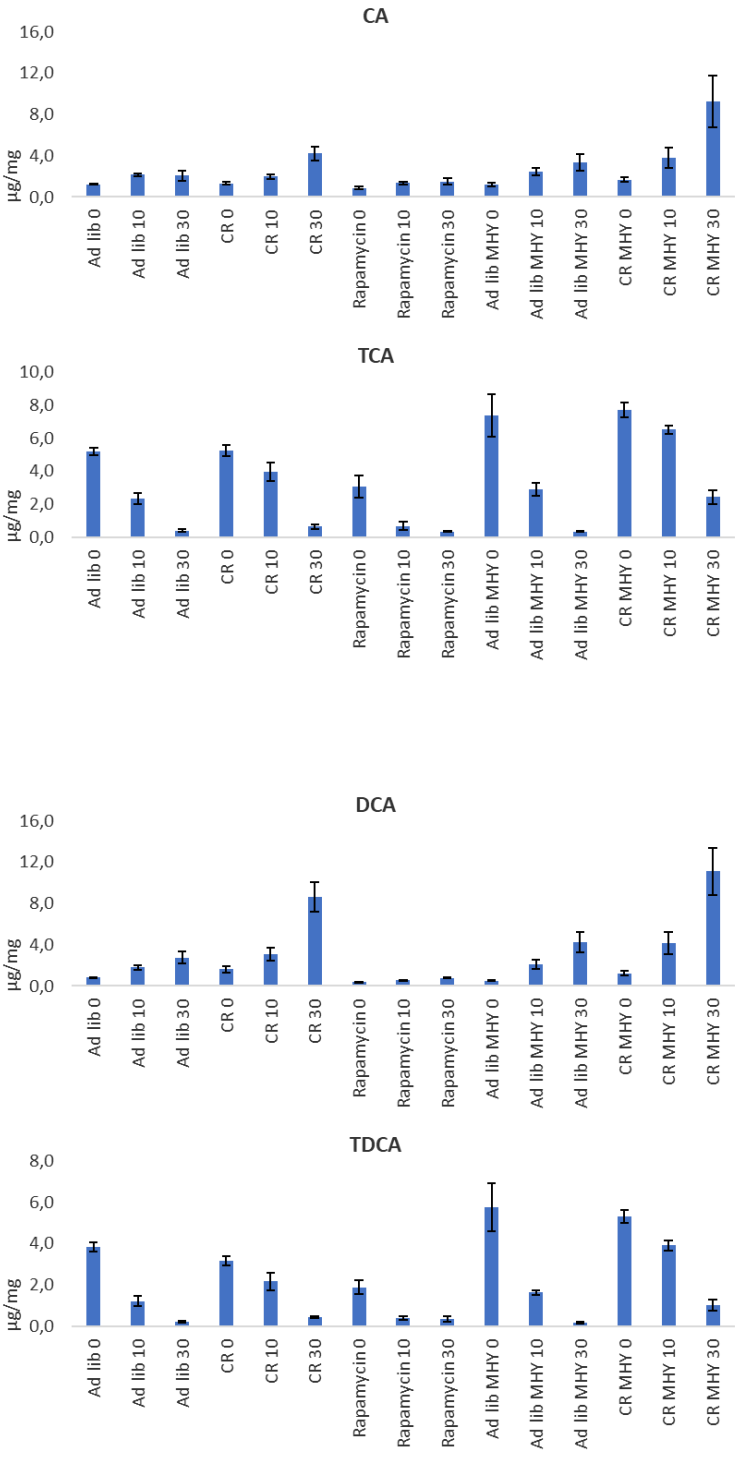


Figure 18 BAs measured over three time points to observe BSH activity in feces; BSHi, BA supp, and Fluvastatin.

The extent of deconjugation of BAs over time was estimated in the BSH assays by comparing the concentration of unconjugated BAs with their taurine-conjugated counterparts. For TCA, a strong decreasing trend was observed in all groups across the three time points. On the other hand, there was an increase for CA over time. A similar downward trend was also observed for TDCA in all groups. There was an increase in concentration for DCA, with the smallest increase observed in the BSHi groups. UDCA and its conjugated counterpart TUDCA showed the same pattern as DCA. The results for CDCA and TLCA were similar to the patterns observed for the other BAs (Figure 18).

6.5.1.2 Ad lib vs CR plus Rapamycin & MHY1485



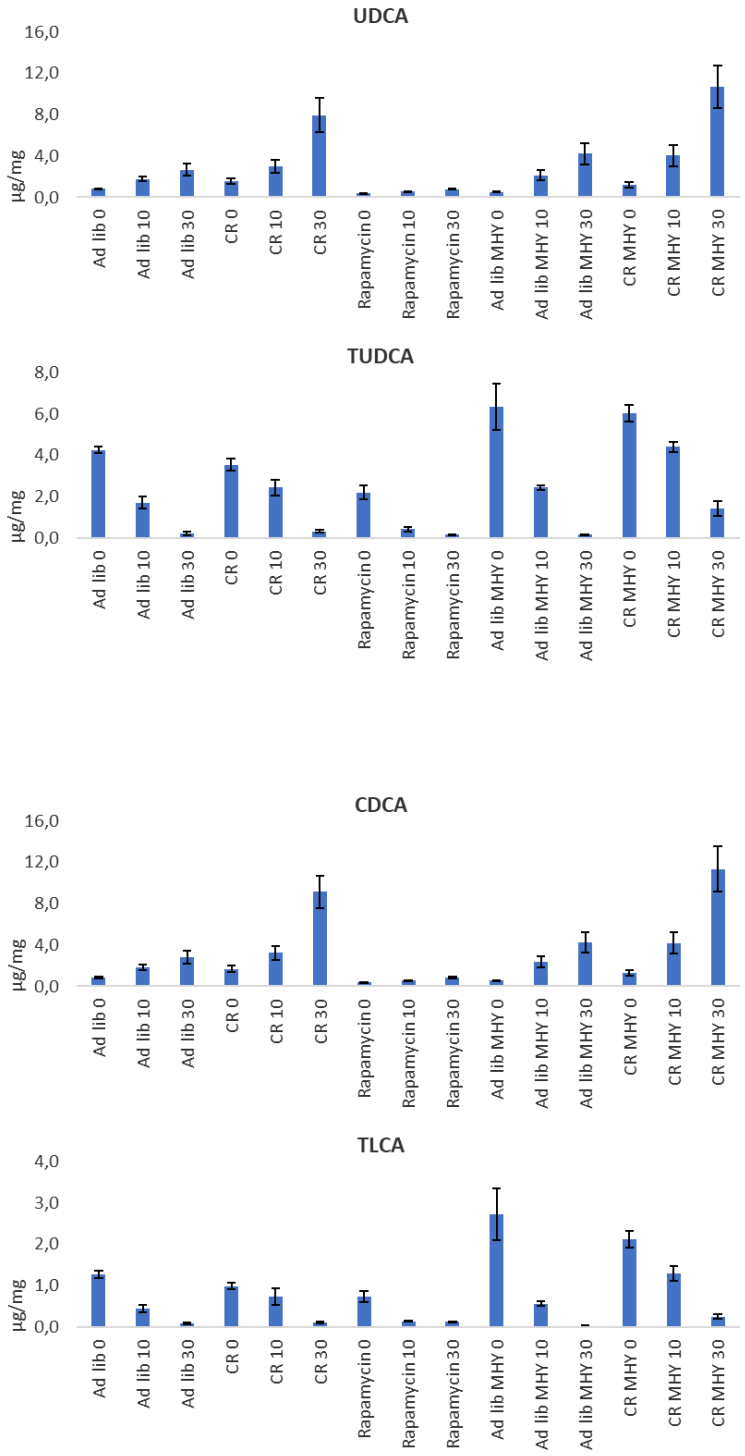
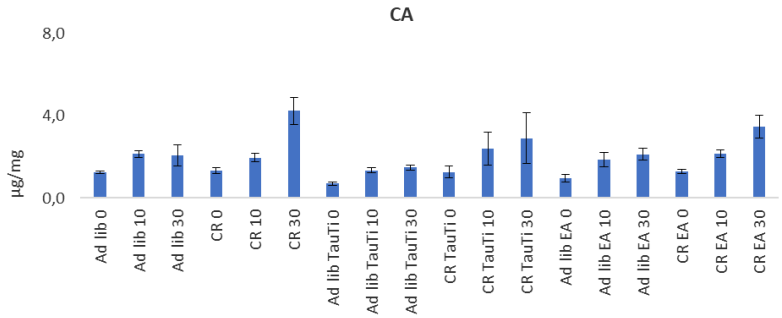
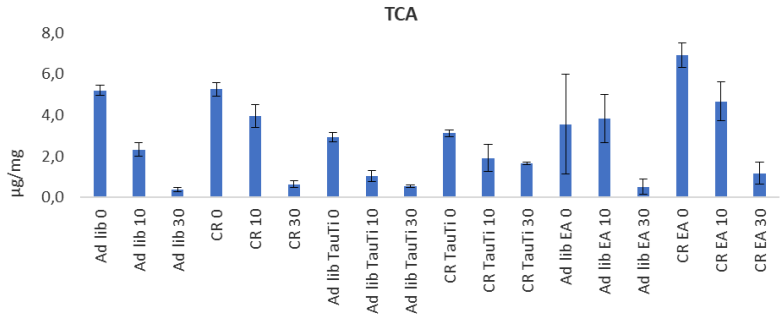


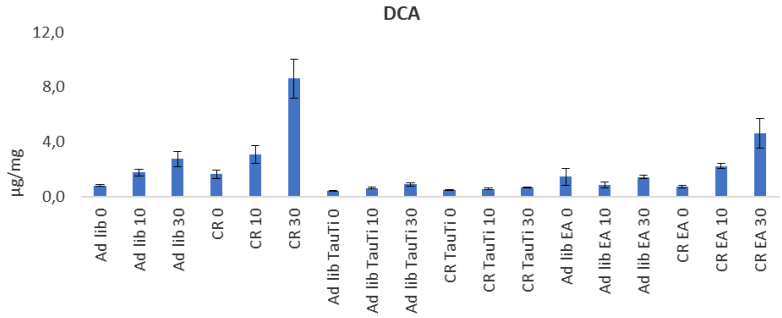
Figure 19 BAs measured over three time points to observe BSH activity in feces; Rapamycin and MHY1485; MHY = MHY1485 (mTOR activator).

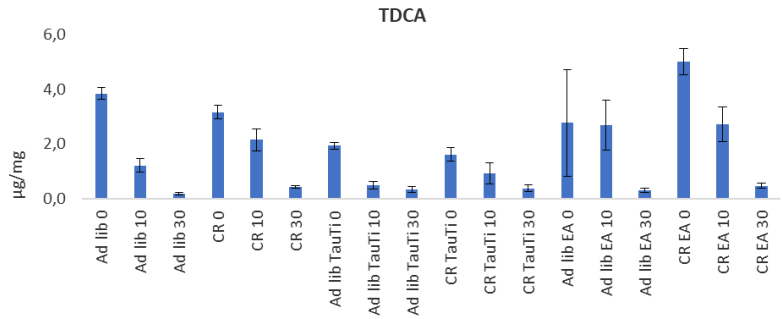
The concentration of TCA was observed to decrease over the three time points in all groups and CA increased on the other hand. A similar trend was observed for DCA and TDCA. The concentration of DCA showed an overall increase, with the smallest increase seen in the Rapamycin-treated group. The pattern for UDCA and TUDCA closely followed the trend observed for CA and TCA. Results for CDCA and TLCA were consistent with the trends observed for the other BAs (Figure 19).

6.5.1.3 Ad lib vs CR plus taurine inhibitor & EA









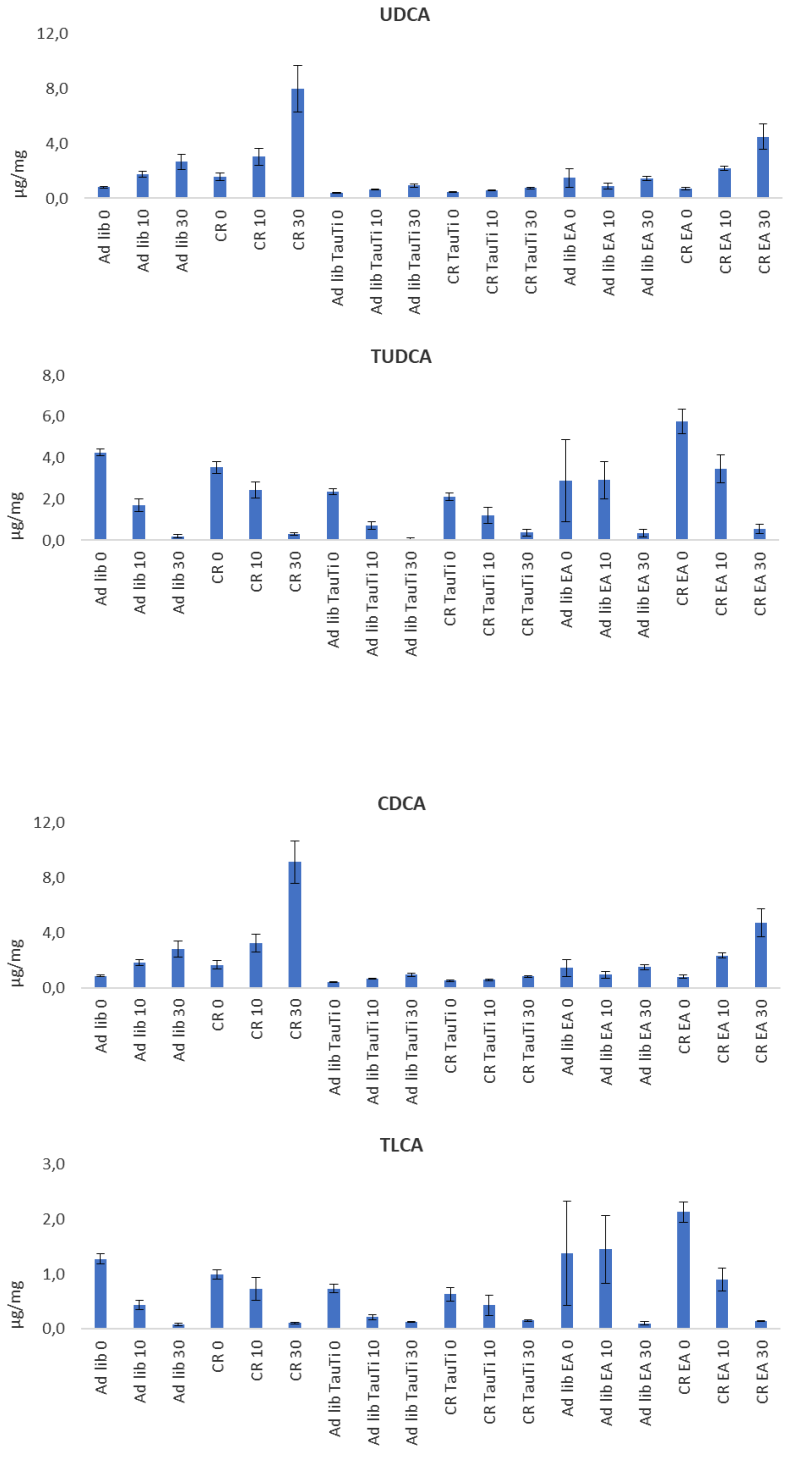


Figure 20 BAs measured over three time points to observe BSH activity in feces; TauTi and EA. TauTi = taurine transporter inhibitor; EA = ethacrynic acid.

The concentration of TCA showed a strong decreasing trend over the three time points for all groups and CA showed an increase on the other hand, with an exception for the Ad lib control, where a slight increase was observed between the second and third time point. TDCA showed a decrease in concentration across all groups. On the other hand, the increase in DCA concentration was only prominently visible in the control groups and the CR EA group. UDCA showed a comparable trend to DCA. The concentration of TUDCA decreased over the three time points in all groups. CDCA followed the same pattern as the unconjugated BAs DCA and UDCA. TLCA showed a similar trend to the other taurine-conjugated BAs with a decreasing concentration over time (Figure 20).

7 Discussion

7.1 BAs and CR

This thesis investigates the impact of CR on BA levels, composition and metabolism. Results from the first experiment indicate that CR treatment increases the BA pool in the ileum, liver, plasma, and proximal colon of mice (Figures 2-5). However, these findings contrast with those reported by Fu et al., who observed an increase in the small intestine and serum but not in the liver or large intestine during CR [64]. In this thesis, all measured BAs increased in the aforementioned tissues, but in the ileum and proximal colon, the difference was not as pronounced for taurine-conjugated BAs. The most significant increase in BAs was observed in plasma, potentially indicating systemic effects. BAs have been shown to act as signaling molecules involved in regulating lipid, glucose, and energy metabolism, drug metabolism, and immune response modulation [11]. These results may suggest higher BA levels due to CR intervention. Increased amounts of BAs may enhance lipid and fat-soluble vitamin absorption to compensate for caloric deficiency. Another reason for the higher BA levels may be attributed to insulin function, as prolonged fasting has been associated with decreased insulin levels and upregulation of CYP7A1, the rate-limiting enzyme for BA synthesis [8]. Changes in gut microbiota composition may also contribute to these results, as gut microbiota plays a role in converting primary to secondary BAs [65].

7.2 Impact of bedding and fecal transplant

According to the findings of Gregor et al., mice have been observed to supplement their diet with bedding, particularly under fasting conditions. This behavior has the potential to impact the profile of their gut microbiota [63]. Fiber is known to bind BAs, resulting in increased excretion of BAs via feces. This binding effect may contribute to the reduction of cholesterol concentrations, as newly synthesized BAs are required to replace those that have been excreted. Thus, the binding of BAs by fiber may represent one mechanism by which fiber exerts its physiological effects [66]. In the second experiment, the BA composition was analyzed in the ileum, liver, and adipose tissue of Ad lib and CR mice, both

with and without bedding. Additionally, CR mice that received a fecal transplant from either Ad lib or CR-treated mice were also evaluated. In summary, the results from the ileum and liver tissue showed that CR generally led to higher levels of unconjugated BA compared to Ad lib despite bedding or not. In the ileum CR mice that received a fecal transplant from CR-treated mice had lower levels of unconjugated BAs than those that received a transplant from Ad lib mice. Additionally, in the ileum, taurine-conjugated BAs were found in the highest concentrations in the Ad lib group without bedding. This could indicate that this group has the lowest deconjugation capability (Figures 6-7). In the liver, BA composition was found to be similar between groups with and without bedding However, in the ileum, the groups without bedding tended to have higher levels of BAs. This observation may suggest that the absence of fiber from bedding consumption results in reduced excretion of BAs through feces (Figure 7). The analysis of eWAT, sWAT, and BAT revealed several differences in BA composition between Ad lib and CR groups. In eWAT, a significant difference was observed between Ad lib and CR groups without bedding for all BAs, while only small differences were found between the Ad lib and CR control groups. The FT groups that received a transplant from CR mice exhibited slightly lower BA levels compared to those that received a transplant from Ad lib mice (Figure 8). In sWAT, the CR transplant groups had lower levels of unconjugated BAs compared to the Ad lib transplant groups. Higher levels of all BAs were observed in CR groups regardless of bedding, with the highest levels found in the CR group without bedding (Figure 9). Overall, the data from eWAT and sWAT showed higher BA levels in groups without bedding compared to those with bedding. This may be due to reduced excretion resulting from a lack of fiber consumption, leading to increased systemic BA levels. Schmid A. et al. have provided evidence for functional BA signaling pathways in adipocytes, suggesting that systemic BAs may regulate white adipocyte physiology, including lipolysis [67]. The analysis of BAT showed that without bedding, the CR groups had higher BA levels, for conjugated and unconjugated BAs. Interestingly, the Ad lib group with bedding, had more unconjugated BAs, except CA, than the CR group. The FT groups showed a strange pattern, with lower BA levels for the

unconjugated BAs in the groups with the CR transplant, but higher levels of conjugated BAs, except TDCA. With the fecal transplant from CR mice, it would be expected to have higher deconjugation capabilities compared to the Ad lib transplant (Figure 10). It has previously been reported that microbiota mediates the effect of CR on metabolic improvements including fat browning, liver health, and better glycemic control [68]. Watanabe et al., showed that the administration of BAs to mice increases energy expenditure in brown adipose tissue, preventing obesity and resistance to insulin [69].

7.3 Influence on microbiota

The antimicrobial properties of BAs are well-documented and may serve to protect the intestinal tract from infection. However, it is also important to note that certain bacterial species, including *Lactobacillus*, *Clostridium*, *Bifidobacterium*, and *Bacteroides*, play a role in modifying BAs. This suggests a symbiotic relationship between gut bacteria and BAs [70]. Furthermore, it has been shown that there is a tight connection between CR and microbiota. Wang et al. provide evidence of a causal relationship between CR and the gut microbiota. Their findings suggest that the microbiota plays a crucial role in mediating the beneficial effects of CR, including reduced body weight, maintenance of a high basal metabolic rate, decreased blood glucose levels, and reduced serum cholesterol levels [71]. Throughout the second experiment, changes in the microbiome in cecum samples were investigated. For *Bacteroides fragilis* and *Lactobacillus*, the CR NB group showed by far the highest levels. *Bifidobacterium* levels were lower in the CR groups and showed similar levels between the Ad lib NB and CR NB groups. *Akkermansia muciniphila* showed an increased level in the CR groups compared to their Ad lib counterpart, regardless of bedding. *Clostridium leptum* showed decreased levels for the CR groups, regardless of the presence of bedding. Overall, the data suggests that the CR NB phenotype has higher levels of *Bacteroides fragilis* and L*actobacillus*, but *Bifidobacterium* seems unaffected by this. Within the CR groups, despite bedding or not, the results showed an increase in *Akkermansia muciniphila* and a decrease in *Clostridium leptum*. Interestingly, BSH levels were slightly lower in the CR groups, which would indicate a reduced deconjugation capability by the microbiota (Figure 12). The results

from the FT groups indicate that those mice exhibited also an increase in *Akkermansia muciniphila* and a decrease in *Clostridium leptum* for the CR groups. In terms of *Bacteroides fragilis*, the CR NB FT CR group had higher levels compared to other groups, consistent with the CR NB phenotype. The FT groups showed slightly higher levels of *Bifidobacterium* compared to the Ad lib and CR control. Interestingly, *Lactobacillus* levels were lower in the CR NB FT CR mice compared to those that received a transplant from Ad lib mice. Additionally, BSH levels were slightly lower in the fecal transplant groups (Figure 13). It has been demonstrated that BSHs have the capability to promote bacterial colonization in the lower digestive system of higher mammals. Nevertheless, the complete understanding of the physiological benefits from BSHs remains incomplete and could differ depending on the bacterial species and groups. A hypothesis proposes that the process of deconjugation could potentially function as a method for detoxifying bile salts [14]. Tannock et al. disputed this hypothesis concerning *lactobacilli*, asserting that unconjugated BAs are more toxic than their conjugated forms [72]. However, in vivo, when other intestinal bacteria perform 7-dehydroxylation on free BAs, the resulting secondary BAs can precipitate (depending on the pH in the lumen) and bind themselves to insoluble fiber or be absorbed across the colonic membrane. Consequently, this leads to lower concentrations of secondary BAs in the bacterial microenvironment [14]. The BSH assay analysis of fecal samples showed deconjugation capabilities in all groups, with an increase in all unconjugated BAs and a decrease over time for all taurine-conjugated BAs. Interestingly, there was a slight increase in taurine-conjugated BAs from the first to the second time point in the CR NB and CR NB FT CR groups, followed by a greater decrease at the third time point (Figure 11). The data indicates that both Ad lib and CR treated mice, with or without bedding, have the ability to deconjugate taurine-conjugated BAs in feces. Similarly, CR mice treated with FT from either Ad lib or CR also exhibited comparable deconjugation capabilities.

7.4 Taurine diets

Taurine is one of the important metabolites of L-cysteine in mammals and is found in high concentrations in various mammalian tissues [73]. Numerous studies have investigated the

physiological role of taurine, with its clearly established function in the formation of taurine-conjugated BAs [74], which aid intestinal digestion and absorption of lipids. Mice that were given the standard chow diet had the ability to process taurine from both cysteine and methionine since these amino acids were present in the diet. However, in the case of the LTD diet, taurine, cysteine, and methionine were not included, which hindered the mice's ability to produce taurine endogenously. When it comes to restricted feeding, taurine has been proposed as a potential supplement to replicate the benefits of CR [29]. In jejunum samples from mice supplemented with taurine in their drinking water, the data did not show any significant differences from the controls. There was a slight tendency for the Ad lib mice supplemented with taurine to have lower levels of taurine-conjugated BAs than the Ad lib control (Figure 14). In the liver, increased levels of CA were observed in CR-treated mice compared to their Ad lib counterparts, regardless of taurine supplementation or restriction. Notably, the highest levels of CA were observed in CR mice receiving taurine supplementation via drinking water. Interestingly, for the taurine-conjugated BAs, the Ad lib LTD mice had higher levels than the Ad lib control, with the exception of TCA, for which levels were comparable between the two groups. For TCA, the most abundant taurine-conjugated BA, the data showed that levels were higher in the Ad lib Tau group compared to the Ad lib control and were comparable to those observed in the CR Tau group. Additionally, TLCA and TUDCA levels were similar between Tau groups. In contrast, the largest differences in TCA and TUDCA levels between Ad lib and CR groups were observed in mice fed NTD diets containing 0.5% taurine. Interestingly, taurine supplementation via drinking water with 4% taurine, resulted in similar levels of most taurine-conjugated BAs between Ad lib and CR groups (Figure 15)

7.5 Transporters, receptors, and transcription factors

In plasma, the highest levels of taurine-conjugated BAs were observed in the CR BA supp group, consistent with their taurine-conjugated BA supplementation. The data indicates that levels of all BAs were elevated in CR groups relative to their respective Ad lib counterparts, irrespective of intervention, suggesting a strong effect for the CR treatment.

Interestingly, fluvastatin treatment was associated with reduced BA levels in both Ad lib and CR groups, consistent with its known ability to reduce cholesterol [75]. Reduced cholesterol availability as a substrate may have led to decreased BA synthesis. In the BSHi groups, lower levels of secondary BAs DCA and UDCA were observed in the CR groups compared with the CR vehicle control group, whereas TUDCA and TCA levels were elevated. These findings suggest that microbiota-mediated modification of primary to secondary BAs may have been reduced (Figure 16). Elevated plasma BA levels may enhance their signaling capacity and facilitate their systemic functions. Analysis of *TauT* expression in cecum revealed upregulation during CR treatment relative to Ad lib treatment. Interestingly, slightly decreased *TauT* levels were observed in the CR group supplemented with taurine-conjugated BAs compared to the CR vehicle control group, but not statistically significant. This would be consistent with previous reports from Tappaz et al., demonstrating downregulation of *TauT* in response to taurine exposure [25]. In contrast, *TauT* expression was comparable between Ad lib and CR mice in the fluvastatin intervention group. The expression from *Ibat* showed no differences between the Ad lib and CR vehicle control, but for the BSHi and BA supp intervention, the expected increase for the CR phenotype was still present. Fluvastatin treatment showed a similar high expression for both Ad lib and CR groups. This is interesting as it may indicate that higher *Ibat* expression could compensate for reduced de novo BA synthesis resulting from lower cholesterol levels. Enhanced *Ibat* expression may facilitate increased BA reuptake in the intestine and prevent loss via feces. Analysis of *Mrp1* expression showed elevated levels in the CR vehicle control and BSHi groups compared to their respective Ad lib counterparts. Interestingly, for the BA supp and fluvastatin groups, the expression of *Mrp1* was downregulated in the cecum of mice treated with CR. CR treatment had a strong effect on *Mgst1* expression levels, as the CR groups had elevated levels in all treatment groups except fluvastatin, which had similarly low levels of *Mgst1* expression in both the Ad lib and CR groups. *Ostα* expression was also found to be increased in CR groups, with the exception of the fluvastatin group, which showed high expression levels but no discernible difference between Ad lib and CR groups. *Tlr3*

expression was higher in the CR-treated groups. BA supp and fluvastatin treatment showed similarly high levels in both the Ad lib and CR groups. *Pxr* expression showed the same pattern as *Tlr3*, with higher expression in the CR groups. The groups treated with BA supp and fluvastatin showed similarly high expression levels for both the Ad lib and CR interventions. *Stat1* expression was downregulated during CR for all interventions when compared to their Ad lib counterpart. The fluvastatin group had the highest expression overall. Previous studies have reported that CR reduces the expression of inflammatory factors along the gastrointestinal tract. For instance, Duszka et al. observed downregulation of *Stat1* and *Tlr3* expression in the jejunum of caloric restricted mice [28]. These findings are consistent with the measured *Stat1* expression levels, but not with those of *Tlr3*. In this thesis, *Tlr3* expression was found to be upregulated in the CR vehicle control and BSHi groups, with only negligible decreases observed in BA supp and fluvastatin CR mice compared to their Ad lib counterpart. Evaluation of the fluvastatin intervention indicated a strong effect on transporter expression, with increased levels of *TauT*, *Ibat*, *Ostα,* and *Mrp1* observed. In Ad lib fluvastatin-treated mice, *Tlr3* and *Pxr* were upregulated to levels consistent with the CR phenotype. However, no corresponding increase in *Mgst1* expression was observed during CR fluvastatin treatment. In contrast, the BSHi intervention did not show a strong effect when compared to vehicle control groups. (Figure 17)

7.6 BSH activity

BSHs catalyze the initial reaction in the BA modification pathway mediated by the gut microbiota. Given that BAs serve as signaling molecules that regulate their own biosynthesis, lipid absorption, cholesterol homeostasis, and intestinal mucosal defenses, microbial BSH activity has the potential to significantly impact host physiology [76]. Several hypotheses have been proposed to explain the importance of deconjugation. As previously mentioned, deconjugated primary BAs can function as signaling molecules that alter the total BA pool. Thus, it is possible that the microbiota has evolved deconjugation mechanisms to further manipulate bile production. Ridon et al. showed that deconjugation also increased the concentrations of the antimicrobial BAs, CA and CDCA, which may induce

shifts in microbiome composition and serve as a form of microbial chemical warfare. BSHs are capable of deconjugating both glycine- and taurine-conjugated primary BAs, although variations in activity may suggest BSH substrate specificity [14]. Additionally, members of the gut microbiota may utilize the released glycine and taurine residues as nutrient sources. In any case, deconjugation is a crucial function of the gut microbiome [70]. The extent of BA deconjugation over time was estimated by comparing the concentration of unconjugated BAs with their taurine-conjugated counterparts. TCA showed a strong decreasing trend over time in all groups, while CA increased. TDCA and TUDCA decreased in all groups, but DCA and UDCA, as unconjugated counterparts, did not increase to the same extent in all groups. The data also showed the same effect for CDCA. The Ad lib BSHi group had the lowest increase in unconjugated BAs, but there was no difference in the deconjugation of the taurine-conjugated BAs. The Ad lib TauTi and Rapamycin treated mice showed the same pattern. The data suggest that the interventions did not significantly impact deconjugation capabilities. However, there was still a notable difference in the increase of the unconjugated counterparts for the Ad lib BSHi, Rapamycin, and Ad lib TauTi treatments (Figures 18-20).

7.7 Further measurements

Some of the measurements yielded ambiguous and differing results, suggesting the need for further experimentation. Muricholic acid (MCA), a key BA in mice, was not included in these measurements for this thesis. Hence, assessing MCA levels and comparing them with other primary BAs can offer valuable information. Since this thesis focuses on BAs and their alterations in mice, it would be advisable to conduct further research involving human studies in this field. Due to differences in BA metabolism, it is not always easy to extrapolate results from animal studies to human metabolism. Therefore, it is essential to conduct more research on BAs and CR in humans. Additionally, it is recommended to reevaluate the measurement of certain transporters, receptors, and transcriptional factors that play a role in BA metabolism to gain deeper understanding. Given these factors, future studies should be conducted with modified experimental designs and the inclusion of additional

parameters to obtain comprehensive results. As such, the generalizability of the current results is limited, and further research is needed.

7.8 Limitations

Attempts were made to measure LCA, a secondary BA, using LCMS. However, the peak detection and resulting concentrations were inconsistent and unclear, leading to the exclusion of LCA from further measurements and its omission from the results. The inclusion of LCA measurements would have been valuable, as DCA was the only secondary BA measured in this thesis. Given that LCA is considered the most toxic BA, the absence of LCA results is particularly noteworthy. Similarly, measurements of DHCA were also inconclusive. Attempts to measure farnesoid X receptor (*Fxr*) expression using qPCR were also unsuccessful. Furthermore, the results from the microbiota measurements were inconclusive for some bacteria.

8 Conclusion

This master thesis aims to study the effects of CR on BAs composition and metabolism in mice. The primary objective was to identify differences in the BA composition across various tissues. The results indicate a distinct CR phenotype in the ileum, liver, plasma, and proximal colon, characterized by an elevated BA pool size. In adipose tissue, CR-induced BA increase was most evident in mice without bedding. Surprisingly, additional dietary taurine or its absence had a negligible effect on the CR phenotype. There are several potential reasons for the increased BA pool during CR, including changes in the expression and activity of transporters, receptors, and transcription factors involved in the synthesis and metabolism of BA, which motivated the second objective of this work. The results revealed enhanced expression of BA-associated transporters in the CR phenotype, particularly *TauT*, the taurine transporter, which showed higher expression levels in the CR groups. Intriguingly, treatment with BSHi, which is intended to inhibit the deconjugation of taurine-conjugated BAs, had no discernible effect compared to the control. *Ibat*, the apical BA transporter in

the gut, was not elevated during CR in the control groups, but with BSHi and BA supp (only taurine-conjugated BAs) treatment, the CR phenotype was still evident. In contrast, *Ostα*, the basolateral BA transporter, showed higher expression levels during CR treatment in the control. The expression of the inflammatory factors *Stat1* was downregulated during CR, whereas *Tlr3* was upregulated. The CR phenotype also exhibited higher expression levels of the detoxification factors *Pxr* and *Mgst1*. Interestingly, mice treated with fluvastatin, an inhibitor of the rate-limiting enzyme in cholesterol biosynthesis, did not show significant differences between Ad lib and CR for those genes, except for the downregulation of *Stat1*, which was comparable to the other treatment groups. Another possibility for changes in the BA pool composition, is that CR alters the gut microbiota, which in turn can affect BA metabolism. In this section of the thesis, changes in the microbiota were observed in the cecum. The data suggests that CR is associated with changes in the levels of specific bacteria, including a decrease in *Clostridium leptum* across all CR treatments and a strong effect from the CR NB phenotype on *Bacteroides fragilis* and *Lactobacillus*. *Bifidobacterium* appeared to be more abundant in mice kept under NB conditions. In the final part of the thesis, fecal BSH activity was estimated by assessing the ability of the microbiota to deconjugate taurine-conjugated BAs. The results showed that the deconjugation capabilities were present in all tested groups, with no differences between Ad lib and CR. However, slight differences were observed in the expected increase of unconjugated BAs as a counterpart, for the BSHi, TauTi, and Rapamycin treated mice, which could indicate further influences on BA modification through gut bacteria. To conclude, CR may strongly affect the BA pool and metabolism, including the associated transporters, receptors, and transcription factors. It appears that there is an interaction between CR, BA composition, and gut bacteria. However, additional research is necessary to fully comprehend the mechanisms underlying the observed increase in BA pool size during CR in mice. Despite extensive studies on BAs, much remains to be discovered.

9 References

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