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Eidesstattliche Erklärung

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Wien, 2022

Index of Abbreviations

ABC	ATP binding cassette
ACOX2	Acyl-CoA oxidase 2
AD	Alzheimer´s disease
Ad lib	Ad libitum
AKR1D1	Aldo keto reductase
BA	Bile acid
BAS	Bile acid-CoA synthetase
BAT	Bile acid-CoA amino acid N-transferase
BMI	Body mass Index
BSEP	Bile salt export pump
BSH	Bile salt hydrolases
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CR	Caloric restriction
CSD	Cysteinesulfinic acid decarboxylase
DCA	Deoxycholic acid
DHEAS	Dehydroepiandrosterone
DMT	Diabetes mellitus typ
DNP	2,4-dinitrophenol
FFM	Fat free mass
GAT	Gamma-aminobutyrate transporte
GGT	γ-glutamyltranspeptidase
GIT	Gastro intestinal tract
GSH	Glutahtion thiol reduced
GSSG	Glutathion dissulfid oxidized
HD	Hungtington disease
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylgrlutaryl coenzyme A reductase
HSD3B7	3β-Hydroxy-Δ^5-C 27-steroid dehydrogenase
ICD	International Codes of Diseases

IFNγ	Interferon gamma
Ig	Immune globulin
IL	Interleukin
LCA	Lithocholic acid
LDL	Low density lipoprotein
MHY1485	4,6-dimorpholino-N-(4-nitrophenyl)-1,3,5-triazin-2-amine
mTOR	Target of rapamycin
NCD	Non communicable disease
NK	Natural killer
PD	Parkinson´s disease
RMR	Resting metabolite rate
SPF	Specific pathogen free
TauT	Taurine transporte
UDCA	Ursodeoxycholic acid
WAT	White adipose tissue
WHO	World Health Organization
Ω-MCA	Muricholic acid

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1 Abstract

Bile acids are produced from cholesterol in a multistep reaction. Their important role is to emulsify and absorb fat and lipids from the intestine. Under certain conditions and in certain organism some bile acids can be beneficial for life extension. In order to elucidate the effect of caloric restriction (CR) on bile acids, mice were subjected to a CR diet for two weeks. During these two weeks, mice were supplemented with fluvastatin, BSH inhibitor, taurine conjugated bile acids, rapamycin and MHY1485 in their diet. The outcomes were evaluated.

Furthermore, a lifespan experiment was done with the nematodes *C. elegans by* comparing the experimental group to a control group. The experimental groups were set on a dose of 0.002 mg/ml and 0.0005 mg/ml mix of taurine conjugated bile acids respectively. Our data showed that there was no significant difference between the experimental groups compared to the control groups in terms of lifespan.

The body weight of the CR fed mice was statistically significant lower compared to the Ad lib fed mice. In this CR experiment the levels of taurine and taurine conjugated bile acids were increased in the liver and in the intestine. The morphology of the small intestine also changed. The muscle thickness and the villi length decrease supplementing bile acid. That leads us to assume that caloric restriction triggered production and release of taurine conjugated bile acids.

2 Zusammenfassung

Gallensäuren werden in einer mehrstufigen Reaktion aus Cholesterin hergestellt. Ihre wichtige Aufgabe besteht in der Emulgierung und Absorption von Fetten und Lipiden aus dem Verdauungstrakt. Unter bestimmten Bedingungen und in bestimmten Organismen können einige Gallensäuren für die Lebensverlängerung von Nutzen sein. Um die Auswirkungen einer Kalorienrestriktion (CR) auf Gallensäuren zu untersuchen, wurden Mäuse zwei Wochen lang einer CR-Diät unterzogen. Während dieser zwei Wochen erhielten die Mäuse zusätzlich Fluvastatin, einen BSH-Inhibitor, Taurin-konjugierte Gallensäuren, Rapamycin und MHY1485 in ihrer Nahrung. Die Ergebnisse wurden ausgewertet.

Darüber hinaus wurde ein Lebensdauerversuch mit dem Fadenwurm *C. elegans* durchgeführt, bei dem die Versuchsgruppe mit einer Kontrollgruppe verglichen wurde. Die Versuchsgruppen erhielten eine Dosis von 0,002 mg/ml bzw. 0,0005 mg/ml einer Mischung aus konjugierten Taurin-Gallensäuren. Unsere Daten zeigten, dass es keinen signifikanten Unterschied zwischen den Versuchsgruppen und den Kontrollgruppen in Bezug auf die Lebenserwartung gab.

Das Körpergewicht der CR-gefütterten Mäuse war statistisch signifikant niedriger als das der Ad lib gefütterten Mäuse. In diesem CR-Experiment waren die Werte von Taurin und taurinkonjugierten Gallensäuren in der Leber und im Darm erhöht. Auch die Morphologie des Dünndarms veränderte sich. Die Muskeldicke und die Länge der Darmzotten nahmen mit der Einnahme von Gallensäure ab. Dies lässt vermuten, dass die Kalorienrestriktion die Produktion und Freisetzung von konjugierten Taurin-Gallensäuren auslöste.

3 Introduction

In the last decades obesity is reaching pandemic proportions. Obesity is a major factor for NCD (Non-Communicable Diseases). Individual long-term effects against obesity could be caloric restriction and physical activity. Caloric restriction (CR) is used as a tool to reduce the caloric intake. If ad libitum represents the 100% of daily caloric intake, CR is a reduced caloric intake by at least 10%. Showing remarkable health benefits in modern medicine, CR is used for weight loss and health maintenance.

The first nutrition studies involving CR were conducted at the beginning of the 20th century but the idea that CR leads to health benefits and extended lifespan is much older. Studies have been conducted across all species. The first CR experiments were performed using mice and rats resulting in the lowered age specific mortality. In mice it was observed that CR changed the physical activity pattern and also affected the metabolism, like reduction of adipokines, insulin and glucose levels.

Taurine is known as an endogenous amino acid with protective properties. It has anti-inflammatory, antioxidative and osmoprotective properties. Bile acids have an important role for emulsification and absorption of fats and lipids. It is known that conjugated bile acids have a lower pKa than non-cojugated bile acids, what leads to higher emulsifying capacity.

3.1 Obesity

In the past few decades' obesity prevalence has increased worldwide. In some regions and countries, like developing countries, it is doubled or tripled compared to the early years of 1975. Obesity causes more than ≥ 70 % of the early deaths worldwide and is a major risk factor for non-communicable diseases (NCD) like cardiovascular diseases, diabetes mellitus, cancer, and premature disability [1]. On the tenth revision of International Codes of Diseases (ICD 10) obesity was added to the category of endocrine, nutritional, and metabolic diseases [2].

There are many factors that can influence obesity, such as western lifestyle, technical revolution (sedentary work), eating behavior, and individual energy expenditure. Behavioral researchers showed that western lifestyle might increase obesity. Pima Indians living in Mexico have a lower prevalence of obesity than those living in the United States. Some gene mutations and single nucleotide polymorphism may cause the BMI variability [3].

In some cultures, increased weight or obesity is considered as beautiful and is intentionally used to make a person attractive for marriage. Being overweight can be beneficial in period of war and famine and is even a protecting factor. All individuals being overweight have better chance to survive these periods. Evolutionary it was a tool to survive periods of undernutrition. In the early decades of the past century, overweight was a symbol of health and wealth [3].

Nevertheless, nowadays with food in excess, obesity is a recurring problem that has not been resolved yet. In 2010 for the WHO "Global Action Plan for the prevention and Control of Non-Communicable Diseases" the target was to stop the obesity or at least to decrease it. Current health recommendations aim at individual restriction of the caloric intake and to change the moving behavior to expend more energy [2]. However, it would also need more support from the government in making policies for the food processing and marketing, education, environment, and agriculture. The benefits for each individual stemming from decreasing obesity and physical activeness in old age is minimizing the risk of getting cancer, diabetes mellitus or other with NCD associated diseases.

3.2 Caloric restriciton

3.2.1 Definition and History of Caloric Restriction

Caloric restriction is defined as a tool to reduce the daily caloric intake. In most studies caloric reductions of 20% - 50% are common. But in some cases, also reductions, higher than 50% are reported. Beneficial effects occur with a caloric restriction from 10% to 50% [4]. Caloric restrictions higher than 50% with or without appropriate medical supervision can end deathly [5].

In the 15th century an Italian nobleman called Luigi Carnaro, proclaimed in his book “La vita sorba” or translated to “The way of living long”, that caloric restriction increases the life expectancy based on his self-experiment. The premise was to eat as little as possible in order to live longer [4].

The first experiment on caloric restriction was conducted on mice and rats at the beginning of the 20th century. Osbourne et al. showed in 1917 that a restriction in food intake has positive effects on the life extension [6].

In one of the first studies examined on white rats, 40% of them under CR, extended the life span up to two times applying the CR at the age of weaning or short thereafter [7]. Nevertheless, there are also detrimental effects of caloric restriction like intensified perception of feeling cold also in warm months, slowing down the metabolism, food obsession, apathy, depression, weakness, edema, lack of ambition and absence of libido [8]. Additionally, the CR effect on lifespan was not positive on some invertebrates like house flies or water strider [4].

3.2.2 Benefits of Caloric Restriction

The apparent effect of the caloric reduction is the loss of weight. In the Minnesota study 36 white male volunteers, that were between 20 and 33 years old, were set for 6 months on a CR after 3 months of control. During the 3 months of control, they were set on a diet with 3150 kcal/day followed by a 1750 kcal/day CR diet for 6 months with a recovery period. The chosen diet was high in carbohydrates (~70 %) and low in fat and proteins to represent the food which characterized the

European famine conditions. The human study showed that from the average weight loss more than 35% was the fat. The rest of it was the fat free mass [8]. In animals CR has the same effect, loss of weight, mostly from the white adipose tissue (WAT) [9]. In another animal study with a quite different food protocol and a standard laboratory chow, CR led to decreases in both fat and the lean body mass. Furthermore, it also led to decrease glucose homeostasis and insulin sensitivity. In animal lifespan studies, nutritional intervention like caloric restriction, time restricted feeding and intermittent fasting are used to slow the progression of age associated symptoms [10]. There are not well controlled long-term studies but short-term CR diets on humans showed beneficial effects on weight loss and could be used to treat obesity [8,11]. Weight loss is not the only result of CR. Effects like the change of body composition, the drop of the RMR (resting metabolite rate) and the decrease of FFM (fat free mass) occurred to save the body fat. The physical activity also changed under CR and is decreased under normal circumstances and increased during the feeding phase [12]. The metabolic biomarkers glucose, insulin and leptin declines and the metabolism changes from carbohydrates to fat metabolism [10].

The lifelong CR intervention in mammals slows the intrinsic rate of aging and improves life quality. As reported from early experimental works, Osbourne et al. showed a prolongation of the lives of CR rats [4,6-7]. Since then, several observations with similar criteria have been done in different organisms like mice, rats, flies, worms, and monkeys. Studies on different model organisms showed that lower body temperature is a biomarker for longevity [13]. Furthermore, an experiment on rhesus monkeys showed that not only low body temperature but also low plasma insulin and high DHEAS (dehydroepiandrosterone) levels could lead to biomarkers. DHEAS is therefore suspected to be a marker of longevity in humans [14]. The pioneer study on humans showed that besides the poor quality of the diet, CR caused changes in metabolic rate, pulse, body temperature [8]. It is common that some populations which are exposed to dietary restriction lack of some macro- and micronutrients. Consequences of it would be short stature, late reproductive maturation, suppressed ovarian function, impaired fecundity, and impaired immune function [15].

In Japan the Okinawa analysis showed that there are 40 time more centenarians living on the island than in the rest of Japan. Furthermore, analysis showed that inhabitants of Okinawa are smaller compared to the rest of Japan. A 14-year-old average girl is about 4 cm smaller and 4,3 kg lighter than one who lives on the mainland of Japan. The energy intake of schoolchildren was more than 35% lower in Okinawa. Also, the total energy intake of the adults was 20% lower than the Japanese national average, but the lipid and protein intake were the same. Only few people in Okinawa had serious diseases before the age of 60 years [16]. People who left the island showed higher mortality rates than those who remained on it [15].

Eight participants, 4 male and 4 female, were sealed for 2 years in the Biosphere 2 project. During the project the participants were set on low caloric but nutrient dense diet with vegetables, nuts and grains but low in dairy, egg and meat. The Biosphere 2 project has shown that all participants lost weight. The loss of weight in combination with a lower renin level lowered the systolic and diastolic blood pressure. Other blood parameters are also decreased under CR like fasting blood sugar, fasting insulin, cholesterol, white blood cells and T3. Blood lipids are sensitive to CR, and both LDL and HDL are lower under CR than under ad lib conditions. Interestingly prolactin is lowered in females and increased in man under CR conditions [17]. One explanation for longevity is lowering the body temperature concerning the hibernation like-response based on the biochemical and physiological akin responses [18]. Except the effects on health and mortality, CR is also beneficial to lower cancer incidence, diabetes mellitus, immune system and many more [4].

3.2.3 Cancer

One of the leading death causes in the world is cancer. It is one of most common NCD in the world presenting a large group of diseases. Cancer is a transformation from normal cells to tumor cells in a multistage process and uncontrolled growth. Generally, it is distinguished between cancer invasion, where neighboring cells and adjacent organs are attacked, and metastasis, which are spread to different sites

of the body. Other common terms used for cancer are neoplasm and malignant tumor [19].

A review from 2005 showed that in 2001 there were estimated 7 million deaths worldwide caused by cancer. About 35% of these deaths were linked to modifiable risk factors, including obesity and unhealthy lifestyle. The most important cancer cause in high-, middle- and low-income countries was the abuse of tobacco and alcohol. It was shown, that for eating related cancers obesity was the most important cancer cause in high income countries. In contrast to that, a low intake of fruits and vegetables was the most common cause of eating related cancers in low- and middle-income countries [20].

Cancer caused deaths rose in the last two decades and in 2020 there were estimated 10 million deaths caused by cancer. In 2012 it was the second leading death in the world with 8.2 million related deaths [19].

The cancer treatment chemotherapy or radiotherapy combined with surgery remained relatively similar in the last 30 years. In the guidelines of American Cancer Society there is a recommendation for patients who are receiving the chemotherapy to increase the caloric and protein intake without clear medical reason [21]. In one of the first cancer research studies on mice, it was shown that transplanted tumors in underfed mice did not grow as well as those in ad libitum fed mice. The protective effect was confirmed in the 1940s and showed the dependency of caloric content and not of the source. On the other side there are some indications that CR could help to lower the cancer incidence. For that, the time of starting and the amount of CR plays a key role, just like the type of fat that is taken. Even the reduction of 10% of the daily intake leads to reduction of cancer incidence. In a case control study, it was conducted that incidence of colon carcinoma is positively correlated to caloric intake [21, 22].

In rodents that were under CR, it was shown, that during their lifetime the incidence of tumors was reduced for both sexes, compared to the control group. The beneficial reduction effects of tumors have been detected in lymphoma carcinoma, pituitary neoplasm, thyroid neoplasm in females and hepatocellular tumors in males. The onset of the tumor has also been delayed in the first 24 months of age

in CR compared to ad libitum fed mice. It was also shown that CR fed mice reduce to develop mammary carcinoma [4].

3.2.4 Diabetes mellitus

In the last 30 years the number of people diagnosed with diabetes mellitus increased consistently and reached the number of 422 million. Only 9 million people are diagnosed with diabetes mellitus type I. Diabetes is a major cause for blindness, kidney failure, heart attacks, stroke, lower limb amputations and was in 2019 the ninth leading death cause. Diabetes occurs when the pancreas does not produce enough insulin, or the body cannot use it effectively. This leads to hyperglycemia which can lead to serious damage in nerves and blood vessels and many more side effects. 95% of diabetes suffers have type 2 diabetes and the cause of it is excess body weight and physical inactivity [23]. Another reason for diabetes mellitus type 2 is the linear decline of functions of the beta cells continuously whit aging. There is strong evidence that type 2 diabetes is inexorably progressive but, in the opposite, its reversible following bariatric surgery. In one study participants were set on CR to show the changes in the body metabolism. After one week of fasting, plasma glucose and triacylglycerols normalized and were stable for the rest of the experiment. The insulin sensitivity increased, and as a side effect participants lost weight of which more than 60% was fat. After the experiment a follow up was done after several weeks. The triacylglycerols remain unchanged, the fasting plasma glucose and body weight increased [24].

In some cases, CR is not an optimal therapy for diabetes and obesity. Here the patients need to undergo a bariatric surgical procedure. Generally, it should be distinguished between the restrictive and malabsorptive method. After a bariatric surgical procedure, which is a, malabsorptive method, the bioavailability of food is limited. In the restrictive method only the food intake is limited. Both have the effect to lower the nutrient intake. In a meta-analysis different impacts of bariatric surgical procedures were examined and compared. The weight loss effects of the bariatric surgery compared to the conventional therapy are significant and more successful. The diabetes remission, lower fasting plasma glucose and the glycated hemoglobin

was lower in the group which underwent bariatric surgery than conventional therapy. Conventional therapy may achieve great results, but low compliance due to strict dietary restrictions is the biggest problem. In the long-term patients achieve 10–16-times better results with bariatric surgery than with the conventional method [25].

3.2.5 Immune system

The effects of CR on the immune system are of high interest, as the energy demand of the immune system is enormous. It is even more important for rodents which are kept under SPF (specific pathogen free) conditions. In case of an infection under CR conditions the immune defense would be limited. On the other hand, humans do not live under such conditions and the immune system might has impact on the efficacy of CR for a life extension strategy. Thus, the focus on investigations for CR on immune system is on T-lymphocytes, macrophages, and natural killer cells (NK cells). T-lymphocytes, that are originated from marrow bone are formed in thymus into CD4 or CD8 receptor positive T-lymphocytes and were activated in the periphery. Further, interleukin 2 (IL-2) or interferon gamma (IFNγ) are produced from CD8 T-lymphocytes, which play an important role in the immune defense and destroying cells infected with virus and cancer cells. CD4 T-lymphocytes are producing interleukins 4,6 and 10 which stimulate the B-lymphocytes to produce the immunoglobulins (IgA, IgD, IgE, IgG and IgM). The importance of immunoglobulins or antibodies is in the defense of extracellular pathogens (bacteria). A side effect of the antibodies during aging might be autoimmune disease or the immunosenescence. During aging mammalian metabolism decreases and therefore also T-cell production decreases due of thymus involution [4].

A study on rodents shows the effect of CR on immune functions. To explore the humoral response of antigens (IgM and IgG) a challenge test on sheep red blood cells was taken. Results showed that CR delayed the maturation of the immune system [26]. CR has a negative effect on NK cells but on the other side it delays thymic involution. The positive effects of CR on immune responses have been

observed in dogs [4]. Another benefit with CR was shown in vaccinated mice. They had a higher immune capacity to virus infection and a delay of maturation in immune system [27].

It might also be important, when to start with CR. It has been shown that CR may bring benefits to the immune system regardless to the onset and species [28].

3.2.6 Neurodegenerative diseases

Aging is a natural process occurring in every organism and the highest risk factor for neurodegenerative diseases (ND). The pathological hallmarks of neurodegenerative diseases like Alzheimer´s (AD), Parkinson´s (PD) and Hungtington disease (HD) have been located in the affected brain regions. While the exact process of protein aggregation is yet unknown, it is assumed that defects in autophagy pathways may contribute to neurodegeneration. Autophagy decreases with aging, which leads to a failure in the principal protein degradation. Some interventions have shown that enhancing autophagy have better protective effects on AD, PD and HD models. Autophagy serves as a housekeeping function and is essential for degrading damaged proteins and organelles, preventing the accumulation of aggregates, providing energy demands and supporting neuronal plasticity. There are three types of autophagy described in mammals: macroautophagy, chaperone mediated autophagy and microautophagy. The interesting one of them is CMA (Chaperone mediated autophagy), which is important in response to nutritional and oxidative stress, but it also diminish during aging [29]. An important role in autophagy signaling plays mTOR, but the mechanism how it regulates is still unclear. Autophagy could be upregulated through lipophilic antibiotic rapamycin which also inactivate the mTOR and activates the autophagy. Some evidence indicate that basal autophagy is essential for maintaining axon homeostasis and morphology, and its loss of function leads to axonal swelling, followed by progressive neurodegeneration. Excessive activation of autophagy is associated with cell death, massive cell elimination and neuronal excitotoxicity [30].

In a cohort study it has been shown that fat intake is highly associated with PD, and that individuals with the lowest daily intake of calories have the lowest risk for PD. This finding suggests that increased animal fat and caloric intake have a higher oxidative reaction and play an important role in the pathogenesis of PD [31]. In order to neurodegenerative diseases and upregulation of autophagy there are some clinical trials on animals with different pharmacological drugs. The 2,4-dinitrophenol (DNP), an already known pharmacological uncoupler, which was used in the past to reduce body weight and stimulates several adaptive cellular stress-response pathways in neurons has shown on animals in low doses positive effects to improve and protect neurons in Alzheimer and Parkinson diseases [32]. There are possibilities with CR to ameliorate age-related cognition deficits and to activate the autophagy [33].

3.2.7 Negative Effects of CR

Physical activity influences the energy expenditure of most organisms. In animals it would be assumed that they reduce their physical activity to make a balance between intake and expenditure. Paradoxically under caloric restriction animals are moving more than under Ad lib conditions. The reason for their higher activity is the searching for food and therefore leaving the area with low food sources. The stimuli could be the leptin which increases in organism during CR. Another signaling system that increases the physical activity could be orexin [4]. In contrast in humans initial stimulation is missing and a decrease in physical activity appears. This shows that CR in animals is unvoluntary and the response is the seeking food behavior. In humans CR is voluntarily, and the response would be lowering the physical activity [34].

The effects of CR are not always beneficial and do not have positive effects on health. The demonstration of CR in the Minnesota experiment, as described in chapter 2.2.2, has shown what happens with undernourished individuals. Extreme low-calorie diet might not deliver the calories and nutrients necessary to maintain homeostasis, which leads to loss of weight also in the lean tissue. In the last few weeks of the experiment, the morale of the CR group declines together with the

physical activity. The voluntary work was done perfunctorily or was even skipped, social relations dwindled, and the education sagged. It was also examined that apathy and depression were spreading among the participants. Symptoms that occurred were muscle soreness, general irritability, inability to concentrate, depression, dizziness, lack of ambition, moodiness, sensibility to noise, muscle cramps and absence of libido. Evident were also the morphological and physiological changes, feeling cold and the perpetual hunger [8].

After a while under CR the libido is decreased or absent in males and females. It could be an evolutionary aspect to reduce the fertility and, not becoming pregnant in time where insufficient food resources are available. Switching off reproductivity could be rapid and insulin signaling pathway seems to play an important role [4, 34].

3.3 Taurine

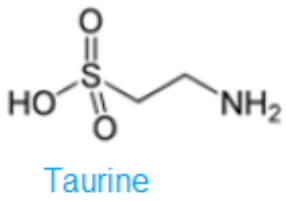

Figure 1: Structural formula of taurine

Taurine is an endogenous protective substance in the eye, protecting the distal retina from toxic levels of glutamate. Also known as 2-aminoethane- sulfonic acid (see figure 1), taurine has a cytoprotective role, it regulate the cell volume (osmolyte), takes part in formation of the conjugated bile acid and modulate the intracellular free calcium concentration. It is an abundant substance with occurrence in tissue all over the body especially in the brain, spinal cord, leukocytes, heart muscle, muscle cells and retina [36].

Its presence is high in algae and the animal kingdom but low or absent in bacteria and plant kingdom. The first isolation and identification of taurine was done in the 19th century by the Austrian scientists Gmelin and Tiedermann. They isolated taurine from an ox bile (Bos taurus), from which it derives its name [37, 38].

An average 70kg person contains ~70g taurine in his body [39]. Depending on dietary intake and nutritional status a healthy adult can synthetize 50-125 mg taurine/day [41]

Instead of the carboxyl group, in the chemical structure, taurine contains the sulfonate group. Taurine is not used in the biosynthesis of proteins. In the human organism taurine is synthesized in the liver from amino acid methionine and cysteine. This is the reason why it is used to call it non-essential or conditionally essential amino acid. The major biosynthesis route (see figure 2) for taurine is from methionine and cysteine over the cysteinesulfinic acid decarboxylase (CSD), which is the rate limiting enzyme [36].

There have been observations which suggest that vegetarian diet is not optimal to maintain the taurine homeostasis in organism, as well it does not deliver enough taurine for the organism, because it is essential nutrient for infants and children. [39] Humans and other primates have limited levels of CSD to biosynthesize taurine. That's why it is important to integrate taurine delivering food, meat, and seafood to the diet [36].

Aside from retina, taurine has potent protective properties against glutamate induced neuronal injury, cytoprotective attributes and functional significance in cell development, and counts to the most essential substances in the body [42]. The dietary taurine has been absorbed in the intestine over Na^+ -and Cl^- dependent transporter. The TauT, GAT2, GAT3 are Na^+ -and Cl^- dependent transporters and PAT1 is a H^+ coupled but Na^+ -and Cl^- independent transporter, of which TauT-transporter has been described as the major transporter under physiological conditions. In any case the absorbed taurine will not be degraded by the intestinal mucosa and will be transported in blood as a free amino acid [38]. Despite the abundance and ubiquitous occurrence of taurine in the body, taurine is not a neurotransmitter. The crucial criterion for a neurotransmitter is the presence of a specific receptor on post synaptic cells [36].

There is evidence that CR is associated with increased levels of free taurine and taurine conjugates in the intestine. It has been shown that multiple taurine conjugates were detected, and their levels were increased in the mucosa of CR animals compared to Ad lib fed animals [43].

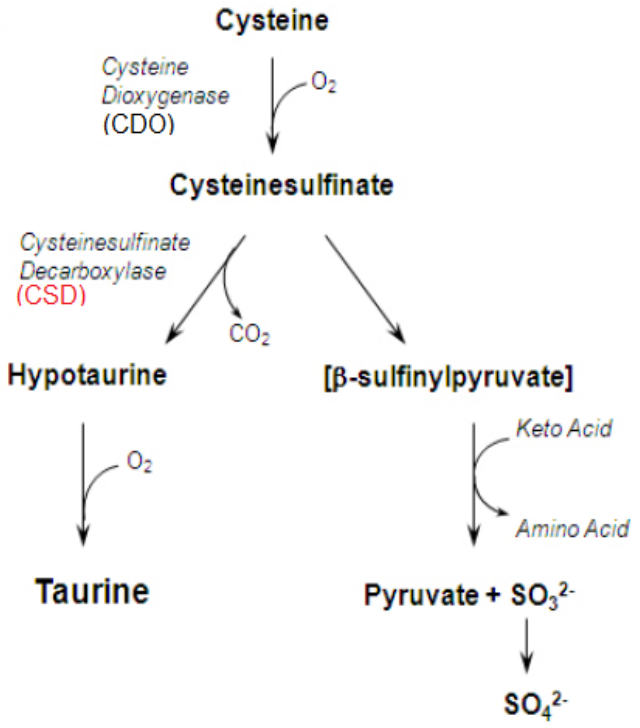

Figure 2: Biosynthesis pathway of taurine

3.4 GSH (Glutathion)

Glutathion (GSH) is a tripeptide, composed of glutamate, cysteine, and glycine present in all mammalian tissue. Glutathion peroxidase (GPx), glutathione S-transferase (GSTs) and GSH forms the glutathione system [44]. Over 90% of the glutathione is present in the reduced form (GSH). GSH has been involved in many physiological processes and plays an important role. The functions can be summarized in three major ways of GSH: serving as an antioxidant, immunity booster and as a detoxifier in eukaryotic organism [45].

In mammalian cells, GSH is found in the cytosol 85%, 10 – 15 % in the mitochondria and a small percentage in the endoplasmic reticulum [46]. GSH was first extracted form yeast and referred as philothion [45]. The origin of GSH is from the liver and it plays an important role in the interorgan homeostasis. A dysregulation of hepatic synthesis of GSH has an impact on the systematically homeostasis in the organism. The only enzyme γ-glutamyltranspeptidase (GGT), which can hydrolase this molecule, is present on the external surface of certain cell types [46].

GSH has been found as a dependent enzyme in the mucosa of the gastrointestinal tract. The concentration in the liver is two times higher than in the gastrointestinal tract [47]. The average concentration in the mammalian tissue is 1 – 10 mM of non-protein thiol [46]. Identified as taurine conjugates, levels of GSH and GSSG has been observed in the jejunum mucosa. It has been shown that GSH and GSSG are decreased in CR compared to Ad lib feed mice [43].

3.5 Bile acids

Bile acids are produced from cholesterol in a multistep reaction and occur mainly in bile. They have an important role for emulsification and absorption of fats and lipids. They also aid in absorption of fat-soluble vitamins and in prevention of cholesterol precipitation. It has also been reported that bile acids can regulate certain enzymes [78].

The synthesis of bile acids involves 17 different enzymes located in the endoplasmic reticulum, mitochondria, cytosol, and peroxisomes of hepatocytes. Each day approximately 500 mg of cholesterol is converted into bile acids in the human liver [48].

3.5.1 Primary bile acids

Bile acids synthetized from the cholesterol in the liver are called primary bile acids. In humans there are two primary bile acids the cholic acid (CA, 3α, 7α, 12α trihydroxy bile acid) and chenodeoxycholic acid (CDCA, 3α, 7α dihydroxy bile acid). In humans, primary bile acids make up about 80 % of all bile acids. In rodents the bile acid composition is different compared to humans. They have cholic acid like humans, but also contain α- muricholic acid (α- MCA, 3α, 6β, 7α-tri hydroxy bile acid) and β- muricholic acid (β-MCA, 3α, 6β,7β-tri hydroxy bile acid) as primary bile acids [49].

3.5.2 Secondary bile acids

Secondary bile acids are formed by primary bile acids in the large intestine by bacterial enzymes. They make up about 20 % of the remaining bile acid pool. Deoxycholic acid (DCA, 3α,12α-dihydroxy bile acid) and lithocholic acid (LCA, 3α-monohydroxy bile acid) are formed of the primary bile acids CA and CDCA by the gut bacterial 7α-dehydroxylase. By isomerization of CDCA, ursodeoxycholic acid (UDCA, 3α, 7β-dihydroxy bile acid) is also formed in the large intestine. In humans UDCA is a secondary bile acid but in rodents a primary bile acid. Furthermore, in rodents the gut microbes convert α-MCA and β-MCA to the ω-muricholic acid (ω-MCA, 3α, 6β,7β-tri hydroxy bile acid) [49].

3.5.3 Conjugated bile acids

Conjugated bile acids are bile acids that are efficiently linked to the amino acids glycine (G) or taurine (T). This process is called conjugation or amidation. It is specifically that conjugated bile acids have a lower pKa than non-conjugated bile acids. At physiological pH conjugated bile acids are ionized, due to their decreased pKa. This leads to a higher emulsifying capacity. Furthermore, conjugated bile acids are resistant to cleavage by pancreatic carboxypeptidase, and they are also highly stable in the intestinal lumina. They are soluble, membrane impermeable and resistant to precipitation at physiological pH [49].

The predominant bile acids in mice are the conjugated bile acid TCA and Tα- MCA and Tβ-MCA [49].

3.5.4 Biosynthesis of bile acids

In the course of bile acids synthesis from cholesterol several reactions have to take part. It is assumed that the entire set of enzymes required for the de novo bile acid synthesis comes from the hepatocytes. Furthermore, the bile acid synthesis is

more active in the pericentral hepatocytes under normal physiology than in the periportal hepatocytes [49].

Comparing cholesterol and cholic acids in figure 3, one of the primary bile acids, it is shown that the carbon skeleton of cholesterol has a planar structure, one 3β-hydroxy group and a double bound at C_5-C_6 position. Whereas cholic acid has kinked A and B rings along the C_5-C_{10} bond and three α hydroxy groups and a carboxyl group that are positioned on the side of steroid nucleus. Therefore bile acids are amphipathic molecules that have physiological detergent properties [49].

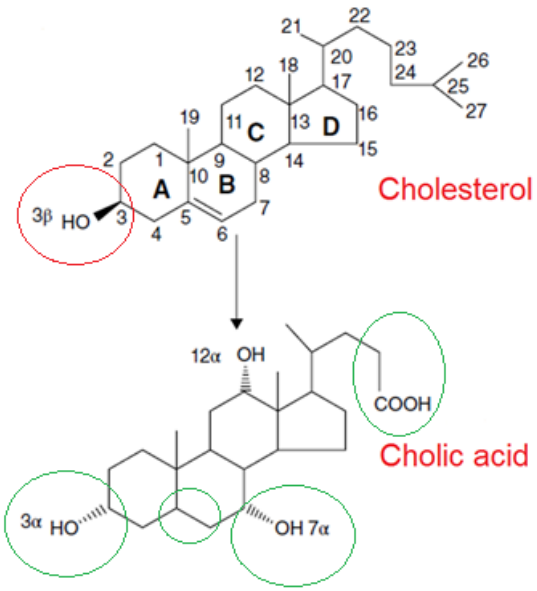

Figure 3: Main differences between cholesterol and cholic acid

There are two major pathways to synthetize primary bile acids (CA and CDCA) in the hepatocytes: the classic or the alternative pathway. There are different contributions of the classic and alternative pathways depending to the species. In humans the classic pathway is the predominant one and contributes to 90% of the hepatic bile acid production compared to the alternative one which contributes to less than 10%. Whereas in newborn, the predominant pathway is the alternative pathway, before they start expressing the enzyme that is initiating the classic pathway. In mice the bile acid synthesis is equally produced by both pathways [49].

In the first step of the classic pathway, also neutral pathway called, the C-7 α position of cholesterol is hydroxylated by the rate limiting enzyme cholesterol 7α-hydroxylase (CYP7A1), which is located in the endoplasmic reticulum. The 3β-Hydroxy-Δ^5-C 27-steroid dehydrogenase (HSD3B7) then converts 7α-hydroxycholesterol to 7α-hydroxy-4-cholesten-3-on (C4). CA will be produced if sterol-12α-hydroxylase (CYP8B1) is hydroxylating the C-12 position of C4. Otherwise CDCA will be produced. Therefore the activity of CYP8B1 affects the ratio of CA:CDCA production. In a cascade of different enzymatic reactions with aldo-keto reductase enzymes and the mitochondrial enzyme sterol 27-hydroxylase (CYP27A1) 3α, 7α, 12α-trihydroxy-5-cholestanoic acid (THCA) the precursor of the CA and, 3α, 7α-dihydroxycholestanoic acid (DHCA) the precursor of the CDCA, are produced. In several enzymatic modifications of the steroid nucleus using two different acyl-coenzyme A (CoA) and other enzymes CA and CDCA are formed respectively. CA and CDCA are then conjugated to taurine or glycine by bile acid-CoA:amino acid N-acyltransferase (BAAT) [49].

The alternative pathway, also acidic pathway called, begins with the hydroxylation and oxidation of cholesterol by mitochondrial CYP27A1. The C-7 position is hydroxylated by oxysterol 7α-hydroxylase (CYP7B1), followed by enzymatic reactions to produce CDCA [49].

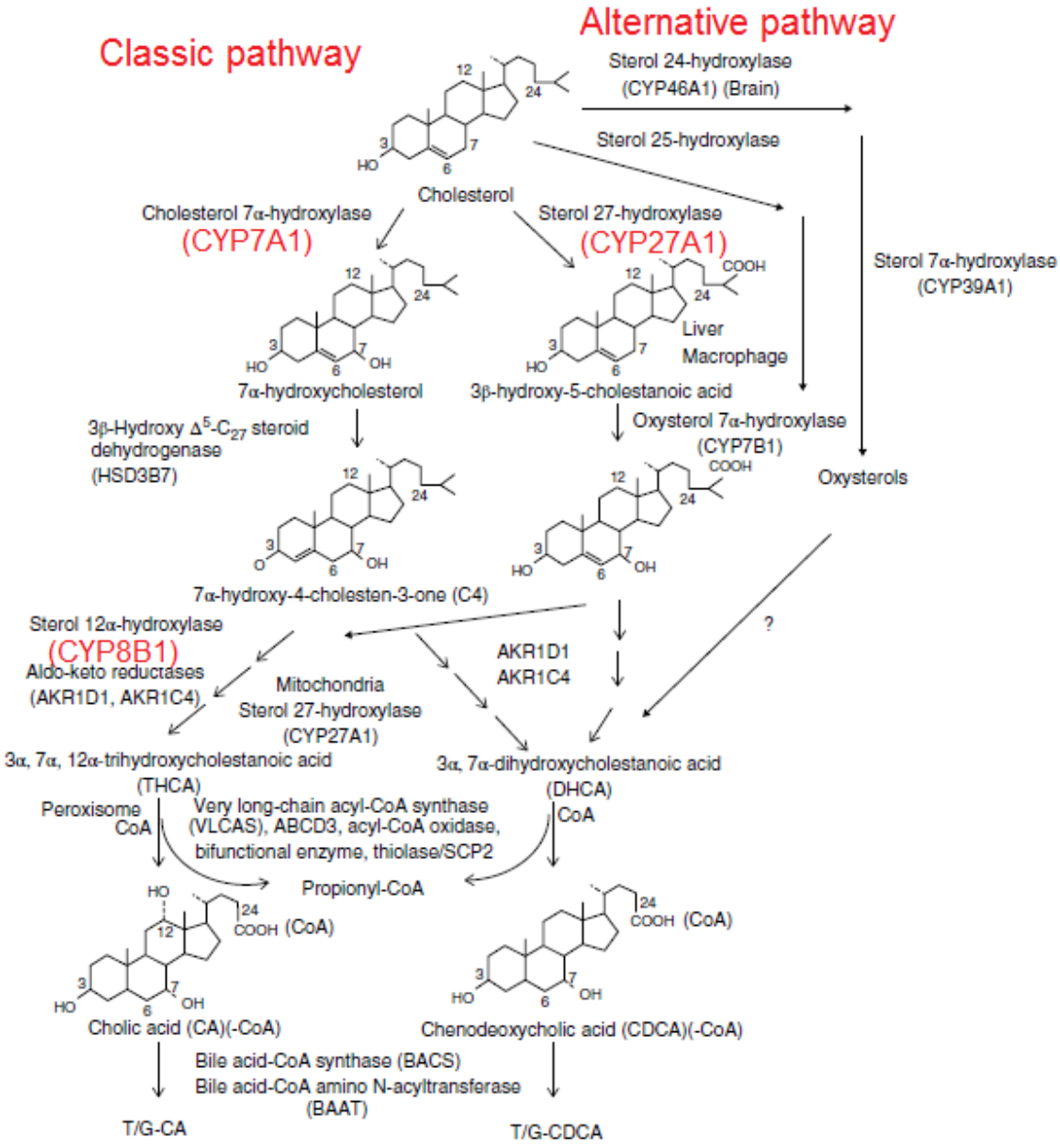

Figure 4: Classic and alternative bile acid biosynthesis pathway

3.5.5 Secondary bile acid synthesis

After the secretion of bile acids in the small intestine, a part of the bile acids is undergoing some complex modifications by bacterial enzymes. The bile acid pathways are remarkably similar between humans and rodents but there are some differences between the species [52].

As shown in figure 5 firstly T/G-conjugated bile acids are deconjugated by bacterial bile salt hydrolases (BSH). Then CA is converted to the predominant DCA, and CDCA is converted to LCA by 7α-dehydroxylase in the colon. Unconjugated primary and secondary bile acids are hydrophobic, of which LCA is the most hydrophobic and toxic one. It is only present in trace amounts in the human body. UDCA is formed by isomerization of the 7α-hydroxy group in CDCA to 7β-position and which is a minor primary bile acid in rodent and secondary bile acid in humans [49].

The difference between humans and mice has been recently confirmed. Mice and rats can synthetize α-MCA and β-MCA using CDCA and UDCA, respectively as substrate. As already mentioned above, α-MCA and β-MCA are primary bile acids in rodents, that can be converted by epimerization of the gut microbes to ω-MCA for fecal excretion. MCA is not formed in humans for any appreciable degree [53].

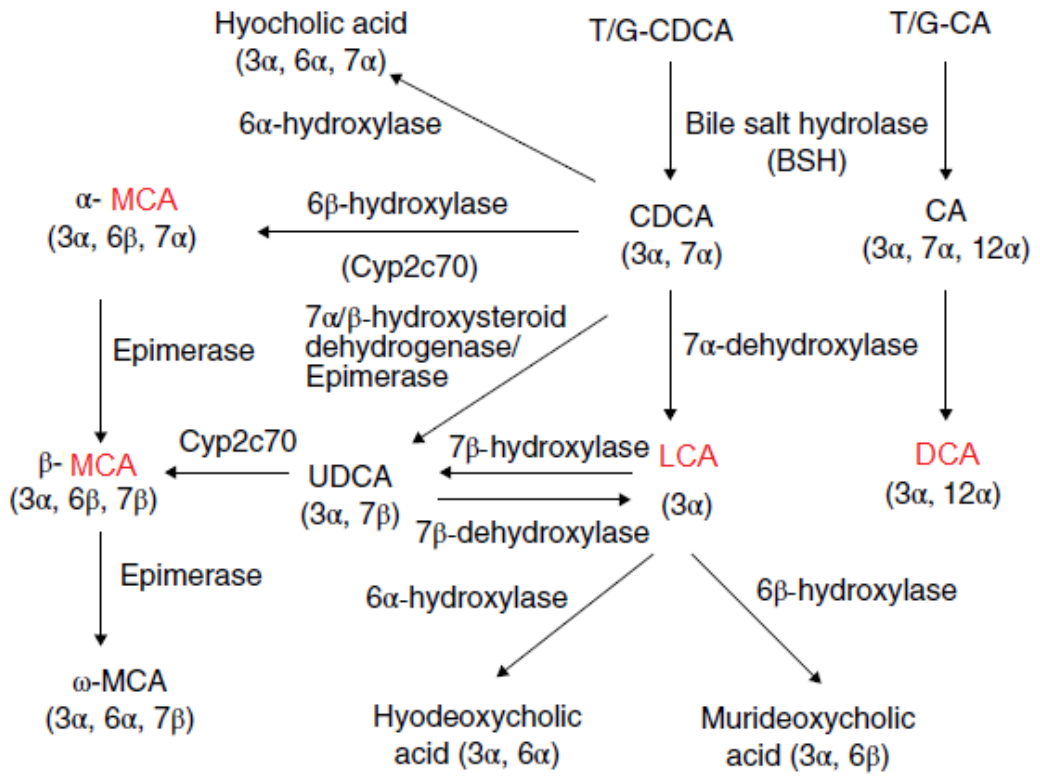

Figure 5: Species different bile acid biotransformation by gut bacteria

3.5.6 Species dependent bile acid conjugation

Another aspect of species-dependent differences is the preferential use of glycine or taurine for bile acid conjugation. Taurine conjugation is predominated in rodents such mice and dogs, while the bile acid pool in humans is more glycine conjugated. Taurine conjugated bile acids are less toxic than glycine conjugated and unconjugated bile acids [54].

The species dependent preference for amino acids glycine and taurine is determined by BAAT and the availability of the amino acids in the peroxisomes. The conjugation of bile acids with the amino acids in the liver is catalyzed by two enzymatic reactions. In the first reaction the bile acid-CoA synthetase (BAS), a microsomal enzyme, is converting bile acids (R-COOH) to an acyl-CoA thioester. In the second reaction, acyl-CoA thioester is linked to glycine or taurine to form the N-acyl conjugate by the bile acid-CoA amino acid N-transferase (BAAT) a cytosolic enzyme. It has been shown that purified hBAAT (human BAT) utilize both glycine and taurine conjugates [55]. Some species like cats and seals tend to be taurine-specific conjugators. It is assumed that it could be because the major dietary source is meat which contain a large amount of taurine. Herbivores and omnivores are associated with the ability to conjugate bile acids with both glycine and taurine. In mouse bile only taurine conjugates were identified. There is also an indication that BAAT is very selective in the choice of amino acid for conjugation of bile acids. In primates the ability of BAAT to utilize glycine and taurine for the conjugation is a recent evolutionary event. Protein sequencing has been performed and the human and mice enzyme was compared. The amino acid sequence of mBAAT is 69% identical and 84% similar to that of hBAAT [56].

3.5.7 Secretion and absorption of bile acids

Hepatocytes are polarized cells with a sodium pump (Na^+ K^+ ATPase) localized on the basolateral membrane similar to other classical epithelia. The apical domain of the hepatocytes represents about 10% - 15% of the cellular surface area. The remaining major part of the cell membrane is the basal

membrane facing blood sinusoids and containing many microvilli, and a membrane that line the intracellular space the lateral membrane. The bile lumen canaliculi is very small (1 µm) and is formed of two adjacent hepatocytes surrounded by tight junctions, which is the only physical blood barrier between blood and canalicular lumen. This membrane of tight junctions is also termed paracellular permeability between blood and bile. As part of the tight junctions claudins and occludins are connected to the cytoskeletal protein to hold the hepatocytes together as well as provide a barrier that prevents bile acids and other large solutes from diffusing from bile. Small ions can passage the membrane and Ca^{2+} is needed to maintain the seal. In the apical by microvilli amplified canalicular membrane area a number of protein transporter are localized. Many of these transporters are ATP dependent and members of the ABC super family (ATP binding cassette). The major canalicular bile acid efflux transporter in the hepatocytes are the ATP binding cassette (ABC) and bile salt export pump (BSEP, ABCB11) [57].

Hepatocytes are located in the functional unit, hepatic lobule, where bile is formed countercurrent to the blood flow. Bile acids generated by hepatocytes are secreted into the network of canalicular, collected and drained into small bile ducts. The bile ducts are formed of cholangiocytes which can modify the bile in the biliary tree by regulated secretion and reabsorption. The bile formation is more present in the periportal than in the pericentral zone of the lobule where the concentration of bile salt is the highest. The secretion of the bile into the canalicular is bile salt independent. The small canalicular lumen increases in size as they approach to the portal tract [58]. They connect then to the canals of Hering which are the initial branches of the biliary tree and drain the bile from the bile canaliculi. The biliary tree contains about 12 branches from the common duct to form the right and left hepatic ducts. Transport proteins of the canalicular secrete bicarbonate enriched fluid and other solutes of the primary biliary secretion to reabsorb the solutes in the fluid. There are some differences determined by species in the functions of cholangiocytes in modifying hepatocyte bile, but the final secretory product flows in the gallbladder and the intestine via common hepatic duct (*Ductus choledocus)* [57].

In the small intestine bile acids are important, together with pancreas lipase and colipase, for the resorption of dietary fat. In the intestine they are forming mixed

micells with fatty acids and monoacylglycerols which are absorbed in the enterocytes, then re-esterified to triglycerides, transported in chylomicrons via the lymphatic system and delivered into the circulation. The reabsorption of bile acids occurs in the terminal ileum where the sodium dependent bile salt transporter is highly expressed. Unconjugated bile acids are reabsorbed in the Intestine via passive diffusion and conjugated via active transport. The extraction rate of the first-pass effect is 60-90 % depending on the bile acid spillover [49].

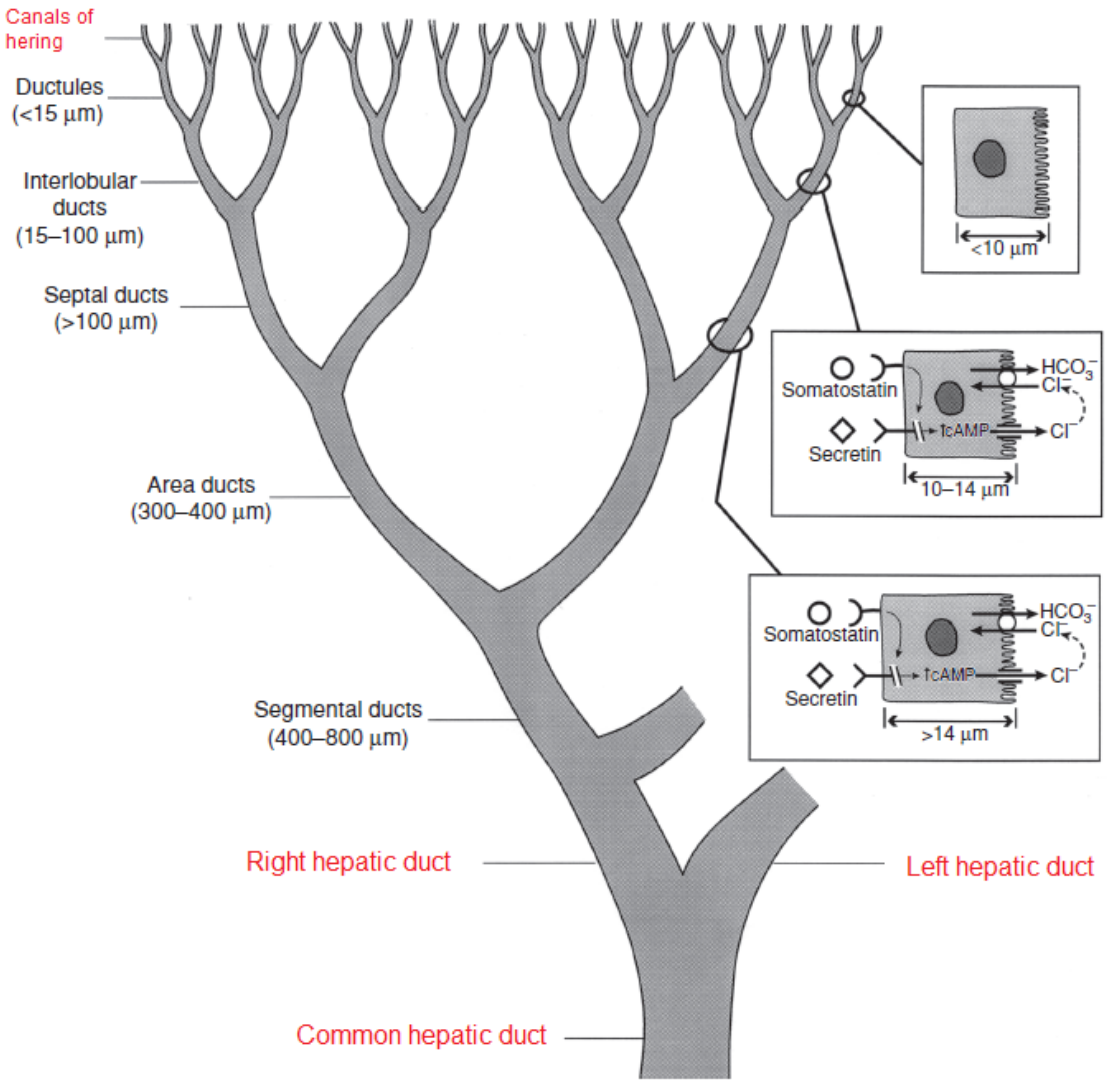

Figure 6: Bile acid secretion and the function of the biliary tree

3.6 Rapamycin

Rapamycin is a macrocyclic lactone discovered as an antifungal metabolite produced by the *Streptomyces hygroscopicus*. The compound that would kill fungi were discovered in the 1960s by Canadian scientists. Rapamycin was named after the place of origin, the Easter Islands (Rapa Nui). The focus was on the antifungal properties, but soon after there was found that rapamycin has also an impact on the growth of eucaryotic cells, immunosuppressive and anticancer properties [59]. At the late 1980s researcher identified the mechanism by which eukaryotic cells can be blocked by Rapamycin. At the beginning of the 1990s the protein target of rapamycin (TOR), which is responsible for rapamycin´s ability to inhibit growth in yeast was discovered [60]. The mechanism and the counterpart of mTOR was identified independently in few separate researches. It was shown that rapamycin binds to the intracellular receptor FKBP12 (designated as FK 506). FKBPs are identified in many eukaryotes from yeast to human and their function is folding proteins chaperons which contain proline residues. Rapamycin has been identified as a potent immunosuppressant that blocks the G_1 cell cycle in antigen activated T-cells in yeast. The binding protein sequence match for mice and human for 98%, and only a region of 133 amino acids is sufficient to bind on the FKBP12/rapamycin complex [61,62]. Breakthrough research from 2009 has reported that gavage of rapamycin in a later life can extend the lifespan of male and female mice. They did two separate studies, in one they begin to feed the mice when they were at 600 days of age and in the another at 270 days of age. In both of them there was a statistical significance compared to the control group [63]. That was the first evidence that a pharmacological agent increases lifespan in mammals. The scientific journal Science declare it as a major scientific breakthrough in 2009 [59].

3.7 MHY1485

4,6-dimorpholino-N-(4-nitrophenyl)-1,3,5-triazin-2-amine (MHY1485) is a synthetic cell permeable activator of mTOR that inhibits autophagy by suppressing the fusion between autophagosomes and lysosomes. In the cells, doubled-membraned vesicles (autophagosomes), are formed to isolate the disposable material in the

cytoplasm. Autophagosome fuse with lysosomes in which the isolate content undergoes distinct degradation by lysosomal hydrolases. Under certain conditions such as starvation and hypoxia when cells need to utilize intracellular nutrients, autophagy is upregulated [75]. In one study it was shown that MHY1485 combined with radiation significantly enhances apoptosis and senescence in tumor cells [76]. In another study with premature ovarian insufficiency (POI), it was shown that MHY1485 helps to increase the effectiveness developing follicles in the ovaria [77].

3.8 Fluvastatin

Coronary heart diseases are casually related with increased plasma cholesterol. In several intervention studies it was shown that lowering the LDL-cholesterol, involving dietary or drug control, could reduce the incidence of coronary heart diseases. To reduce the plasma cholesterol, it was shown that fluvastatin, a synthetic 3-hydroxy-3-methylgrlutaryl coenzyme A (HMG-CoA) reductase inhibitor, has this ability to reduce cholesterol in patient with hyperlipidemia [71]. The crucial role in the synthesis of cholesterol plays the enzymatic conversion of HMG-CoA to mevalonic acid and the cholesterol feedback which suppresses the synthesis [72]. The active forms of HMG-CoA reductase inhibitors block the cholesterol biosynthesis completely or reversibly by inhibiting the activity of HMG-CoA reductase. This inhibition results in decrease of cholesterol biosynthesis and increase in the expression of high affinity receptors for LDL on hepatocyte membrane. This leads to decreased serum concentration of total cholesterol and LDL-C [73]. In an animal model study was shown that fluvastatin is a strong inhibitor of HMG-CoA reductase and cholesterol biosynthesis. Fluvastatin was able to decrease the HMG-CoA reductase in rat liver up to 50% [74].

3.9 Model organism

3.9.1 Ceanorhabditis elegans

A foresight of the Nobel laureate Sydney Brenner lead to the application of *Caenorhabditis elegans* (Nematode) as genetic model for understanding questions of developmental biology and neurology. Over the time *C. elegans* has become an important organism in modern biology and in studies of the basic functions. *Caenorhabditis elegans (C. elegans)* is a small worldwide free-living nematode which grows up to 1 millimeter in length. The main food source of nematodes in the wild is the bacterial strain *Escherichia coli*. *C. elegans* has a short life cycle, lasting 3 days at about 25°C from egg to adult worm. The nematodes begin feeding to develop through four larval stages (L1-L4). Under unfavorable conditions and when food is scarce, a so called dauer larval stage may develop, in which the nematodes can survive for several months. In this process, L2 larvaes activate an alternative life cycle and develop from L2 larvae into an alternative L3 stage [63]. Due to its short lifespan and generation time, *C. elegans* is a frequently used model organism and is mainly used to study aging processes [64]. *C. elegans* is also widely used in science as an experimental model organism concerning important features of higher organisms, such as an epidermis, a nervous system, a gastrointestinal tract (GIT) or a reproductive system. Due to the presence of a gastrointestinal tract, *C. elegans* is a suitable model organism for intestinal barrier research [64].

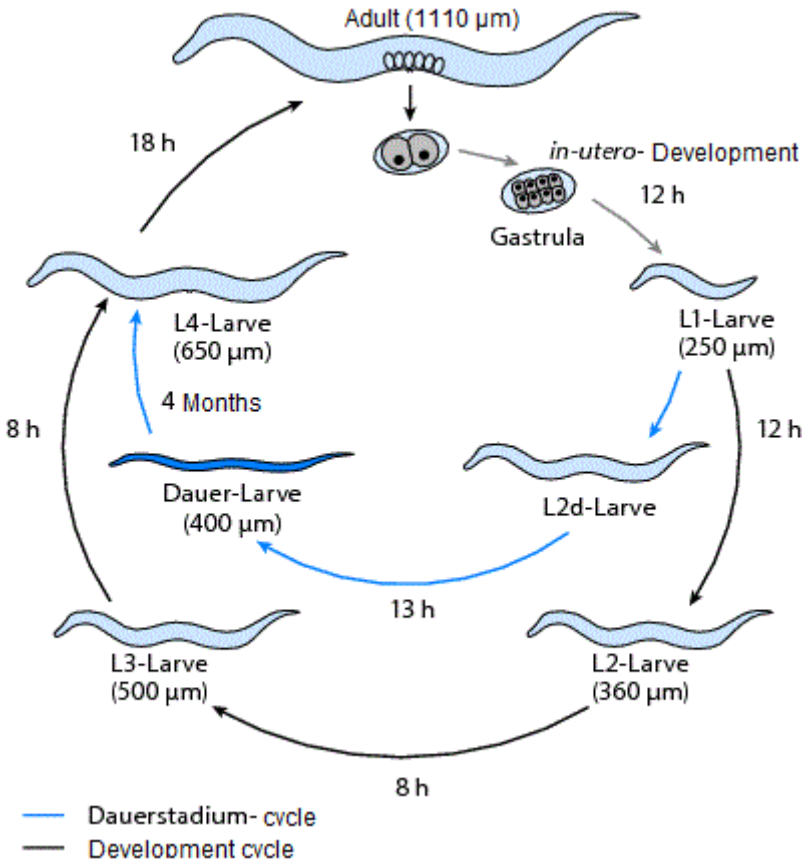

Figure 7: [67] Life cycle of the C. elegans

In the laboratory, the dissecting stereomicroscope is used to observe the movement, feeding, development, reproduction, and egg-laying of nematodes on agar plates or in liquid media inoculated with a layer of the bacterium *Escherichia coli*. Wild-type nematodes have two types of sexes: the self-fertilized hermaphrodites and males. Hermaphrodites are females whose gonads intermittently produce sperm before oocytes. The small size of about 1 mm of the animals has the advantage that they are easy to handle and cultivate. The nematodes can be kept between temperatures of 11 °C - 25 °C. *C. elegans* grows 2.1 times faster at 25 °C than at 16 °C and 1.3 times faster at 20 °C than at 16 °C. The typically use is at 20 °C [66].

3.9.2 Mice C57BL/6

The introduction of inbred strains in the biology was of high importance for the scientists to avoid confusions in the interpretation of their research. The rate of

inbreeding depends on the degree of relationship of the individuals. The closest relationship that is usually possible with mammals is full brother and sister mating also known as full-sib mating. Other regular mating system that leads to high level of inbreeding include half-sib and cousin matings. The Inbreeding is expressed relative to an arbitrary starting point and the inbreeding coefficient never reaches 100 percent. In 1952 The Committee on Standardize Nomenclature for Mice decided as a minimum level of 20 generations of full-sib mating is required before the strain can be designated as inbred. In practice it means all individuals within an inbred strain should be genetically identical [68].

Pioneer in this field was C.C. Little who established in the 1920s the C57BL strain. This strain is widely known and used as a general-purpose strain and a genetic background for spontaneous and induced mutant mice. In the mid-1930s C57BL/6L and C57BL/10 were established, and in the 1970 at least 9 strains were added. The phenotypic differences of C57BL/6 substrains related to behavior, glucose tolerance, alcohol and drugs has been reported. Also demonstrated in the study are the differences between the C57BL/6J and C57BL/6N substrains at eleven SNP loci [69].

In this research C57BL/6 mice were chosen and kept under a 12h light /12-h dark cycle in standard specific-pathogen-free (SPF) conditions with a free water access.

4 Aims of the master thesis

In former studies of Gregor et al. higher concentrations of conjugated bile acids were found under CR mice. Another interesting fact is that in several studies CR showed a life extending effect. In this study the aim was to figure out, if only conjugated bile acid supplementation has a positive impact on the lifespan. Therefore as model organism the popular *C.elegans* were taken, as their life expectancy is predictable.

Another topic of this master thesis was to determine if CR can be mimicked only by supplementing different substances without any reduction of the daily caloric intake. Therefore, mice were split in different supplementation groups, which were subdivided in a CR and an Ad lib group and then compared to a control group. MHY1485 was used as it is a mTOR activator that inhibits autophagy. As another supplement rapamycin was used as it is known to inhibit aging, and therefore it was of interest if CR can be imitated. The BSH inhibitor CAPE was also supplemented in order to determine if a higher concentration of conjugated bile acids can be found. On the other hand, taurine conjugated bile acids were directly supplemented. Fluvastatin was also of interest as it reduces the serum cholesterol level, which is important for the bile acid synthesis in the liver. The aim was to see if fluvastatin has an impact on the bile acid concentrations. For this experiment liver and small intestine samples were measured and bile acid concentrations were determined in order to see if there are statistically significant differences between the supplementation and control groups. Furthermore, taurine and taurine conjugates were measured as it was shown in former studies that concentrations of taurine and taurine conjugates increased during CR. Therefore, it is of interest to determine the impact of the different supplementations on taurine and taurine conjugates. Additionally, the activity of glutathion-S-tranferase from small intestine was measured, to determine if there are significant differences between the supplementation and control groups.

In addition to the determination of bile acid concentrations measured with LC-MS/MS, muscle thickness, villi and crypt length and the amount of goblet cells were determined in tissue samples of small intestine and proximal colon. The aim of

these measurements was to determine whether there are changes during CR or increased bile acid concentrations.

5 Materials and Methods

5.1 Equipment

- **Arium®pro ultrapure water systems;** Sartorius Lab Instruments GmbH & Co KG (Goettingen, Germany)
- **Centrifuge 5418 R;** 100 rpm – 14 000 rpm; 0 °C to + 40 °C; Eppendorf AG (Hamburg, Germany)
- **Centrifuge 5810R;** 200 rpm – 14 000 rpm; -9 °C to + 40 °C; 0,2 mL to 85 mL; Eppendorf AG (Hamburg, Germany)
- **LCMS-8040 Liquid Chromatograph Mass Spectrometer;**
  Shimadzu Corporation (Kyōto, Japan)
    - High Pressure Switching Valve FCV – 20AH2
    - UV/VIS Detector SPD-20A
    - Column Oven CTO-20AC
    - Autosampler SIL- 20AC HT
    - Liquid Chromatograph LC-20 AD
    - Degassing Unit DGU-20A5
    - Kinetex® 5 µm EVO C18 100 Å LC Column 150 x 3.0 mm
    - Lab Solution (Software)
- **LCMS-MicrOTOF-Q Liquid Chromatograph Mass Spectrometer;**
  Bruker Daltonik GmbH (Bremen, Germany)
    - Apollo II ion funnel ESI Electropsray source (1 µl – 1 ml/min)
    - Compass Data Analysis (Software)
    - Mass range 20 – 40 000 m/z
- **Lauda Alpha A6 thermostatic bath**; temperature range between 25 °C – 100 °C; LAUDA DR. R: Wobser GmbH & Co. KG (Lauda-Königshofen, Germany)
- **Paraffin Embedding center MICROM AP250**; MICROM Laborgeräte GmbH (Ulm, Germany);
    - 3 Units (Thermal, dispensing, cooling console)

- **Microtome RM2245 – Semi Motorized Rotary Microtome;** 0.5 µm – 100 µm; Leica Biosystems Nussloch GmbH (Mannheim, Germany)
- **Microscope CH2 - B Olympus;** Objectives (4x, 10x, 40x and 100x); OLYMPUS OPTICAL CO:, LTD. (Tokyo, Japan)
- **Mini – Rocker Shaker MR-1; 5 to 30 rpm;** LTF Labortechnik GmbH & Co. KG (Wasserburg, Germany)
- **HYDRO TISSUE FLOAT BATH Hydro H 2 P**; for stretching sectioned tissue specimens; 25 °C to 80 °C; LAUDA DR. R. WOBSER GmbH & CO. KG (Lauda-Königshofen, Germany)
- **Sartorius Entris® 224i – 1S analytical balance**; Sartorius Lab Instruments GmbH & Co KG (Goettingen, Germany)
- **Sample Concentrator EVA-EC1-S for 24 Samples;** VLM GmbH (Bielefeld, Germany)
- **Vortex mixer/shaker,** Heidorph REAX 2000; Heidolph Instruments GmbH & CO. KG (Kelheim, Germany)
- **Rocker – Shaker Fisherbrand Sea Star;** Fisher Scientific GmbH (Schwerte Germany)

5.1.1 Accessories

- **Centrifuge tubes**; 15 mL, 50 mL; Corning Inc. (Corning, New York, USA)
- **HPLC vial; 2 mL**; Sigma – Aldrich Chemie GmbH (Steinheim, Germany)
- **Microcentrifuge tubes;** 1.5 mL; Eppendorf Austria GmbH (Vienna, Austria)
- **Tips TipOne®;** 10µl, 200µl, 1000µl; STARLAB International GmbH (Hamburg Germany)
- **Shell style inserts Rotilabo® 100µl,** borosilic. gl.; Carl Roth GmbH + CO, KG (Karlsruhe, Germany)
- **Plates 60 – 90 mm;** Sarstedt AG and Co. KG; (Nümbrecht, Germany)
- **Microscope slides;** Thermo scientific; Gerhard Menzel GmbH (Braunschweig, Germany)
- **Low Profile Microtome Blades;** Leica DB80 LS; Leica Biosystems Nussloch GmbH (Nussloch, Germany)

5.2 Reagents and Chemicals

5.2.1 C. elegans

Agar	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
Sodium chlorid (NaCl)	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
Peptone	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
Dipotassium hydrogen phosphate (K_2HPO_4)	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
Potassium hydrogen phosphate (KH_2PO_4)	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
Magnesium sulfate ($MgSO_4$)	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
Calciumchlorid ($CaCl_2$)	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
FUDR (5-flourodeoxyuridine)	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
Ampicillin	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
Tetracyclin	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
Nystatin	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
Streptomycin	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
EMSURE® Ethanol for analysis	Merck KGaA, Darmstadt Germany
Cholesterol	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
LB Media	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland

Yeast extract	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
Glycerin	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
ddH_2O	Produced in the lab with Arium®Pro
Sodium hydrogen (NaOH)	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
TLCA (Taurolithocholic acid)	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
TUDCA (Tauroursodeoxacholic acid)	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
TCA (Taurocholic acid)	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
TDCA (Taurodeoxycholic acid)	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany

5.2.2 Mice C57BL/6J

Taurine	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
Acetonitril	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
GSH (Glutathion)	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
EDTA	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
DMEM (Dulbecco´s Modified Eagle Medium)	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
PBS (potassium buffered saline)	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
EMSURE® Ethanol for analysis	Merck KGaA, Darmstadt Germany
Xylene	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany

Methanol	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
Paraffin	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
1%-Alcian blue solution	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
1%-Periodic acid	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
Schiff´s Reagenz	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
Mayer´s Hematoxylin solution	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
Eukit® Quick-hardening mounting medium	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
Formic acid	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
TLCA (Taurolithocholic acid)	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
TUDCA (Tauroursodeoxycholi acid)	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
TCA (Taurocholic acid)	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
TDCA (Taurodeoxycholic acid)	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
CA (Cholic acid)	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
DCA (Deoxycholic acid)	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
CDCA (Chenodeoxycholic acid)	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
UDCA (Ursodeoxycholic acid)	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany

LCA (Lithocholic acid)	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany
DHCA	Sigma Aldrich CHEMIE GmbH, Steinheim, Germany

6 Animals and experimental protocol

6.1 Caenorhabditis elegans maintaining protocol

6.1.1 Preparation of Luria Bertani media for *E. coli* OP50

For 1 l of LB solid growth media, 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl and 15 g of agar were weighed into a 1 l glass bottle. After adding 1 l deionized water the bottle was shaken well and put it into the autoclave. It was taken care, that the cap of the bottle was not screwed to tight so that it did not burst. It was autoclaved for 30 to 45 minutes and then the bottle let cool for 1 h in a 55 °C water bath. When transferring the bottle from the autoclave to the water bath thermal gloves were used as the bottle and content were hot. Before pouring petri plates 2 ml of 50 mg/ml ampicillin and 2 ml of 5 mg/ml tetracyclin were added.

Working under the fume hood and using sterile technique approximately 20 ml of the LB media were added to pour a 0,5 cm thin layer into each Petri plate and then let cool to solidify. The dishes can be stored at 4 °C for up to 1 month.

6.1.2 Preparation of food source Escherichia coli OP50 (E. coli OP50)

E. coli OP50 were used as food source, as they are antibiotic resistant bacteria. As *E. coli* OP50 are uracil auxotroph, their growth is limited on NGM plates, and therefore they were prepared on LB media plates.

The *E. coli* OP50, which were stored in liquid LB media in the freezer, were firstly thawed. Then they were pipetted and spread with a cell spreader all around the LB plate in order to make a streak plate. The streak plate was incubated overnight (~16 h) at 37 °C.

On the next day a sample was taken from the lawn with a pipette tip for the inoculation of the liquid LB media. Then the bottle was incubated overnight (~16 h) on a rocket shaker with ~ 30 oscil/min at 37 °C. The rocket shaker was used, so

that the bacteria become fresh air. The clear liquid media turned to cloudy, as the cultures have saturated the liquid.

Both the streaking plates and the liquid media bottles can be stored for several months in a cold room at 4 °C.

The next step was the preparation of the *E. coli* lawn on the NGM plates.

6.1.3 Inoculation of the NGM plates

For the inoculation the *E. coli* OP50 liquid media was used. To inoculate the NGM plate, first the 10x concentrated solution of *E. coli* OP50 was prepared. Therefore 10 ml of the solution was centrifugated at 3500 g for 10 minutes. Then 90 % of the supernatant was removed and the rest was resuspended with a vortex-shaker in order to get a 10x concentration of the bacteria.

100 µl of the 10x concentrated *E. coli* OP50 solution was pipetted on the middle of the NGM plate. This step had to be repeated for all NGM plates. In order to spread the OP50 all over the surface of the plate it was either swirled gently or a cell spreader was used. At this step it was avoided to spread the *E. coli* OP50 lawn to the edges of the plate, because the worms tend to spend most of the time in the bacteria. If the lawn would go to the edges the worms would crawl on the walls of the plate and dry out.

The plates were let at room temperature for ~ 8 h outside or under the fume hood to let the culture grow and the lawn to dry out. They are usable for 2-3 weeks when storing them at 4 °C.

6.1.4 Preparation of nematode growth media (NGM)

For 1 L of solid NGM growth media, 3 g of NaCl, 2.5 g peptone and 20 g of agar were weighed into a 1 l glass bottle. After adding 1 l of deionized water everything was shaken vigorously by hand and then put into the autoclave for 1 h. The growth

media was then cooled for 1 h in a 55 °C water bath. Then 1 ml of 1 M $MgSO_4$, 1 ml of 5 mg/ml cholesterol, 1 ml of 1 M $CaCl_2$ and 25 ml of 1 M KH_2PO_4 were added.

Depending on the purpose additionally nystatin, streptomycin, ampicillin or FUDR had to be added.

6.1.4.1 NGM for *C.elegans* maintenance

To 1 l of NGM 5 ml of 10 mg/ml nystatin and 2 ml of 15 mg/ml streptomycin were added. Using sterile technique, working under the fume hood 10 ml of NGM was poured into each 90 mm petri plate to get a constant amount of NGM agar.

It was important to use the same amount of agar, as the microscope did not need to be refocused every time when switching from one plate to another. While transferring the NGM into the petri plates it was avoided that no bubbles were formed as the worms could crawl into them. After pouring the petri plates they were left under the fume hood with lids on for 8 hours to let the NGM dry and solidify. Then they were usable for 2-3 weeks by keeping them in a cold room at 4 °C.

6.1.5 Transferring C. elegans

6.1.5.1 Types for transferring

To visualize the *C. elegans* a dissecting microscope with a light source and more objectives with magnification are needed. The common use is between 0.6x and 5x magnification, for this case 4x is enough. There are 3 different ways how to transfer worms.

The first one is to use a sterilized filter paper which was cut into a 6 cm long and 1 to 2 cm wide stripes. The filter paper should be set on the surface of the agar where it absorbs the moisture and pick up the worms. The worms are then transferred on a fresh plate. The worms will come of the paper. After use the filter paper has to be discarded.

The second one is to “chunk” the agar, and then to transfer the agar-chunk to a fresh plate. It is a quick and convenient method. For this method a sterile scalpel is needed to cut a large piece of the agar, to catch a lot of worms. After cutting the scalped is used to transfer the piece into the fresh plate. The worms will crawl out on the new plate. On the chunk there are hundreds of worms, and therefore this method is used for transferring homozygous stocks.

The third method and the hardest one to practice is “picking” the worms with a worm picker. For this method a worm picker is needed. It can be made by mounting a 0.2 mm platinum wire into a Pasture pipet or to plug in into a bacteriological loop holder. Platinum has the best properties: it heats and cools quickly, and this is important to avoid contamination of the stocks. The platinum wire needs to be cut to an optimum length for the picking. For the bacteriological loop holder ~3 cm are enough. The end of the wire needs to be flattened with a hammer and polished with an emery cloth or sandpaper. This is important for easier picking worms and not to damage the agar or poke holes, because the worms will crawl into the agar. To pick up worms, the tip of the pick is lowered to the agar surface and then swiped gently to the middle of the body of the worm to lift it up. To pick up more worms with one pick this step is repeated several times by gently swiping. The worms are then transferred to the fresh plate. While transferring to the fresh plate the tip was lowered and waited until the worms crawl off from the pick. While picking it should be taken care that the worms should not stand too long on the pick as they can desiccate and die.

In these experiments the “chunking” and “picking” methods were used.

6.1.5.2 Transferring frequency

The transferring frequency of the worms is depending on the genotype, the temperature at which they were kept and the plan what to do whit them. For the heterozygotes and the mating stocks it is good when they would be transferred every 1-3 generations, best before they start starving. To transfer the individual worms the best way is to chunk the agar and transfer it to a fresh plate. The worms will crawl out of the chunk, and then the individual worms can be picked up.

When the worm reaches the *dauer* stage leave them for one or two days on the fresh plate, they will recover, and then pick them.

The environmental temperature is also an important factor in maintaining of *C. elegans*. The usual temperature on which *C. elegans* are maintained is between 16 °C and 25 °C, the most common one is 20 °C. Variation in temperature also affects the variation in growth. *C. elegans* grows 2.1 times faster at 25 °C than at 16 °C, and 1.3 times faster at 20 °C than at 16 °C. A stock at 25 °C will starve sooner than the stock at 16 °C. To have worms on different stages of development alternatively they can be transferred every 1-2 days onto a new plate.

6.1.6 Cleaning contaminated stocks of C. elegans

Stock of *C. elegans* may become contaminated with different MO (Microorganism) like other bacteria, yeast or mold. Most of the contamination is not affecting the worms, but it is easier to collect the worms when the stocks are clean. Mold can be removed by chunking, bacterial and yeast contaminants can be removed by treating it with a hypochlorite solution. It will kill all the contaminants and most of the worms, but the eggs will survive. The worms in the eggs are protected by the eggshell.

6.1.7 Freezing and recovery of C. elegans

L1 and L2 sage larvae are most suitable for freezing. Well grown worms, adults and eggs would not survive the freezing.

6.1.7.1 Preparation of the freezing solution

For the freezing buffer first a S-Buffer was prepared. Therefore 64.5 ml of 0.05 M K_2HPO_4, 429.5 ml of 0.05 M KH_2PO_4 and 1,925 g of NaCl were mixed. Then 3 ml of Glycerol were added to 17 ml of the S-Buffer. Before preparing the freezing

solution, all solutions were autoclaved before (Glycerol, K_2HPO_4 – and KH_2PO_4-solution).

The worms were chunked at least on 5 medium NGM plates. After approximately 1 week the plates were starved, and lots of L1/L2 larvae were on the plates. It was also taken care that no OP50 was left on the plates. With 1.5 to 2 ml freezing solution the plates were washed, and the liquid was collected in cryotubes. The cryotubes were then but into a cryobox and put in a freezer for 24 h at -20 °C. On the next day the cryotubes were transferred into a -80 °C freezer.

To check if the freezing process was successful, one of the tubes was thawed. The process was successful if the worms were wiggling.

The recovery of stocks for this process is about 30 – 35 %. This also means that the worms can´t be stored infinitely long, as the number of surviving worms decrease with each passing year.

6.2 Life span protocol

For the life span protocol the same substances were taken as for the maintenance. For 1 L of solid NGM growth media, 3 g of NaCl, 2.5 g peptone and 20 g of agar were weighed into a 1 l glass bottle. After adding 1 l of deionized water everything was shaken vigorously by hand and then put into the autoclave for 1 h. The growth media was then cooled for 1 h in a 55 °C water bath. Then 1 ml of 1 M $MgSO_4$, 1 ml of 5 mg/ml cholesterol, 1 ml of 1 M $CaCl_2$ and 25 ml of 1 M KH_2PO_4 were added.

6.2.1 NGM for life span measurement with bile acids

For life span measurement 330 µl of 150 mM FUDR and 1 ml of 100 mg/ml ampicillin were added and additionally 2 mL taurine conjugated bile acids (concentration 0.002 mg/ml) TLCA, TUDCA, TDCA and TCA and 500 µL of conjugated bile acids (concentration 0.0005 mg/ml). Using sterile technique,

working under the fume hood 5 ml of NGM was transferred into each 60 mm petri plate to get a constant amount of NGM agar on each plate.

6.2.2 Synchronization of the population

For the experiments it was important to synchronize the worm population. Therefore, a maintaining stock plate was taken, which was kept in the incubator and which was containing plenty of worms and eggs, and washed with hypochlorite solution. All the contaminants and worms died, only the worms in the eggs were protected, because of the egg shell. After 6 hour all worms hatched, and after 2 days they grew to L4 stage larvae. At this time the worms were ready to be picked for the experiment.

6.2.3 Conditions for the lifespan protocol

After pouring plates the plates were left on the bench under the fume for 24 h to dry and solidify. 50 µl of 2x concentrated *E. coli* OP50 was added and left for several hours to dry and build a lawn. The plates can be stored for 2-3 weeks.

The worms grew very fast because the incubator was set at 25 °C for the whole experiment. At the beginning it was important to transfer the worms every day or every second day to differentiate the worms from the hatching larvae. When several larvae are present, it is an indicator that the worms are transferred as young adults instead of L4 larvae and can lay eggs before the FUDR took effect.

6.3 Protocol of mice C57BL/6

For the experiment C57BL/6NRj male mice were used, housing in Tecniplast IVC system cages (cage typ 2 L, blue line) under SPF standard conditions. Humidity and temperature were controlled electronically and supervised constantly. The light-dark cycle was set to 12 hours. The mice had free access to food and water and were set in groups with maximum four per cage. Per experiment the mice were

set in two groups: the CR and the Ad lib group. These groups where then set into subgroups by the method of supplementation: control, BSH inhibitor, BA supplementation, fluvastatin, MHY 1485 and rapamycin. The concentrations of the supplements were:

- CAPE BSH inhibitor (6,5 mg per mice)
- BA supplementation with TCA, TDCA, TLCA, CA, DCA, CDCA, UDCA, LCA, DHCA and TUDCA (0.002 mg/ml)
- Fluvastatin (0,02 mg/g mice weight)
- MHY 1485 in drinking water (14µl/50ml H_2O)
- Rapamycin (2,14 mg/100 ml H_2O).

The ad libitum group was fed with commercial chow ad libitum. The caloric restricted mice were separated and put into single cages with one of the chosen methods of feeding. After the separation the mice were fed for two weeks. The CR mice became 75 % (2.8 – 2.9 g chow) of the daily consumption between 4 and 6 pm daily.

6.3.1 Collecting the tissue

On the 15th day of CR, mice were anaesthetized with isofluran and euthanized. The cardiac blood was taken and 10 µl ethylenediaminetetraacetic acid (EDTA) was added to ensure blood in fluid form. Before centrifugation for 10 minutes at 3.600 rpm 4 °C, 20 µl of aprotinin was added. Then the plasma sample was taken and stored at – 80 °C.

Following further components of the dissected mice were collected: small intestine (separated in 3 parts), liver, feces, lysate, and gallbladder. The histology samples were taken from all groups only not from BSH Inhibitor ad lib and BSH Inhibitor CR group. During the dissection, some samples were lost.

To prepare the lysate samples, a lysis buffer was needed. Also a GT solution was prepared by dissolving 885 mg GSH and 360 mg taurine in 15 ml distilled water. After the lysate tissue sample was transferred in a 1,5 ml tube, the sample weight was noted. Then lysis buffer, that was 5 times the sample weight was added. Then

the tissue was homogenized by quickly aspirating and expelling with a 1 ml syringe. The lysate sample was then used for further reactions (A and B). For reaction A 195 µl of lysis buffer, 10 µl GT and 5 µl of the lysate sample, and for the reaction B 150 µl lysis buffer, 10 µl GT and 50 µl of the lysate sample were pipetted into reaction tubes. The tubes were put in a heat block with 37 °C. After 10 minutes the first samples and after 20 minutes the other samples were collected and put into a sample box and stored at -20 °C.

For the preparation of the small intestine samples, they were divided into 3 even parts duodenum, jejunum and ileum (Si1, Si3 and Si 5) after dissection. From each part a small piece was directly snap frozen and stored at -80 °C for further experiments. The Si parts were flushed with PBS, the ends tied with a thread and filled up with 200 µl of taurine (220 mg/ml) and GSH (61,5 mg/ml). The sacs (small tightly closed intestine) were collected and put into prewarmed (37 °C, water bath) falcon tubes filled with 10 mL Dulbecco´s Modified Eagle Medium (DMEM). For the measurement of the taurine transport every 30 minutes 200 µl of sample extract was taken. In total 5 sample extracts per sac were taken: at 0 min, 30 min, 60 min, 90 min and 120 min. They were stored at -20 °C in a plastic box prior to further preparation.

Histology samples of proximal colon and small intestine were taken of the freshly dissected mice. The samples were cut, collected into tissue cassettes and immediately stored in 4 % PBS buffered formalin to counteract the degradation. Further processing steps followed later.

The remaining components were collected, snap frozen and stored at -80 °C for other experiment.

6.3.2 Further Sample preparation and measurement

6.3.2.1 Intestinal sacs sample preparation

For the analysis of the sacs samples, they were thawed at 4 °C. Then 50 µl of the sacs extract was transferred into a microcentrifuge tube. During the whole process

the microcentrifuge tube was cooled on ice. 150 µl methanol was added and the solution homogenized on a vortex-shaker, and then put on a shaker for 10 min at 155 rpm. The tubes were afterwards centrifuged for 10 min at 12000 g and 4°C. The supernatant was transferred into a new microcentrifuge tube and then dried on a SpeedVac concentrator for 20 min at 60 °C. The residue was then resuspended in 60 µl methanol, transferred into an HPLC vial and put into the autosampler for measurement.

10 µl of sample was injected into the LC-MS-ESI-TOF-system and measurement was carried out in negative mode. For measurement a HPLC UltiMate 3000 (Thermo Fisher Scientific, Waltham, MA, USA) was coupled to a MicrOTOF-Q II (Bruker Daltonik GmbH,Bremen, Germany). For separation a T3 3 µm column (2.1 x 150 mm, Waters, Milford. MA, USA) was used. The temperature of the column was set to 40 °C. Mobile phases consisted of 0,1% formic acid in water (eluent A) and 0,1 % formic acid in acetonitrile (eluent B). The gradient was maintained at an initial 5% for 2.5 minutes to 30 % at 8 minutes, and then was set back at minute 9 with a hold for one minute.

6.3.2.2 Lysate sample preparation

20 µl of the thawed lysate samples, that were prepared in (chapter 3.5.1), reaction A and B, were added to 60 µl MeOH. Then the samples were homogenized on the vortex-shaker and then shaken on the shaker for 10 min at 155 rpm. Afterwards samples were centrifuged for 10 min at 4 °C and 12000 g. The supernatant was transferred into a microcentrifuge tube and then dried on the SpeedVac concentrator at 60 °C for 10 min. The residue was dissolved in 60 µl of methanol and then transferred into HPLC vials. The HPLC vials were put on the autosampler at 4 °C for measurement.

10 µl of sample was injected into the LC-MS-ESI-TOF-system and measurement was carried out in negative mode. For measurement a HPLC UltiMate 3000 (Thermo Fisher Scientific, Waltham, MA, USA) was coupled to a MicrOTOF-Q II (Bruker Daltonik GmbH,Bremen, Germany). For separation a T3 3 µm column (2.1 x 150 mm, Waters, Milford. MA, USA) was used. The temperature of the column

was set to 40 °C. Mobile phases consisted of 0,1% formic acid in water (eluent A) and 0,1 % formic acid in acetonitrile (eluent B). The gradient was maintained at an initial 5% for 2.5 minutes to 30 % at 8 minutes, and then was set back at minute 9 with a hold for one minute.

For the lysate assay taurine and GSH, that were added during preparation, and their product taurine-GSH conjugate, that was catalyzed by glutathione-S-transferase, were measured.

6.3.2.3 Bile acid sample preparation – liver tissue

For measuring the bile acids, the liver tissue was cut into a sample size of 7 to 10 mg, put into cryotubes, and about 10 ceramic beads (1.4 mm) were added. A proper amount of methanol was added (9 times as much as the tissue sample). The tubes were then put in the precellys machine for homogenization at the slowest program. After that the samples were mixed on the vortex-shaker and then vigorously shaken on the shaker for 10 min at 4 °C. Then the samples were centrifuged for 10 minutes at 4 °C and 3000 g. Afterwards the supernatant was transferred into new tubes and again centrifuged for 10 minutes at 4 °C and 1500 g. The supernatant was transferred into HPLC vials and put into the autosampler (4 °C) for measurement.

Samples (10µl injection volume) were measured on a LCMS-8040 in positive mode. Column temperature was set to 30 °C. For mobile phase A water and for mobile phase B, acetonitrile and methanol (3/1, v/v) were used. Both mobile phases were containing formic acid and ammonium acetate in a concentration of 20 mM.

6.3.2.4 Sample preparation bile acid measurement in duodenum, jejunum and ileum

For measurement, the small intestine tissue samples were thawed at a 37 °C hot water bath and then immediately put on dry ice and 70% ethanol. A proper amount

of methanol was added (9 times as much as the tissue sample) and about 10 ceramic beads (1.4 mm) were added. The tubes were then put in the precellys machine for homogenization at the slowest program. After that the samples were mixed on the vortex-shaker and then vigorously shaken on the shaker for 10 min at 4 °C. Then the samples were centrifuged for 10 minutes at 4 °C and 15000 g. Afterwards the supernatant was transferred into HPLC vials and put into the autosampler (4 °C) for measurement.

Samples (10µl injection volume) were measured on a LCMS-8040 in positive mode. Column temperature was set to 30 °C. For mobile phase A water and for mobile phase B, acetonitrile and methanol (3/1, v/v) were used. Both mobile phases were containing formic acid and ammonium acetate in a concentration of 20 mM.

6.3.3 Preparation (sectioning) and conservation of the tissue samples

6.3.3.1 Day 1

As mentioned, the tissue samples were collected into tissue cassettes and stored immediately in a 4% PBS buffered formalin for at least 48 hours. Afterwards the samples were washed under running water for 15 min. The water was emptied from the sample container and 75 % of ethanol was poured in it and left for 1 h. During this process the container was swiveled every 10 min to avoid bubbles. After 1 h the solvent in the container was changed to 85 % ethanol and the container was again every 10 min. After another hour the solvent in the container was changed to 95 % ethanol and 5 % methanol. The container was left overnight. Furthermore, the oven was set at 60 °C and enough paraffin was left to melt overnight for the sectioning.

6.3.3.2 Day 2

The solvent in the container was changed with 100 % ethanol and left for at least 1 h. The container was swiveled again every 10 min. After 1 h the solvent is changed again with fresh 100 % ethanol and left for 1 h. In total, the container should be washed 3 times with 100 % ethanol. Next ethanol is exchanged to xylene and left for 45 min. Then again the solvent in the container is exchanged with fresh xylene and left for 45 min in the oven at 60 °C to reach the same temperature as the paraffin. Then xylene is exchanged with paraffin and left for 30 min in the oven. This step is repeated two further times with fresh paraffin.

6.3.3.3 Embedding

The embedding machine was turned on in time to reach the needed working temperature. The machine has 3 units: thermal unit for the samples, dispensing unit (for melting paraffin and embedding the sections) and the cooling unit.

The section samples were put into the warm chamber of the thermal unit to melt the rest of paraffin for better embedding. Then the section was taken from the cassette and a small amount of melted paraffin was poured into a mould and then the tissue put into it to fix the tissue sample. After the paraffin hardened the whole tissue was covered with liquid paraffin. The cassette was put on the sample and afterwards the samples were put on the cooling plate to harden. After preparing all samples they were cooled in a freezer at -20 °C before cutting.

6.3.3.4 Sectioning

The sectioning was done on Microtome RM2245 a Semi Motorized Rotary Microtome, 0.5 µm – 100 µm, Leica Biosystems Nussloch GmbH (Mannheim, Germany). At the beginning the knife was either replaced or tested before sectioning, to ensure good cutting quality. Then the machine was set in trim mode (16 µm) and the excess of paraffin was removed. After that cutting mode (4 µm) was set and the sample cut. For every section 3 samples were cut. The cuts were

put into daminozide water. After sectioning samples were dried for 1-2 days at room temperature on slides.

6.3.3.5 Alcian blue Staining

To prepare the alcianblue solution, 1 g were dissolved in 100 ml of a 3 % acetic acid solution, and then filtrated. The solution is stable for 2-6 months.

The sections were dewaxed and rehydrated as follows: The slides were put into xylene for 4 min, then put into 95 % of ethanol also for 4 min. Afterwards the glass slides were put for 2 min into 70 % ethanol, and in the end into deionized water for 4 min. The paraffin was wiped away from the slides with paper wipes by not touching the samples.

Then about 100 µl of acianblue solution were pipetted onto each slide and left in the humid chamber for 30 min. Then samples were washed under running water for 2 min and then for 10 s in deionized water. 100 µl of 1 % periodic acid were pipetted onto the slides and left in the humid chamber for 10 min. The slides were again washed under running water for 2 min and left it in deionized water for 10 s. Afterwards 100 µl of Schiff´s solution were pipetted onto each slide, then left in the humid chamber for 15 min and washed under running water for 2 min and further 10 s in deionized water.

Next the samples were put into hematoxylin stain solution for 90 s for small intestine and 120 s for proximal colon samples and then washed for 5 sec in tap water 1, 15 s in tap water 2 and stopped for 2-3 s in deionized water. After that samples were left first for 2 min in 70 % ethanol, then 4 min in 95 % ethanol and last for 4 min in xylol. In the end on each tissue sample a drop of entellan was added and the slides were covered with a cover glass. Samples were then dried for 1 day under the fume hood.

6.3.3.6 Analysis of pictures of histology sections

Tissue pictures were taken on a Karl Zeiss microscope and software with a 5x, 10x and 20x magnification. For measuring the muscle thickness, the crypt depth and villi length ImageJ software was used. The 5x magnification was used to measure the muscle thickness, the 10x magnification to measure the villi length and crypt depth, and the 20x to count the goblet cells.

6.4 Statistical data analysis

For the statistical analysis, the data was set in two groups CR and ad libitum which were compared using a two-sided student´s t-test to verify the statistical significance. As statistically significant, a p-value lower than 0.05 was considered. Bonferroni correction was done for groups to compare them to each other.

7 Results

7.1 Bile acid impact on C. elegans life span

Results (figure 8) show that there was no statistically significant difference between the control and the bile acid supplemented group. Neither a bile acid concentration of 0.0005 mg/ml per plate nor a bile acid concentration of 0.002 mg/ml showed an effect.

The median life span of *C. elegans* was 15 days, when more than 50 % of the worms were alive. The last worms died on the 31st day of the experiment.

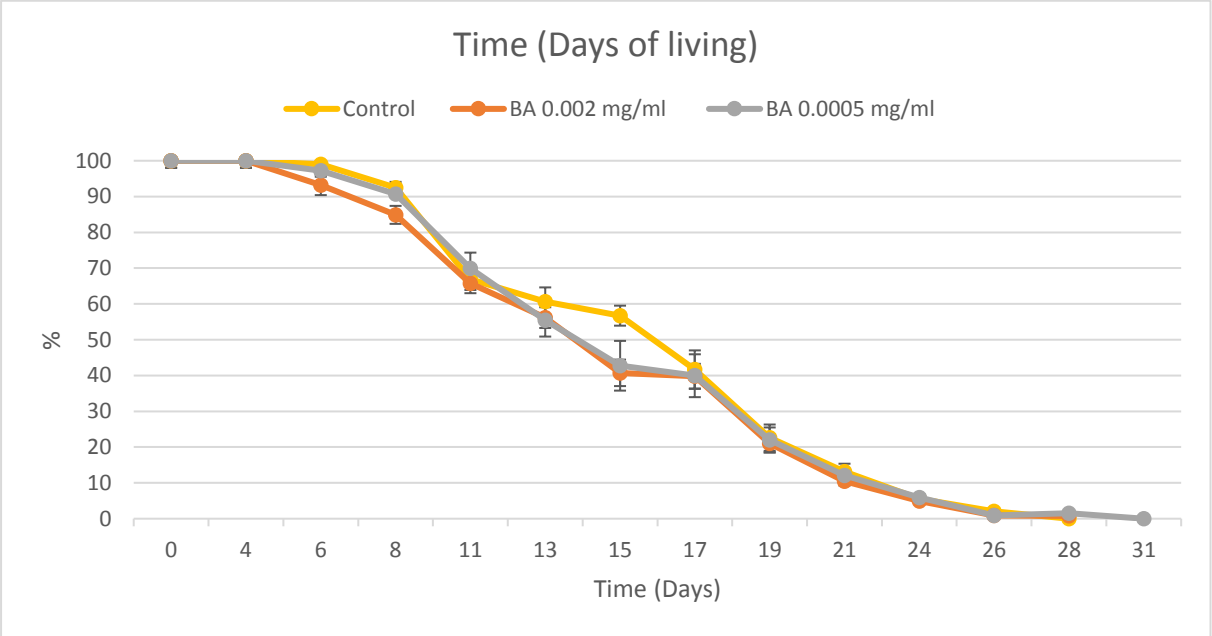

Figure 8: C. elegans lifespan experiment. Median lifespan for experimental and control group was 15 days.

7.2 Bile acid, Taurine and GSH Impact on the Gastrointestinal Tract in Mice

7.2.1 Body weight

The body weight of all groups was measured at the day of dissection. Mice were first euthanized and then set on scale.

As in figure 9 shown, there was a statistically difference found between both control groups (Ad lib and CR). Furthermore significant differences in body weight were found between the MHY1485 (Ad lib) and MHY1485 (CR) group. There was also a statistically significant difference between Rapamycin and the CR control group.

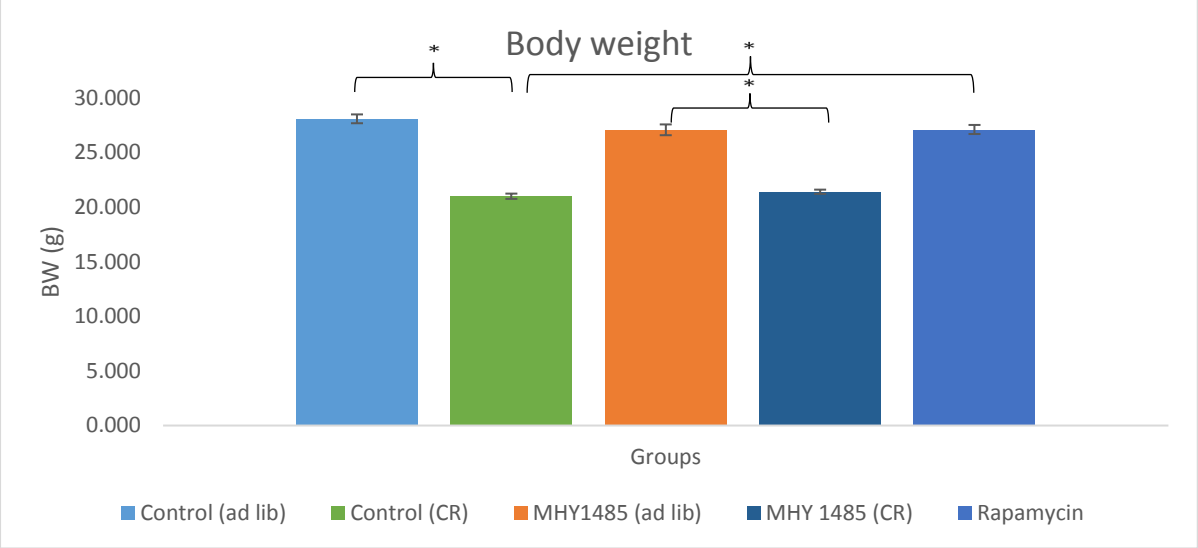

*Figure 9: Caloric restriction impacts on body weight. Mice in the groups: MHY 1485, Rapamycin and Control. * Statistically significant difference p < 0,05.*

As in figure 10 shown a statistically significant difference in body weight was measured between all Ad lib and CR groups. Within the Ad lib group there was a statistically significant difference between the Fluvastatin and control group. Furthermore a significant difference was measured between the BA sup. (CR) and the control group.

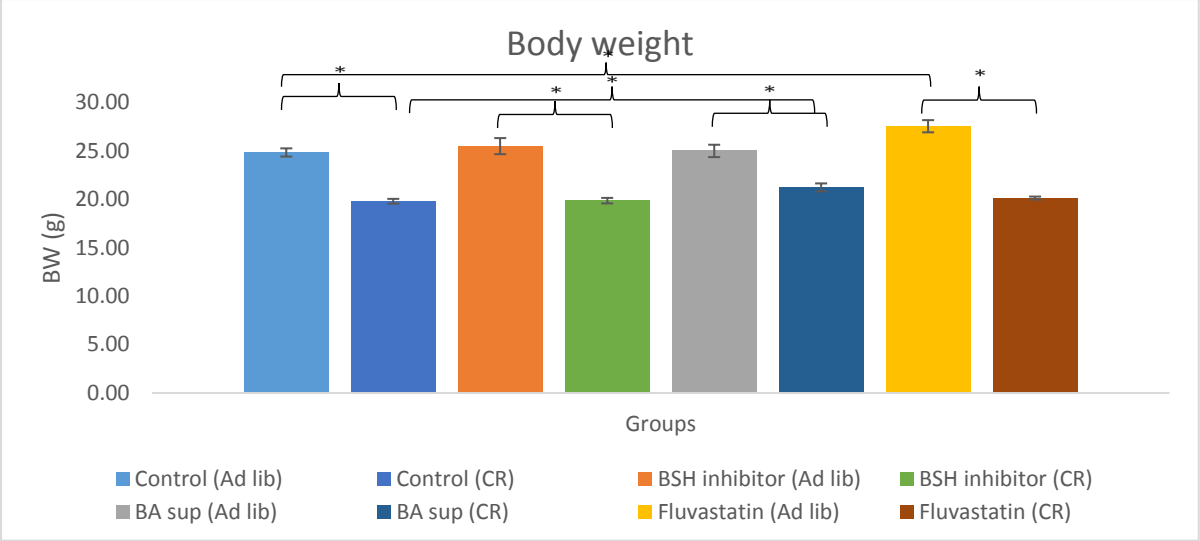

*Figure 10: Caloric restriction (CR) impacts on body weight. Mice in the groups: BSH inhibitor, BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05.*

7.2.2 Bile Acids in Liver

7.2.2.1 MHY1485 and Rapamycin

As shown (figure 11) there is a statistically significant difference for the primary bile acid CA in the liver between the MHY1485 and the Ad lib control group. There is also a statistically significant difference for the other primary bile acid CDCA between the MHY1485 (Ad lib) and MHY1485 (CR) experimental group (see figure 12).

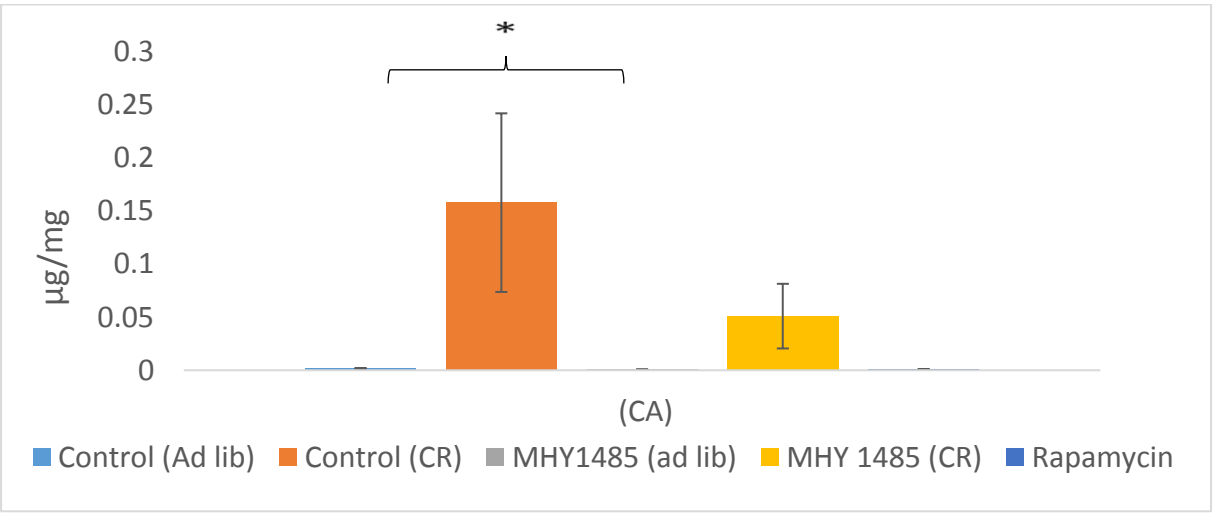

*Figure 11: Liver CA. Mice in the groups: MHY1485, Rapamycin and Control. * Statistically significant difference p < 0,05*

For UDCA (figure 12), which is a minor primary bile acid in rodents, a statistically significant difference was found between the CR control group and the rapamycin group. Also the experimental group MHY 1485 showed significant differences between Ad lib and CR.

For the secondary bile acid DCA there is a statistically significant difference between CR control and MHY 1485 (CR), as well as for CR control and the rapamycin group.

For DHCA no statistically significant differences were measured.

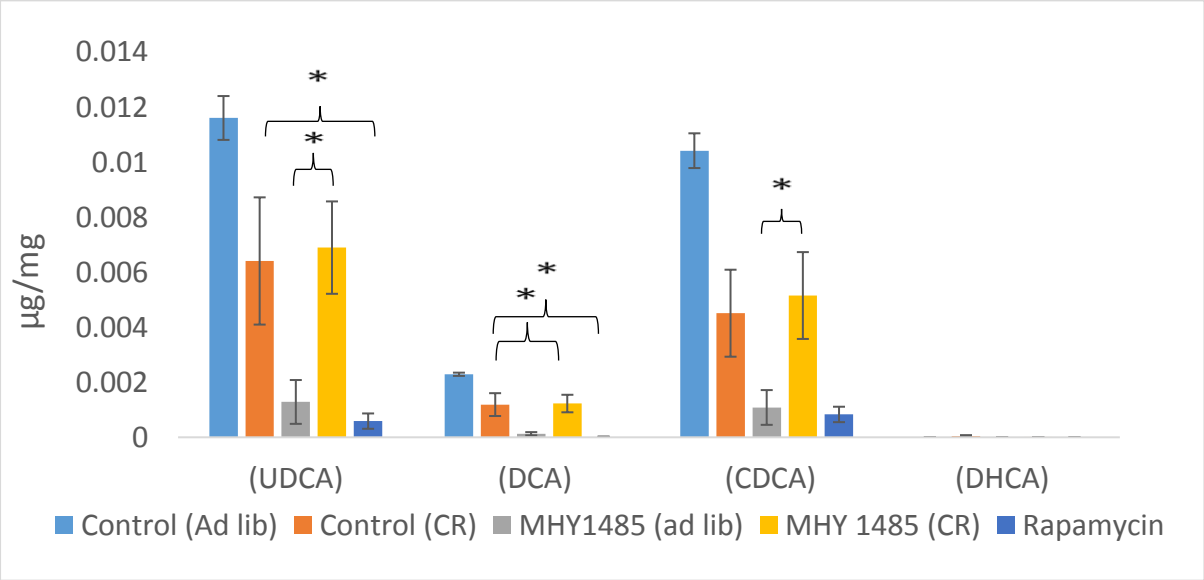

*Figure 12: Liver UDCA, DCA, CDCA and DHCA. Mice in the groups: MHY1485, Rapamycin and Control. * Statistically significant difference p < 0,05*

The results for measurement of the conjugated bile acid TCA (figure 13) that there is a statistically significant difference between the Ad lib and CR control group. Within the CR group, there is a significant difference between the control group and MHY 1485 group and also between the control group and rapamycin group.

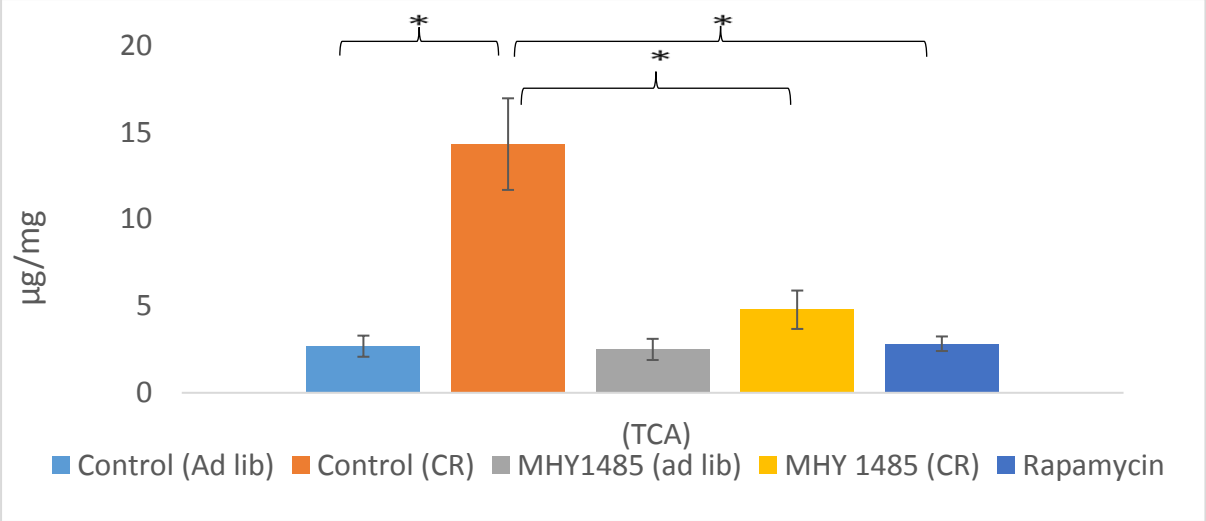

*Figure 13: Liver TCA. Mice in the groups: MHY1485, Rapamycin and Control. * Statistically significant difference p < 0,05*

For TDCA (figure 14) there a statistically significant difference was measured between the Ad lib and CR control groups. Furthermore, statistically significant differences are shown between CR control group and rapamycin group. In the experimental group MHY1485 there is a significance between Ad lib and CR.

No significant differences for TUDCA were found between the groups.

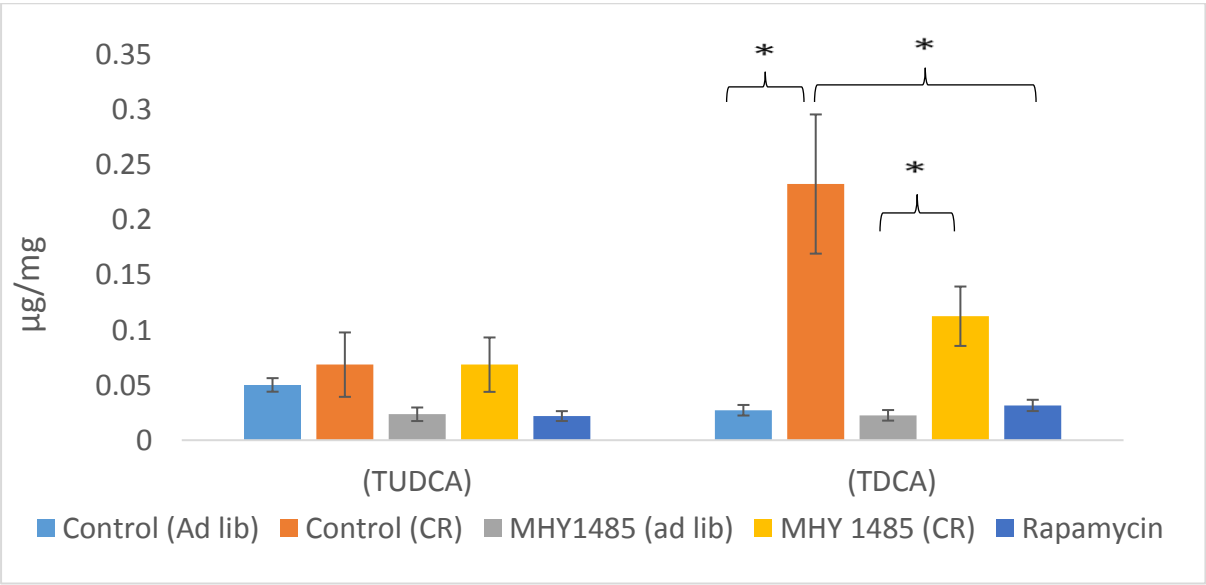

*Figure 14: Liver TUDCA and TDCA. Mice in the groups: MHY1485, Rapamycin and Control. * Statistically significant difference p < 0,05*

For TLCA (figure 15) there is a statistically significant difference between the Ad lib and CR control group and within the MHY 1485 group there is a statistically

significant difference between Ad lib and CR group. Furthermore there is a significant difference between rapamycin and the control group for TLCA.

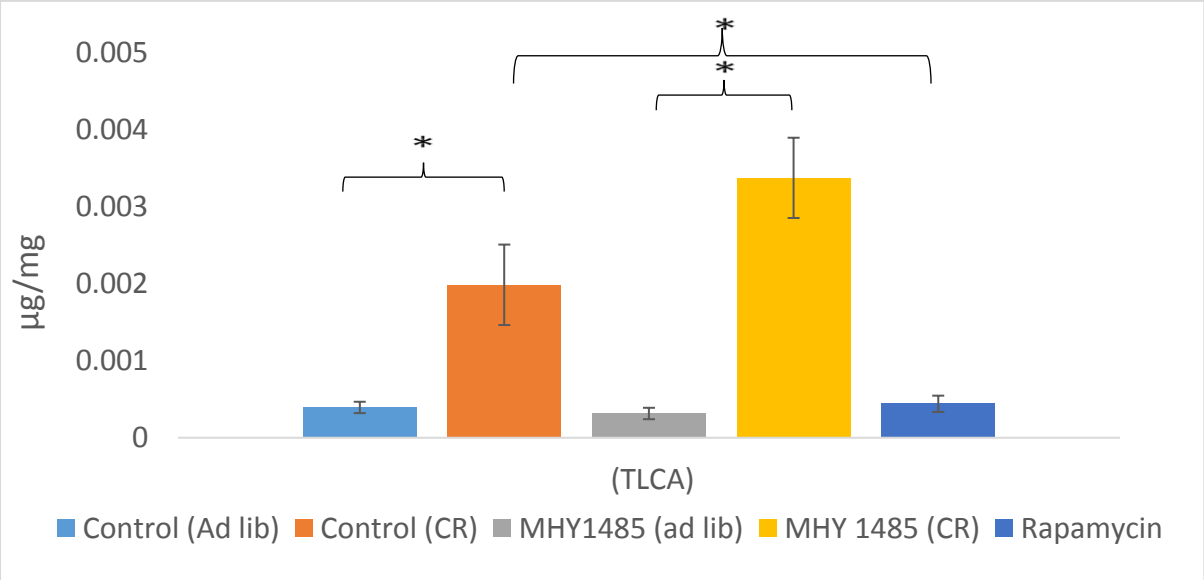

*Figure 15: Liver TLCA. Mice in the groups: MHY1485, Rapamycin and Control. * Statistically significant difference p < 0,05*

7.2.2.2 Fluvastatin and BA Supplementation

In the experimental group with Fluvastatin and bile acid supplementation (figure 16) there is a statistically significant difference for the primary bile acid CA between the experimental groups BA sup. (Ad lib) and BA sup. (CR) and also between Fluvastatin (Ad lib) and Fluvastatin (CR).

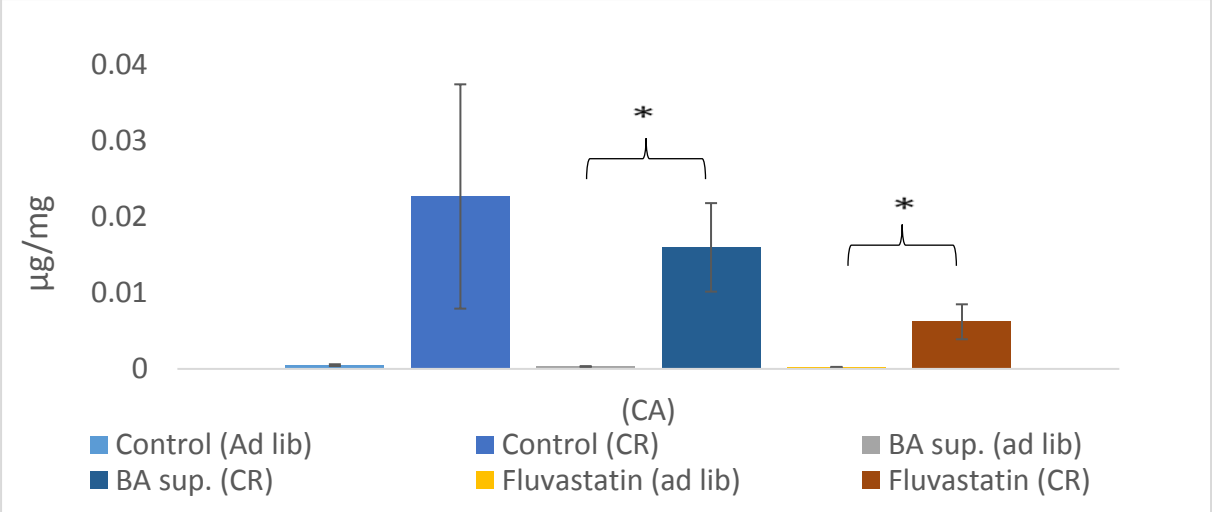

*Figure 16: Liver CA. Mice in the groups: BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05*

For the bile acid UDCA (figure 17), a statistically significant difference was measured between Fluvastatin (CR) and the control group. No significant differences were shown for the bile acids DCA, CDCA and DHCA.

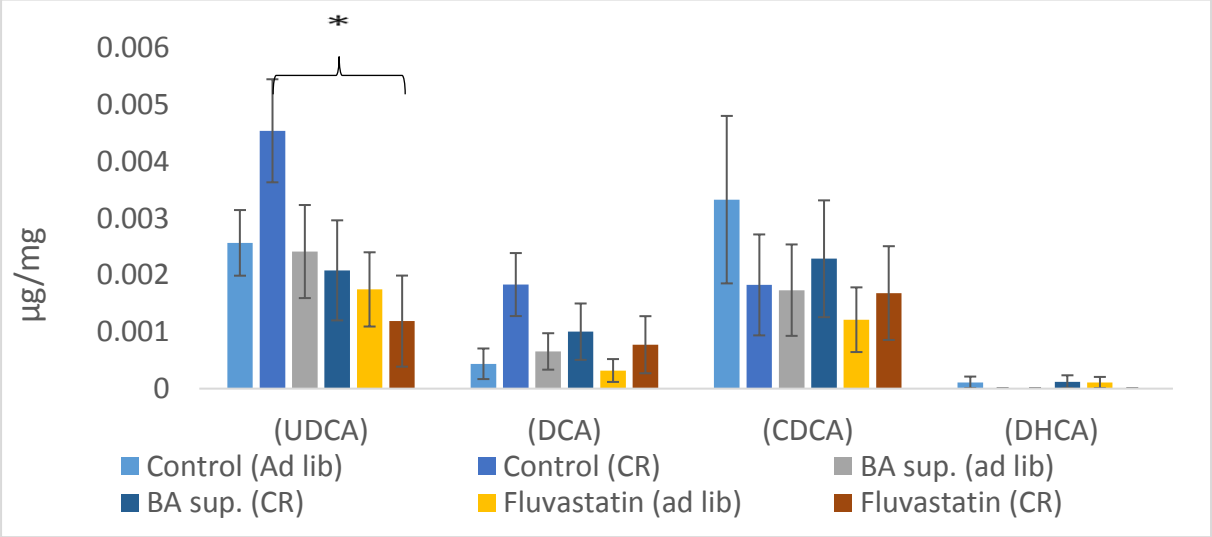

*Figure 17: Liver UDCA, DCA, CDCA and DHCA. Mice in the groups: BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05*

For the conjugated bile acid TCA (figure 18), a statistically significant difference was shown between the BA supplemented (Ad lib) and BA supplemented (CR) group.

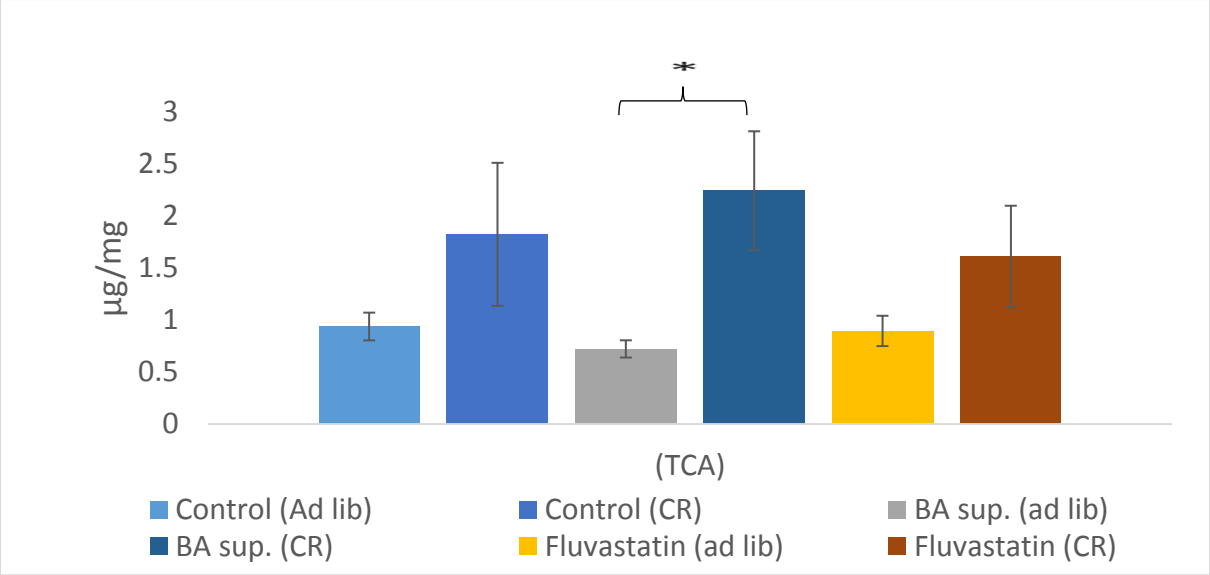

*Figure 18: Liver TCA. Mice in the groups: BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05*

For TUDCA a highly statistically difference was shown (see figure 19) between the experimental groups BA sup and the control group respectively for Ad lib and CR.

For TDCA a statistically significant difference was measured within all three groups between Ad lib and CR, respectively: control, BA sup. and Fluvastatin

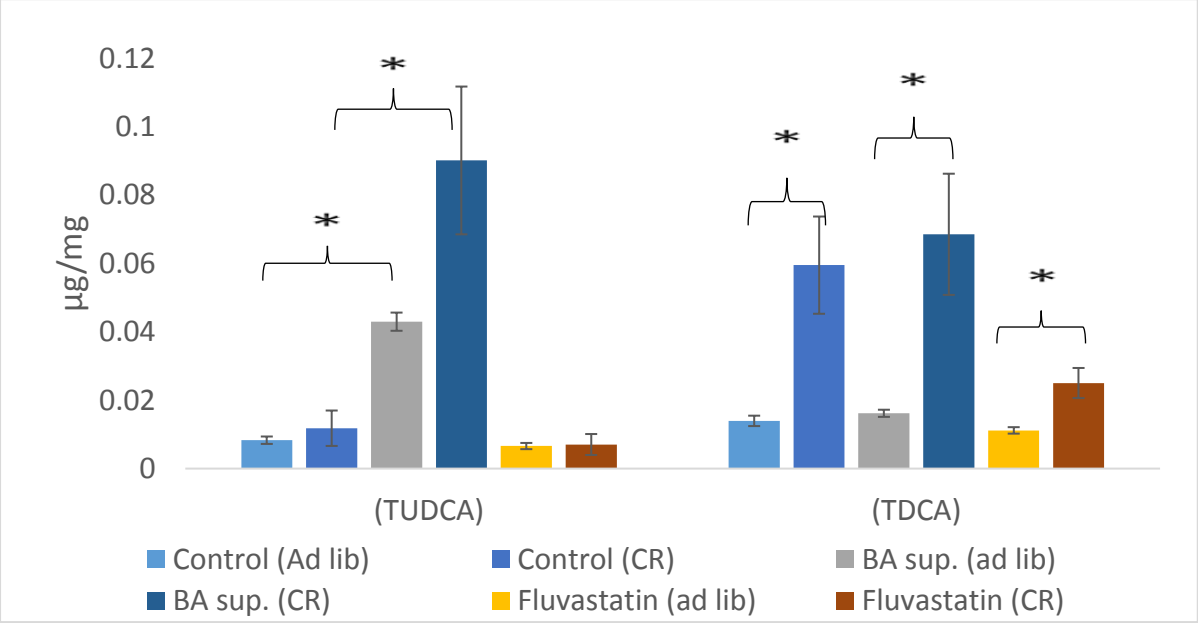

*Figure 19: Liver TUDCA and TDCA. Mice in the groups: BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05*

Measurements also showed that TLCA has statistically significant differences between the control groups Ad lib and CR (see figure 20). Also, significant differences were shown within either Ad lib and CR group between control and BA sup. Furthermore a significant difference between CR control group and the Fluvastatin (CR) group was also shown.

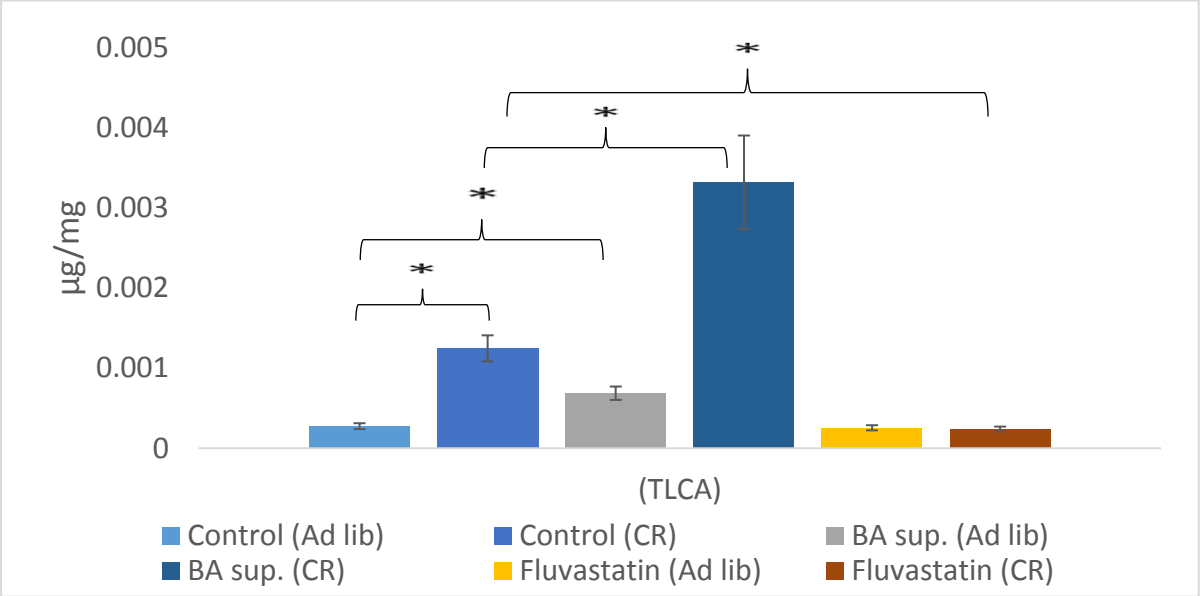

*Figure 20: Liver TLCA. Mice in the groups: BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05*

7.2.2.3 BSH inhibitor

Another group was compared to the control group: the bile salt hydrolases inhibitor (BSH i.). For CA, there is a statistically significant difference between the BSH i. (CR) and the control group (see figure 21).

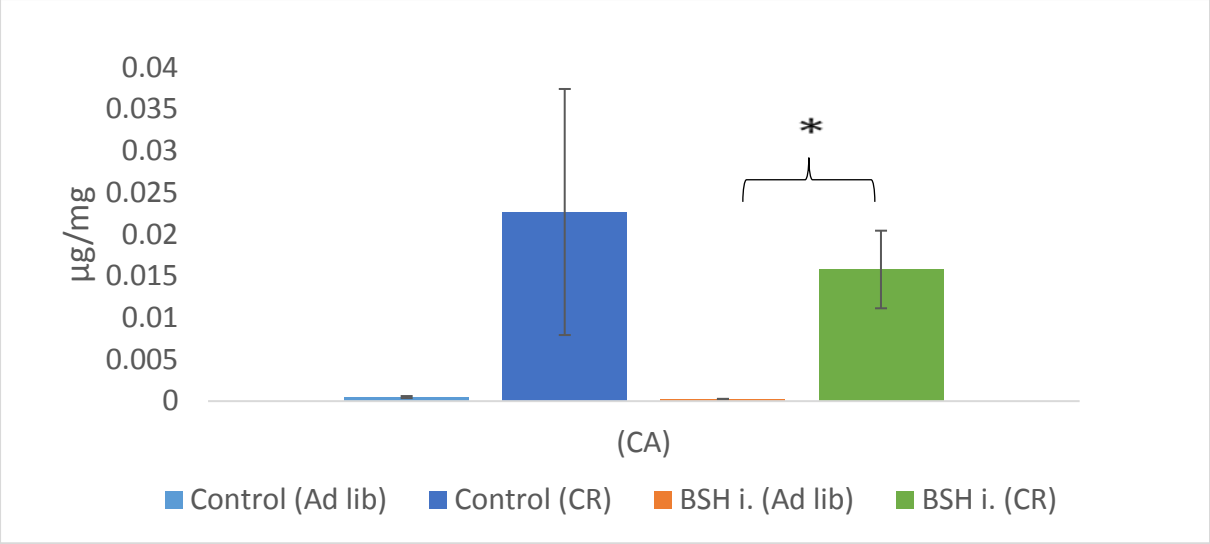

*Figure 21: Liver CA. Mice in the groups: BSH inhibitor and Control. * Statistically significant difference p < 0,05*

For CDCA there is no difference between the groups (see figure 22). For the secondary bile acid UDCA there is a statistically significant difference between BSH i. (Ad lib) and the control group and for DCA a significant difference between the BSH i. and the control group was found (see figure 22).

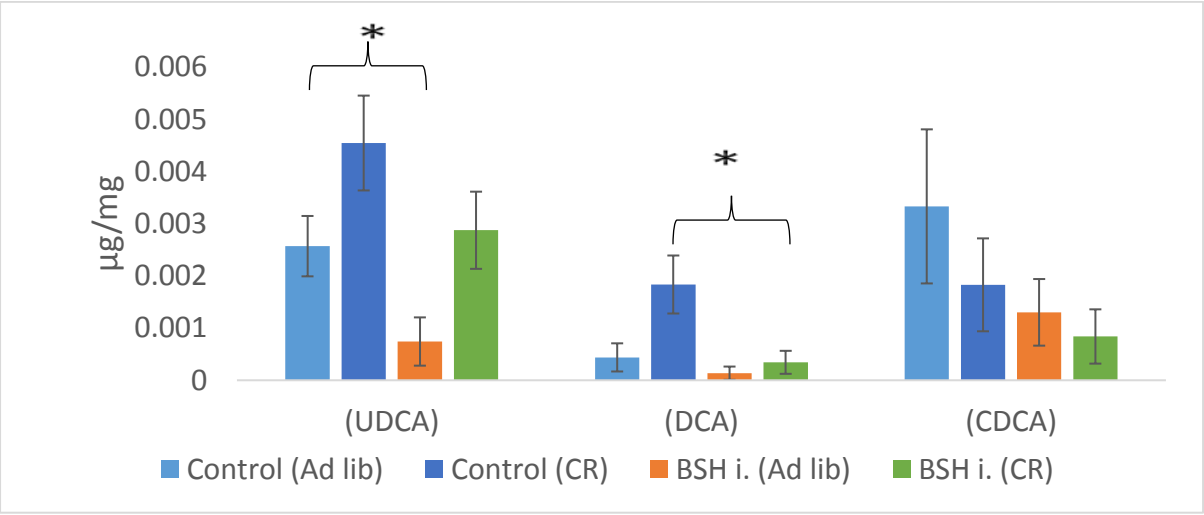

*Figure 22: Liver UDCA, DCA and CDCA. Mice in the groups: BSH inhibitor and Control. * Statistically significant difference p < 0,05*

For the conjugated bile acid TLCA a statistically significant difference between Ad lib and CR control groups and between BSH i. (CR) and the control group was measured (see figure 24). For TUDCA measurements showed significant differences between the BSH i. (ad lib) and control group (see figure 25).

Furthermore for TDCA a significant difference between the ad lib and CR group was found.

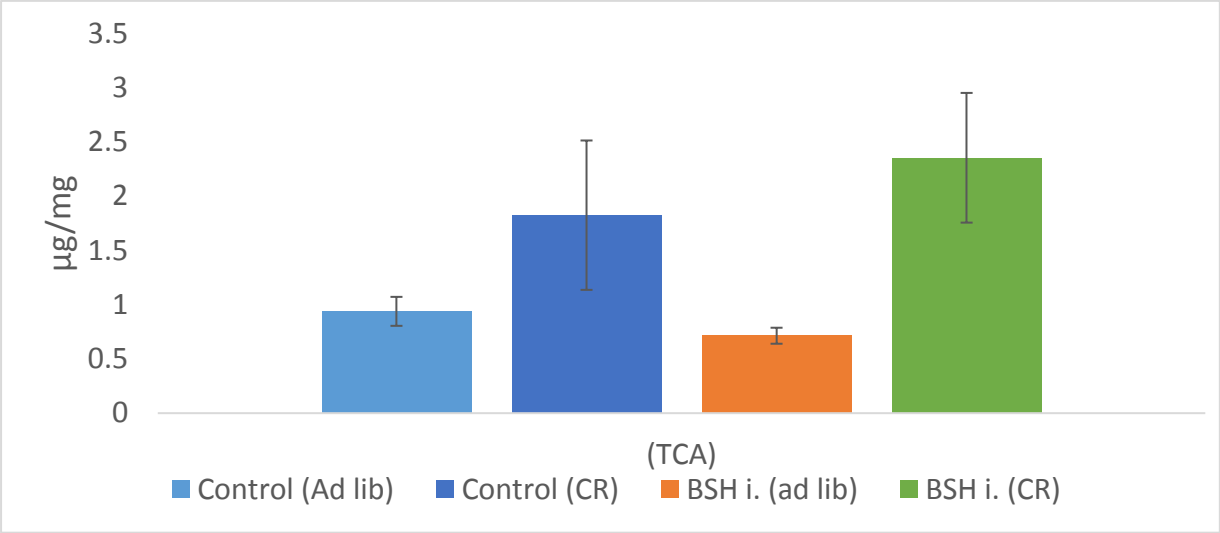

*Figure 23: Liver TCA. Mice in the groups: BSH inhibitor and Control. * Statistically significant difference p < 0,05*

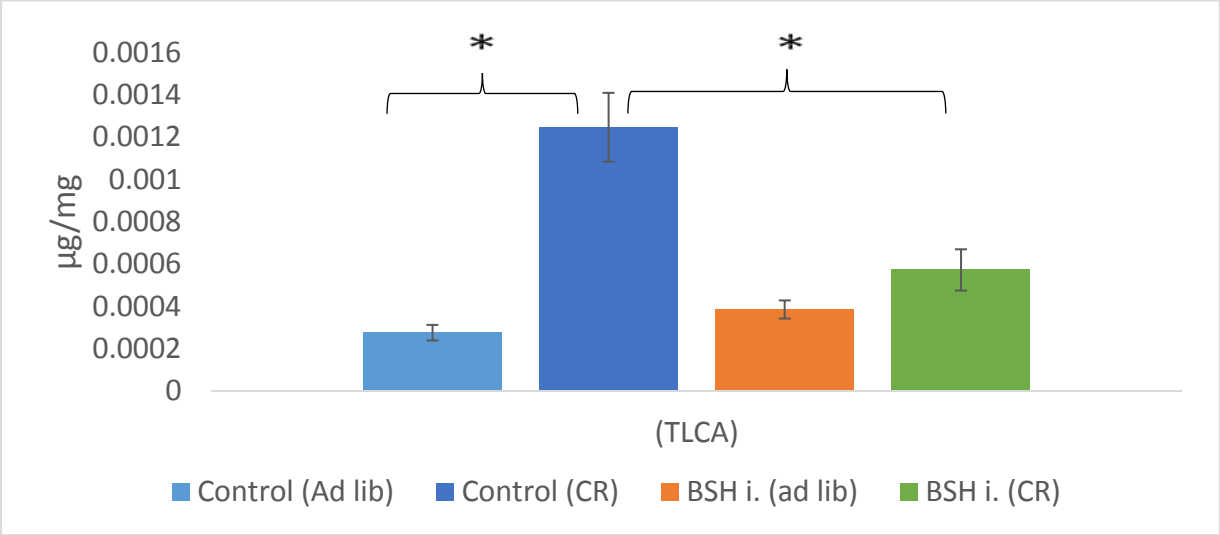

*Figure 24: Liver TLCA. Mice in the groups: BSH inhibitor and Control. * Statistically significant difference p < 0,05*

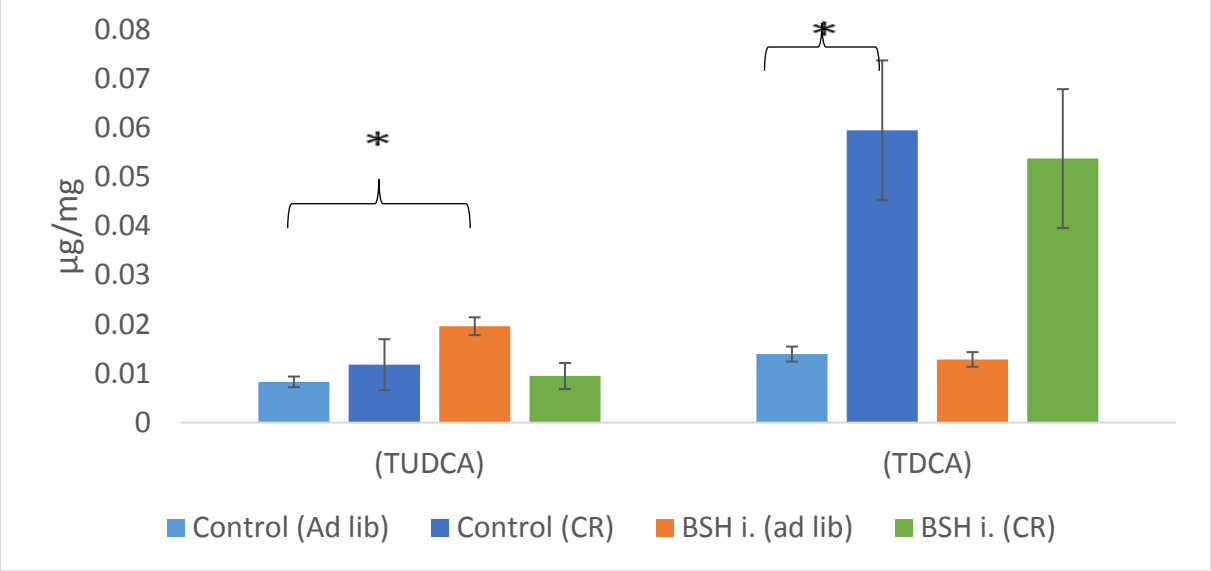

*Figure 25: Liver TUDCA and TDCA. Mice in the groups: BSH inhibitor and Control. * Statistically significant difference p < 0,05*

In table 1 all statistically significant differences measured in liver are listed.

		Control		MHY1485		Rapamycin	BA supplementation		Fluvastatin		BSH inhibitor	
		Ad lib	CR	Ad lib	CR		Ad lib	CR	Ad lib	CR	Ad lib	CR
Primary bile acids	CA	x		x			x	x	x	x	x	x
	CDCA			x	x							
	UDCA	x	x x			x				x	x	
				x	x							
Secondary bile acids	DCA		x x x		x	x					x	
	DHCA											
Conjugated bile acids	TCA	x	x x x		x	x	x	x				
	TUDCA	x x	x				x	x			x	
	TDCA	x	x x	x	x	x	x	x	x	x		
	TLCA	x x	x x x x	x	x	x	x	x		x		x

Table 1: Bile acids measured in liver. Summary of statistical significance between the groups. Colors were used to distinguish between groups. yellow: MHY1485, purple: rapamycin, blue: BA supplementation, red: fluvastatin, green: BSH inhibitor.

7.2.3 Bile Acids in Ileum

The same measurements were performed for the ileum as for the liver. The same experimental and control groups and parameters were measured.

7.2.3.1 MHY 1485 and Rapamycin

For the primary bile acid CA, a statistically significant difference was measured between the MHY 1485 (ad lib) and MHY1485 (CR) group (see figure 26).

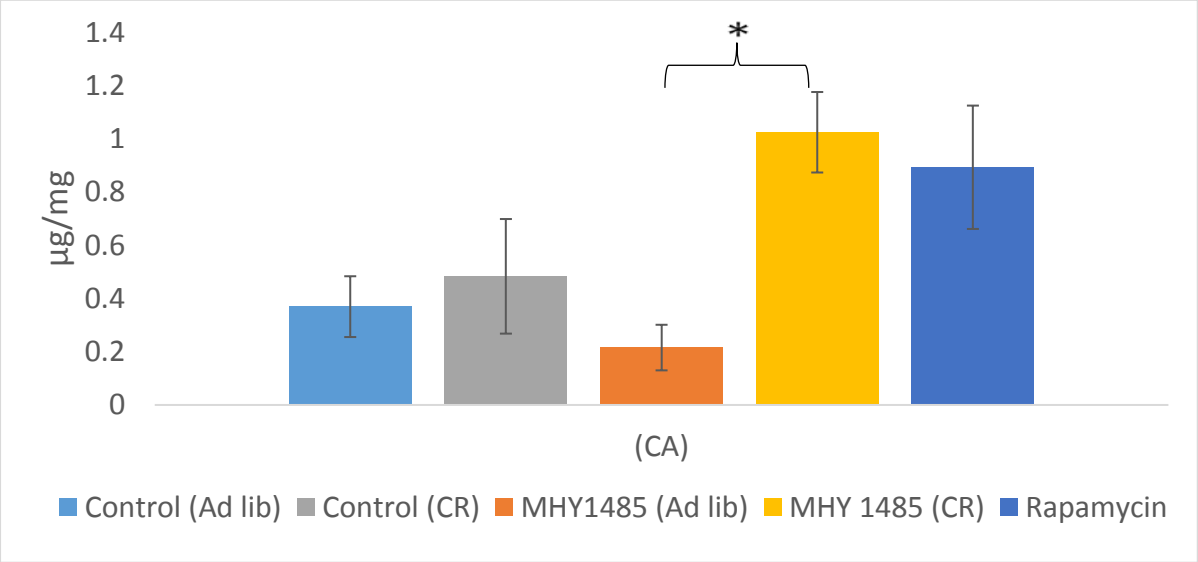

*Figure 26: Ileum CA. Mice in the groups: MHY1485, Rapamycin and Control. * Statistically significant difference p < 0,05*

For the bile acid CDCA (see figure 27) a significant difference between the MHY 1485 (CR) and the control group was found. In UDCA and CDCA there was a statistically significant difference between the MHY1485 (CR) and control group and between the MHY1485(Ad lib) and MHY1485(CR) group. No significance in the DHCA group was shown (see figure 28).

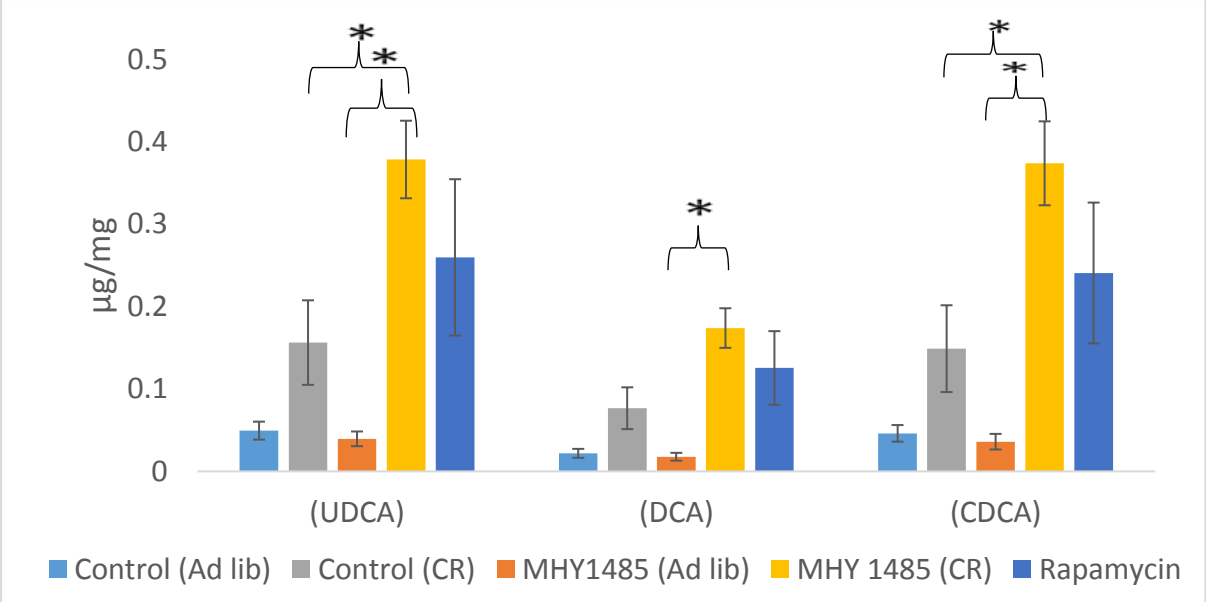

*Figure 27: Ileum UDCA, DCA and CDCA. Mice in the groups: MHY1485, Rapamycin and Control. * Statistically significant difference p < 0,05*

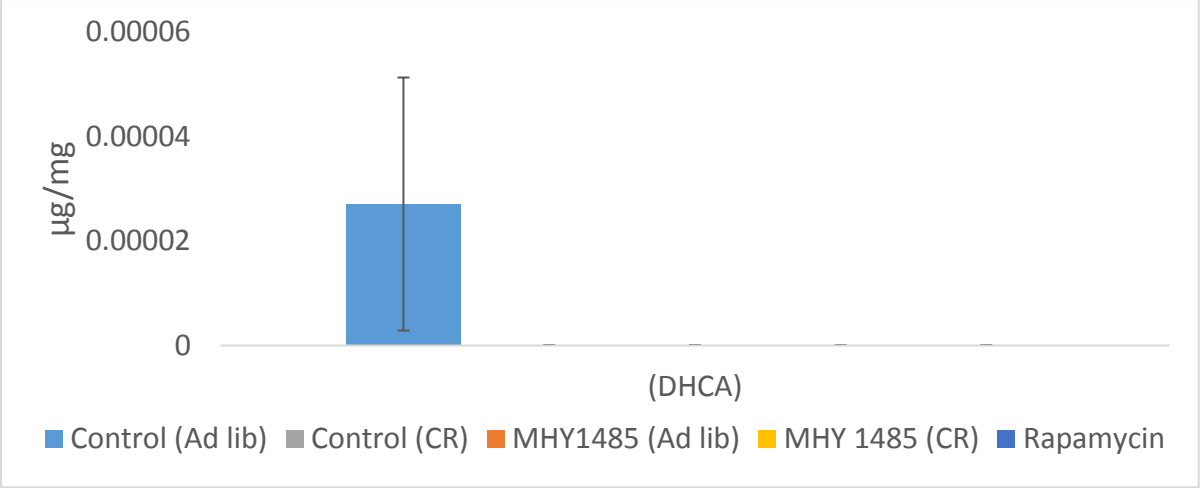

*Figure 28: Ileum DHCA. Mice in the groups: MHY1485, Rapamycin and Control. * Statistically significant difference p < 0,05*

For the conjugated bile acids TCA, TLCA, TUDCA and TDCA no significant difference between the groups was measured.

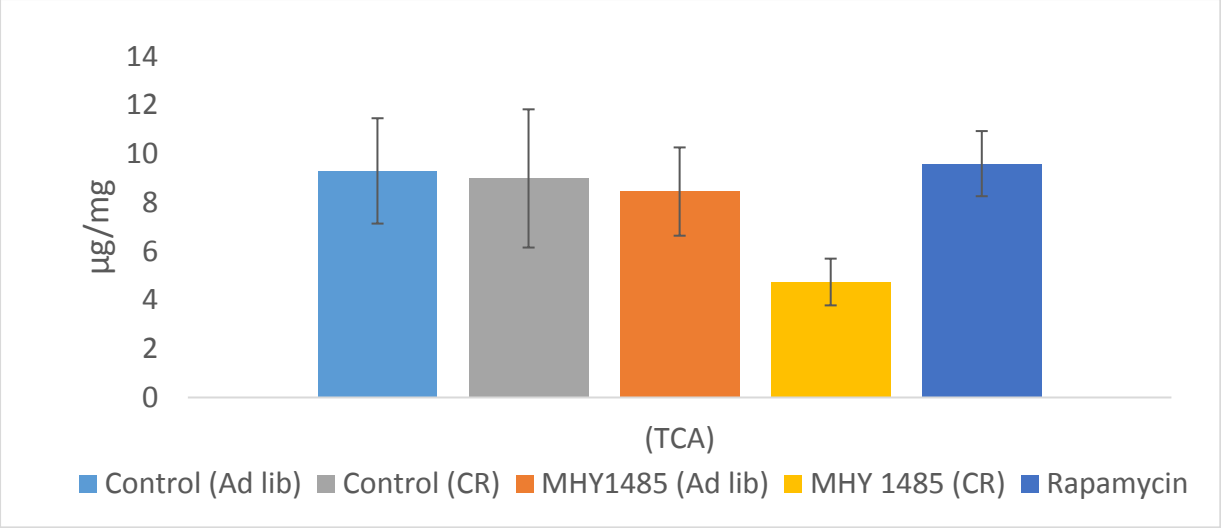

*Figure 29: Ileum TCA. Mice in the groups: MHY1485, Rapamycin and Control. * Statistically significant difference p < 0,05*

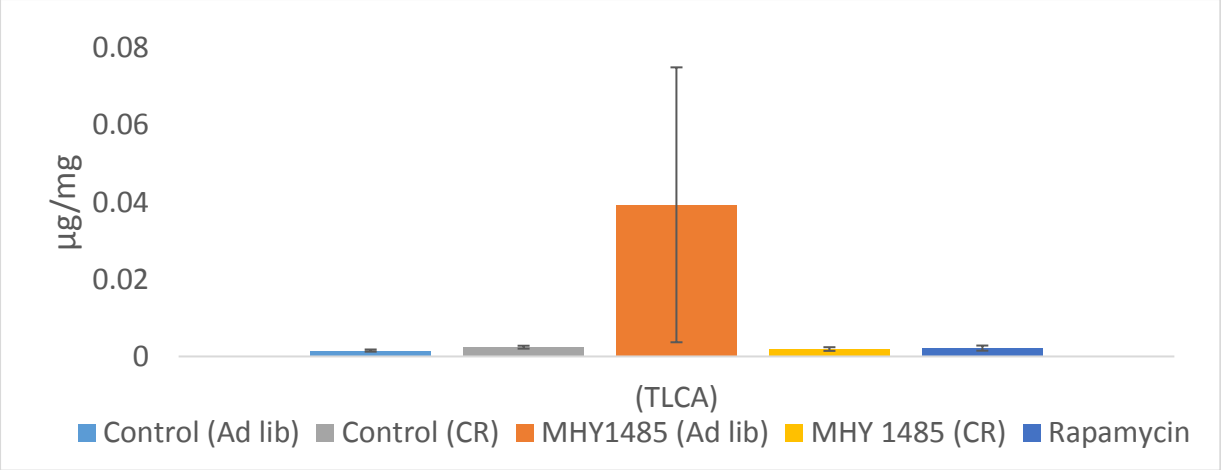

*Figure 30: Ileum TLCA. Mice in the groups: MHY1485, Rapamycin and Control. * Statistically significant difference p < 0,05*

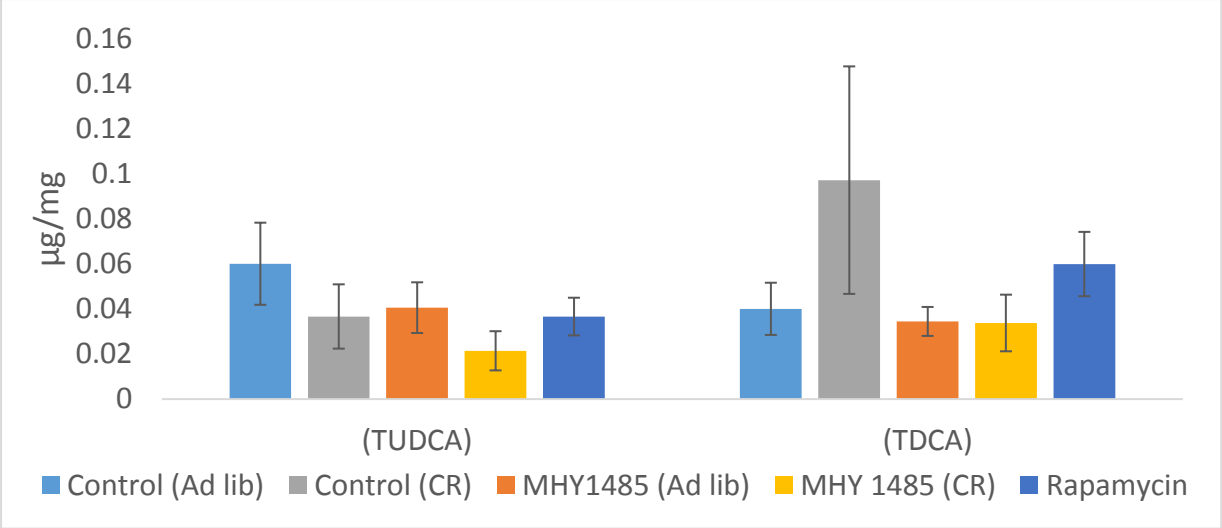

*Figure 31:Ileum TUDCA and TDCA. Mice in the groups: MHY1485, Rapamycin and Control. * Statistically significant difference p < 0,05*

7.2.3.2 Fluvastatin and BA Supplementation

For CA, CDCA, DCA and UDCA, there was a statistically significant difference for all four bile acids between the ad lib and CR and also the Fluvastatin (Ad lib) and Fluvastatin (CR) groups (see figure 32 and 33).

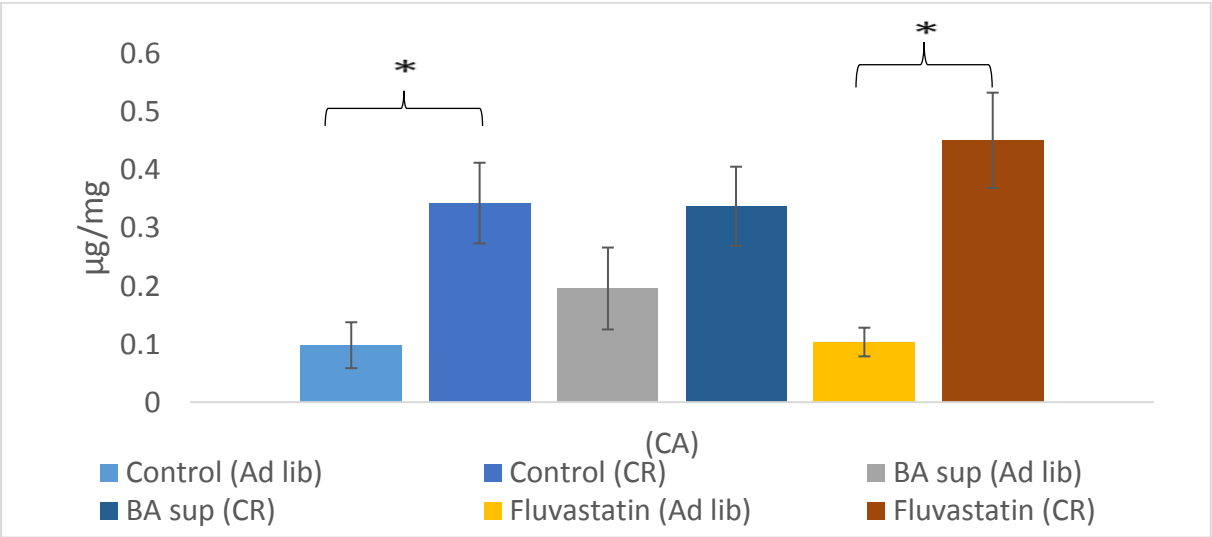

*Figure 32: Ileum CA. Mice in the groups: BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05*

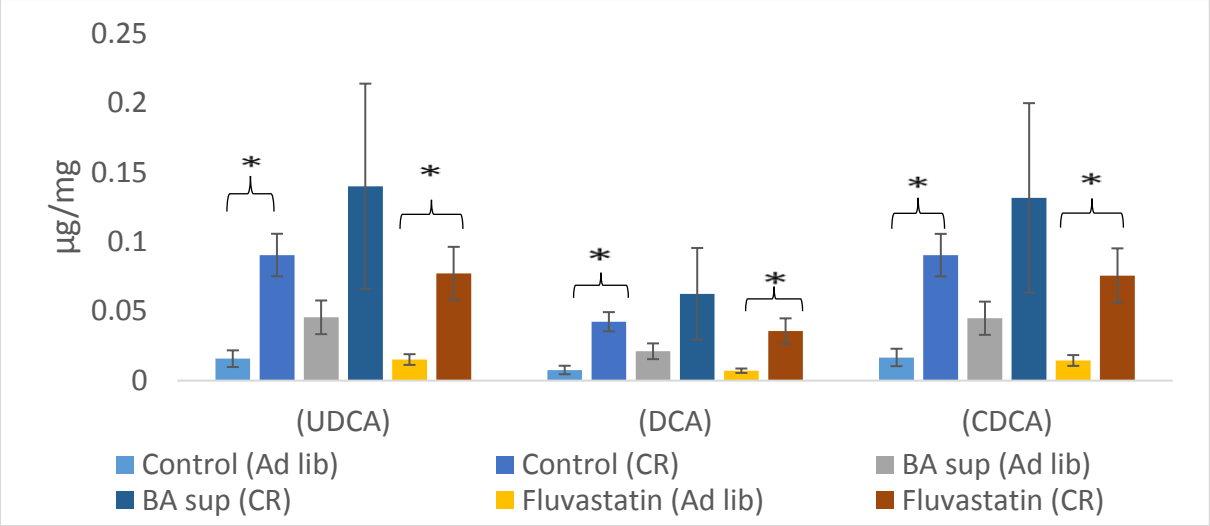

*Figure 33: Ileum UDCA, DCA and CDCA. Mice in the groups: BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05.*

For the bile acid DHCA a statistically significant difference between the BA sup. (Ad lib) and the BA sup. (CR) group was found (see figure 34).

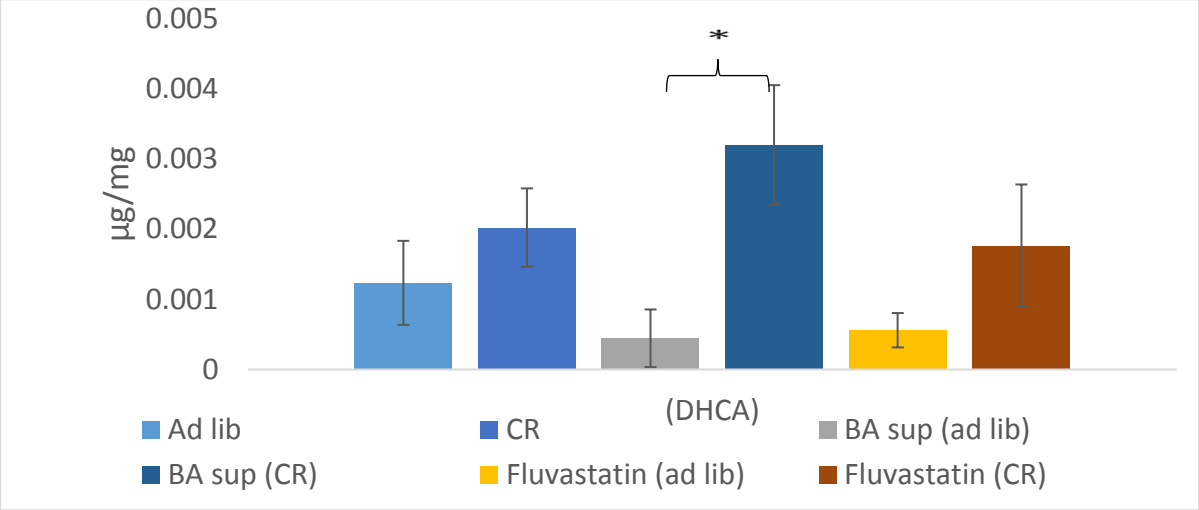

*Figure 34: Ileum DHCA. Mice in the groups: BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05.*

For the conjugated bile acid TCA there was a statistically significant difference between Fluvastatin (Ad lib) and the corresponding control group and also Fluvastatin (CR) and the corresponding control group (see figure 35).

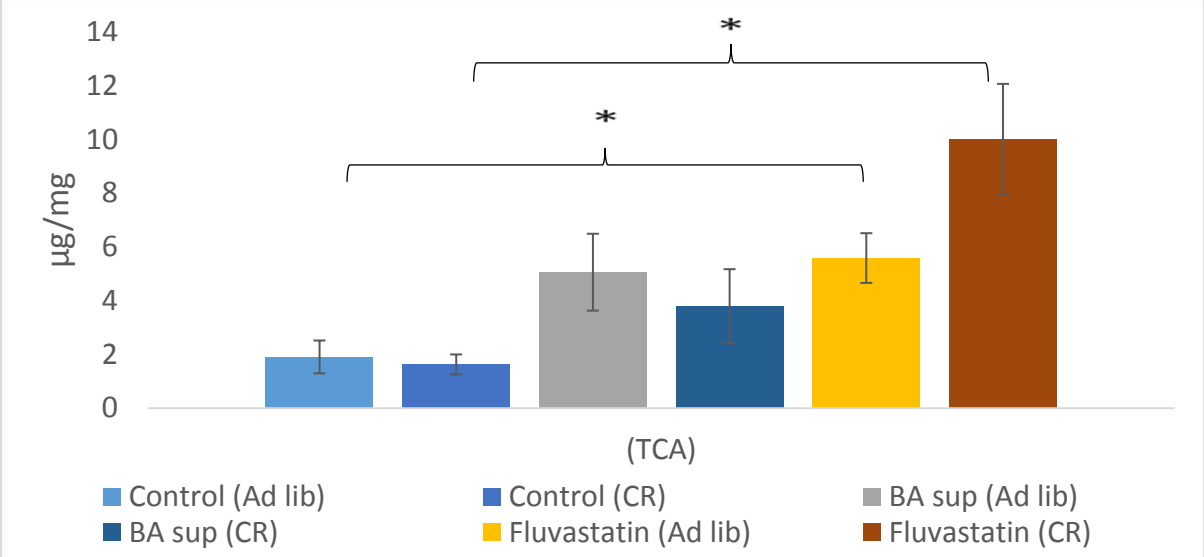

*Figure 35: Ileum TCA. Mice in the groups: BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05.*

Furthermore for the bile acid TLCA there was a significant differnce between the BA sup. and control group, Fluvastatin (Ad lib) and Fluvastatin (CR) and between the Ad lib and CR group (see figure 36).

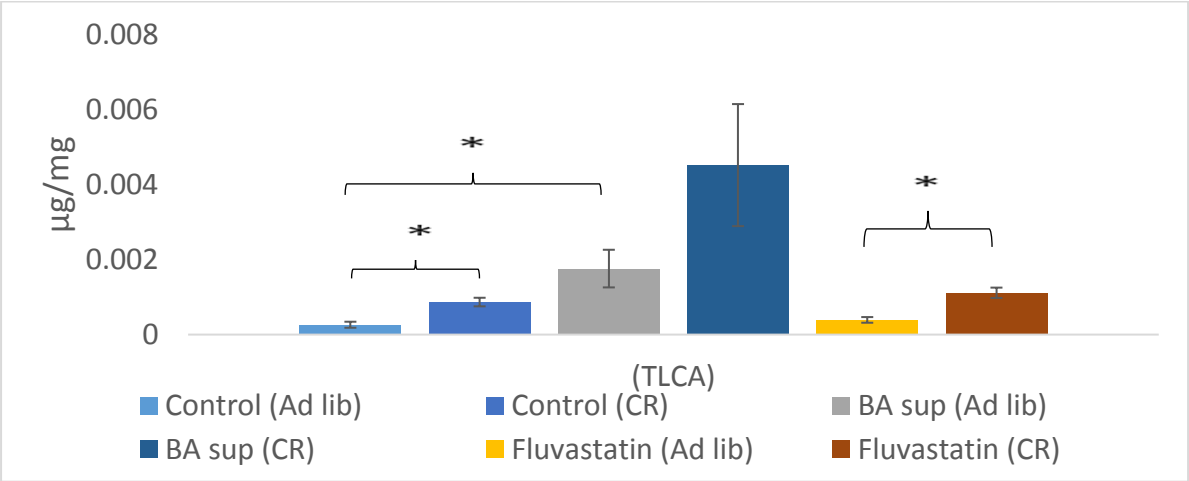

*Figure 36: Ileum TLCA. Mice in the groups: BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05.*

For TUDCA measurements showed statistically significant differences between the BA sup. and control group and Fluvastatin and control group in both groups Ad lib and CR. There was also a statistically significant difference between the BA sup. (Ad lib) and BA sup. (CR). TDCA showed statistically significant diffrencens between the Fluvastatin and control group in both groups Ad lib and CR.

Furthermore significant diffrenences between the BA sup.(Ad lib) and control group (Ad lib), and Fluvastatin (Ad lib) and Fluvastatin (CR) were found (see figure 37).

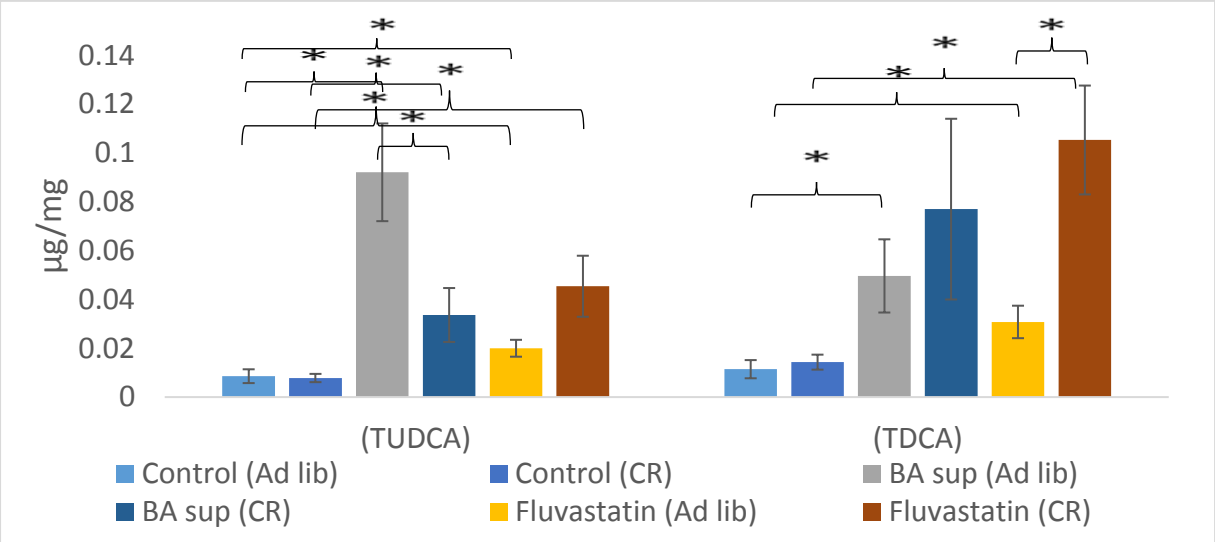

*Figure 37: Ileum TUDCA and TDCA. Mice in the groups: BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05.*

7.2.3.3 BSH Inhibitor

The primary and secondary bile acids CA, CDCA, DCA and UDCA showed statistically significant differences between the Ad lib and CR control group (see figure 38 and 39). For DHCA there was no significance between the groups (figure 40).

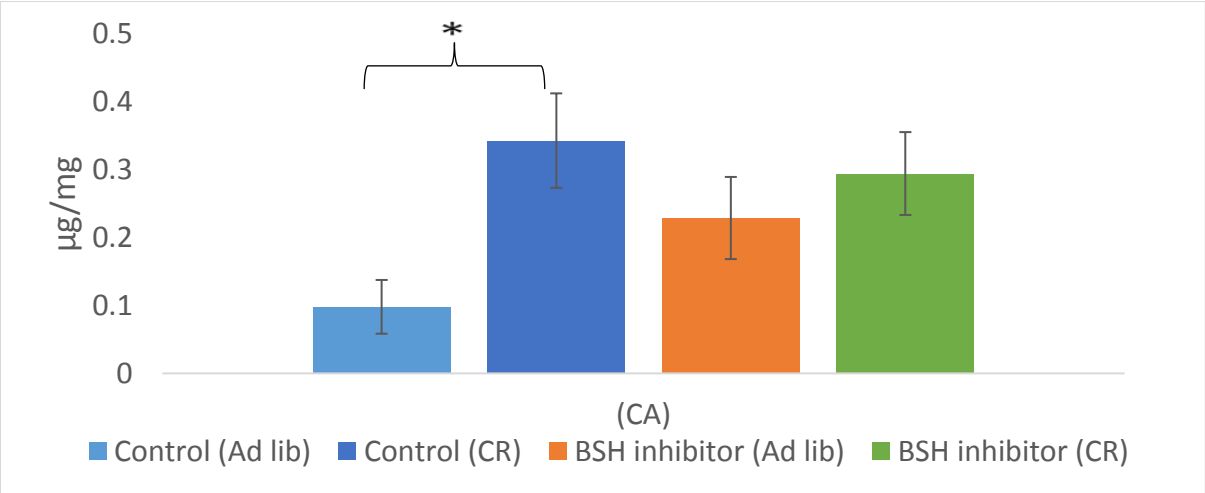

*Figure 38: Ileum CA. Mice in the groups: BSH inhibitor and Control. * Statistically significant difference p < 0,05.*

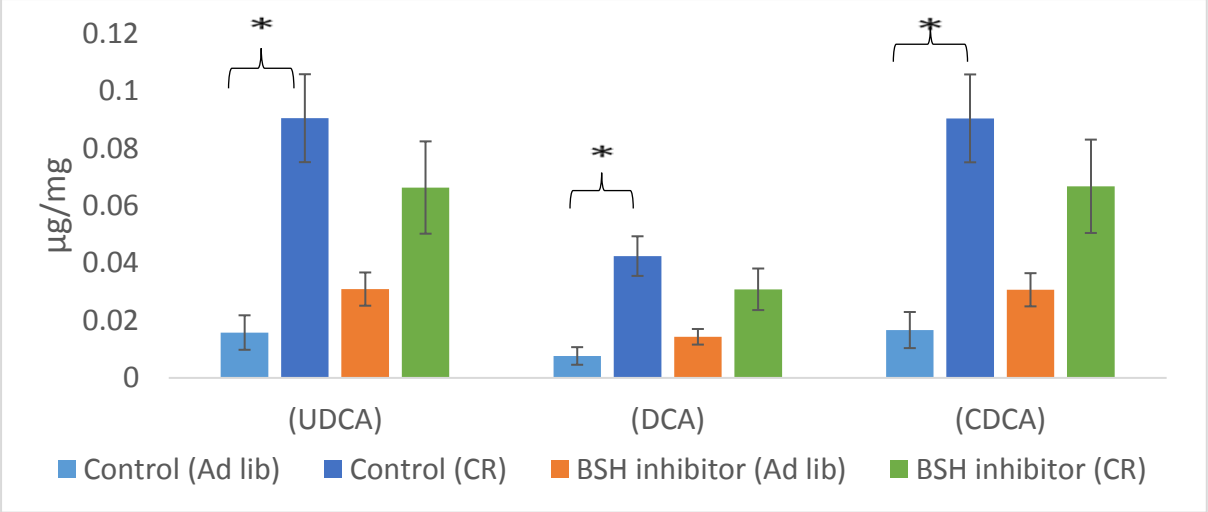

*Figure 39: Ileum UDCA, DCA and CDCA. Mice in the groups: BSH inhibitor and Control. * Statistically significant difference p < 0,05.*

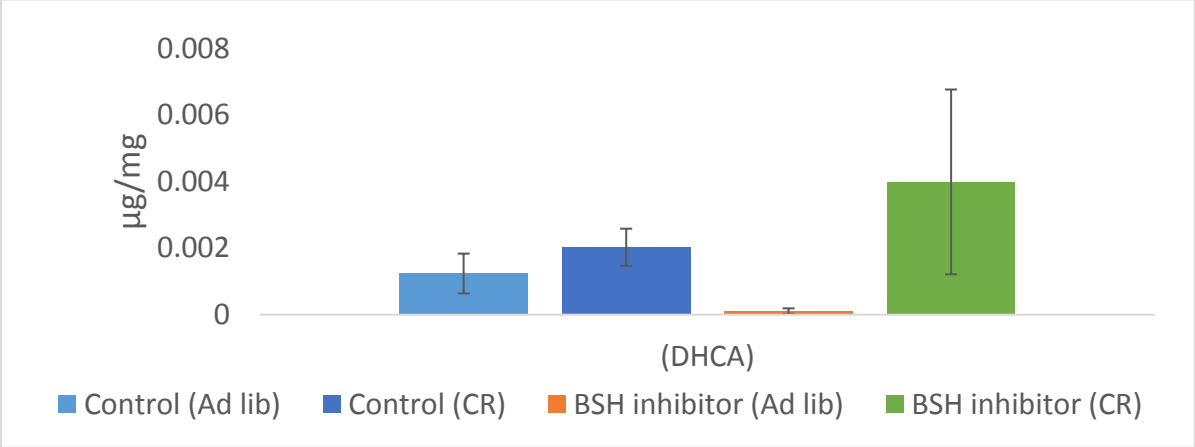

*Figure 40: Ileum DHCA. Mice in the groups: BSH inhibitor and Control. * Statistically significant difference p < 0,05.*

Statistically significant differences for TLCA between the BSH i. (Ad lib) and control group and between the Ad lib and CR group were measured (see figure 42). For TDCA only a significant difference BSH i.(Ad lib) and corresponding control group was shown (see figure 43). In TCA and TUDCA there was no significance between the groups (see figures 41 and 43).

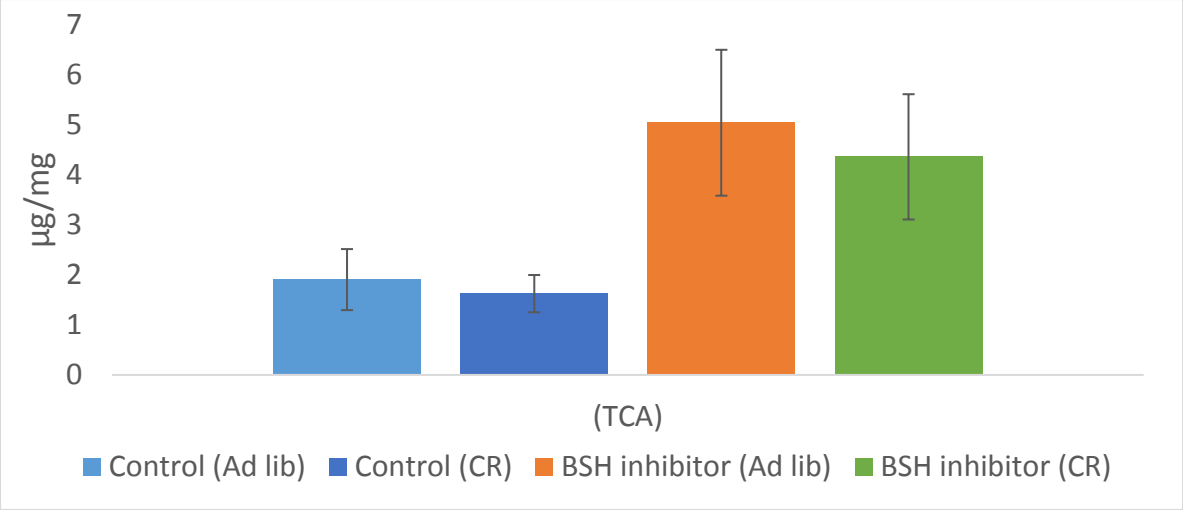

*Figure 41: Ileum TCA. Mice in the groups: BSH inhibitor and Control. * Statistically significant difference p < 0,05.*

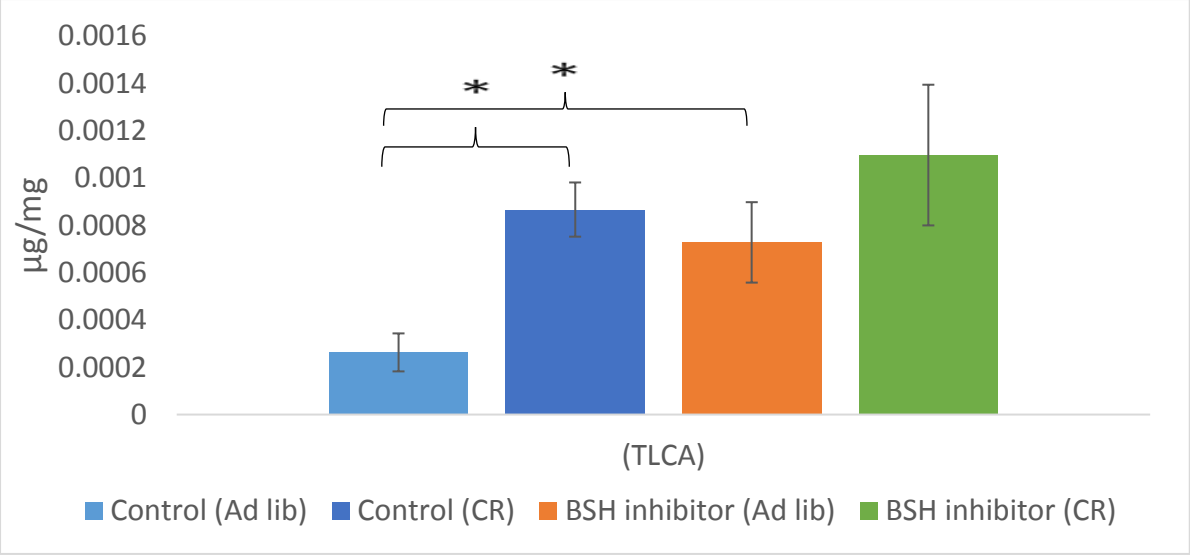

*Figure 42:Ileum TLCA. Mice in the groups: BSH inhibitor and Control. * Statistically significant difference p < 0,05.*

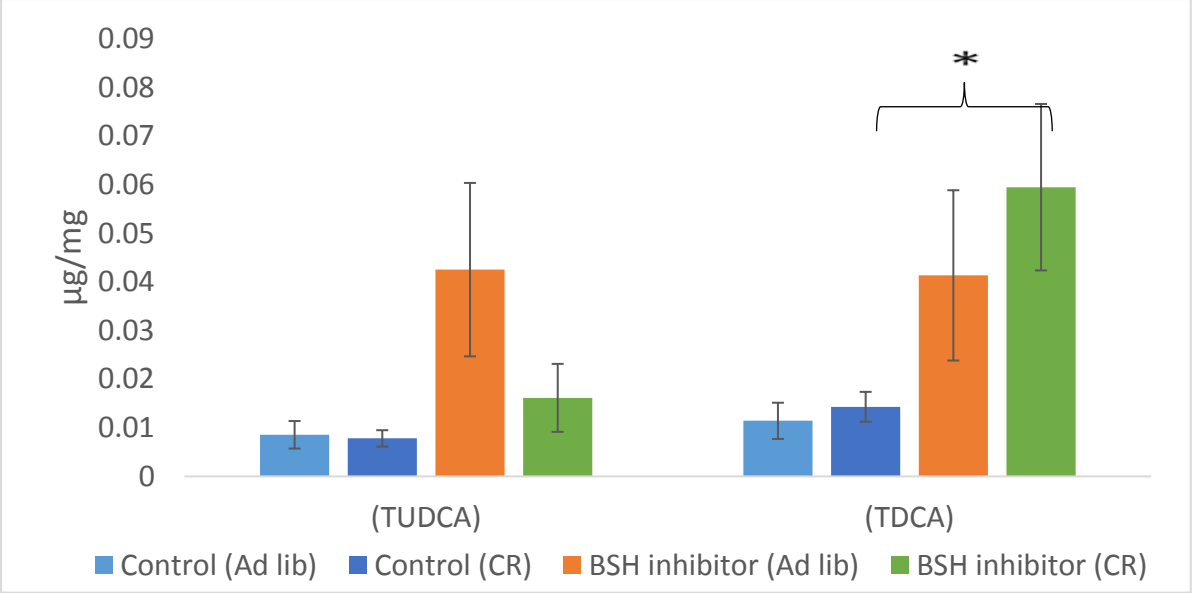

*Figure 43: Ileum TUDCA and TDCA. Mice in the groups: BSH inhibitor and Control. * Statistically significant difference p < 0,05.*

In table 2 all statistically significant differences measured in ileum are listed.

		Control		MHY1485		Rapamycin	BA supplementation		Fluvastatin		BSH inhibitor	
		Ad lib	CR	Ad lib	CR		Ad lib	CR	Ad lib	CR	Ad lib	CR
Primary bile acids	CA	x	x	x	x				x	x		
	CDCA	x x	x	x	x x				x	x		
	UDCA	x x	x	x	x x				x	x		
Secondary bile acids	DCA	x	x	x	x				x	x		
	DHCA						x	x				
Conjugated bile acids	TCA	x	x						x	x		
	TUDCA	x x	x				x x	x	x	x		
	TDCA	x x	x x				x		x	x x		x
	TLCA	x x x	x				x		x	x	x	

Table 2: Bile acids measured in ileum. Summary of statistical significance between the groups. Colors were used to distinguish between groups. yellow: MHY1485, purple: rapamycin, blue: BA supplementation, red: fluvastatin, green: BSH inhibitor

7.2.4 Taurine and GSH uptake in the Intestine

In this part the free taurine and GSH uptake were measured ex vivo from the intestinal sacs. The intestinal sacs were infused with a mix of taurine and GSH to measure the taurine uptake. The samples were collected at five time points over 2h from the surrounding medium (DMEM) in order to determine the taurine and GSH concentration.

7.2.4.1 Determination of Taurine

As shown in the figure 44 the concentration of free taurine increased over the time and reached a saturation at 120 minutes. Statistically significant differences were found between the Ad lib and CR control groups. Within the CR group there a statistically significant difference was shown between the experimental groups and the control group. Within the ad libitum group, there was no statistically significant difference, except the control and Fluvastatin group, only at the 60 minutes timepoint. Furthermore, statistically significant differences were measured between the BA sup (Ad lib) and BA sup (CR) and Fluvastatin (Ad lib) and Fluvastatin (CR) groups over all measured timepoints.

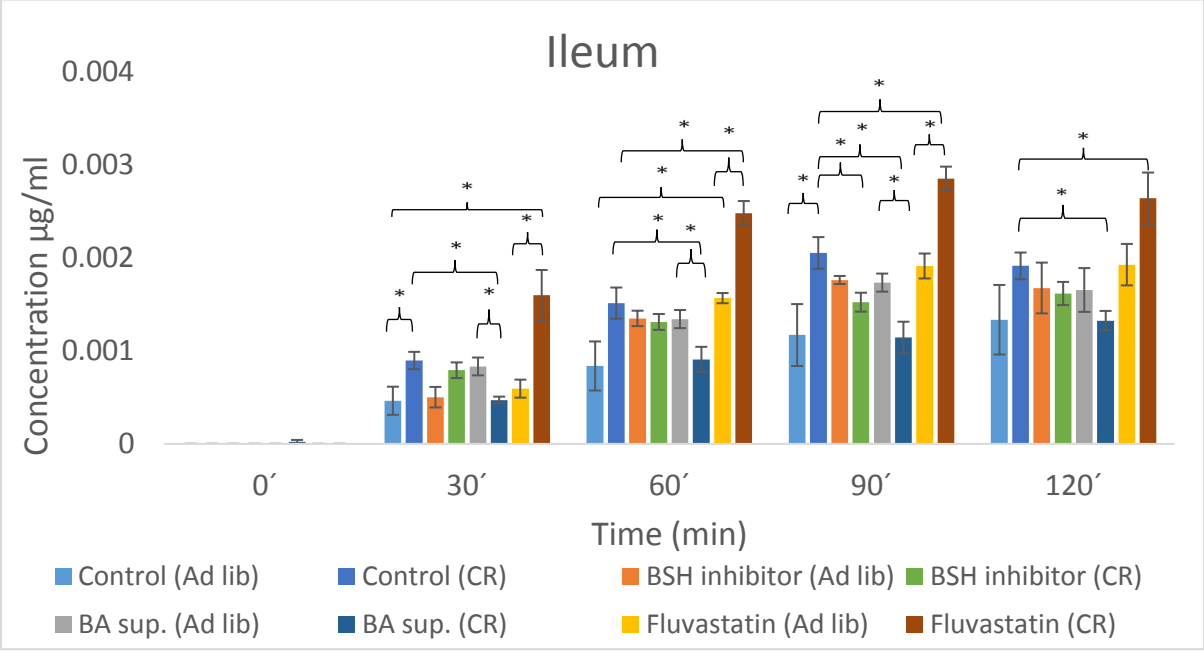

*Figure 44: Increased free taurine concentration in the Ileum. Mice in the groups: BSH inhibitor, BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05.*

7.2.4.2 Determination of GSH

There is a significance inside the CR group between the CR control group and BA supplementation and Fluvastatin group. There is a significance between the BA sup and the control group and the Fluvastatin and control group in three time points. On one time point there is a significance between the BSH inhibitor (Ad lib) and BSH inhibitor (CR) and BA sup. (Ad lib) and BA sup. (CR). A continuous significance was shown in three timepoints between Fluvastatin (Ad lib) and Fluvastatin (CR). There was no significance between the Ad lib and CR and no difference compared the experimental Ad lib group to the control group (see figure 45).

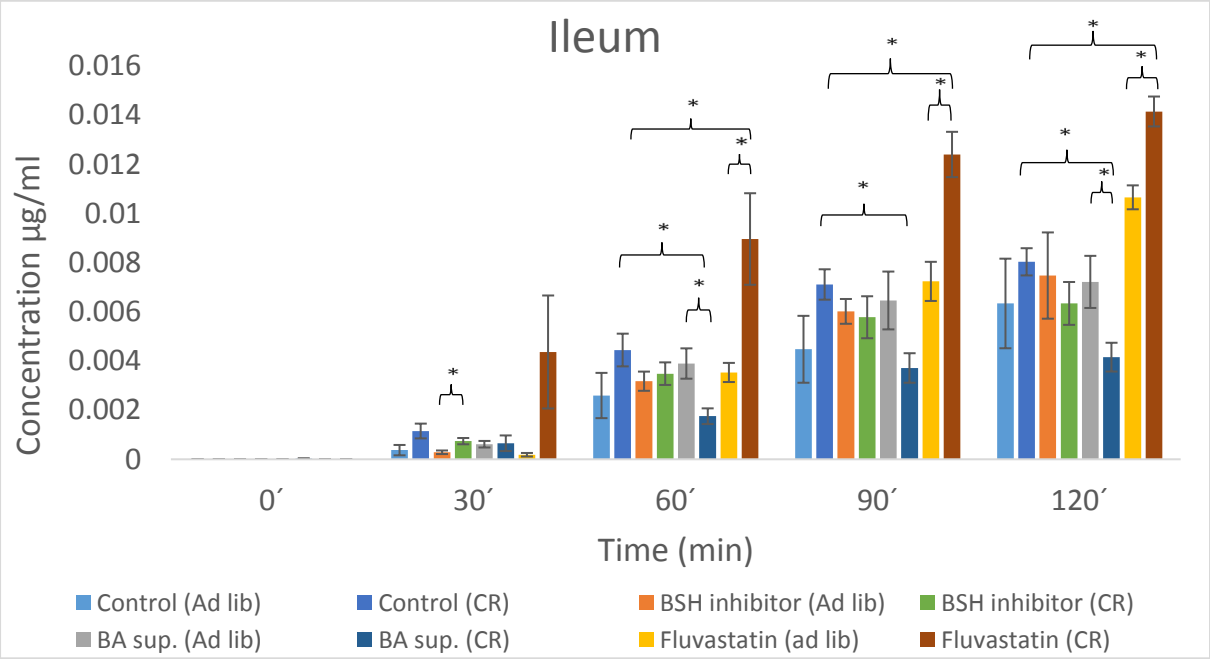

*Figure 45: CR affected GSH levels in the Ileum. Mice in the groups: BSH inhibitor, BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05.*

7.2.5 Taurine and GSH in the lysate

7.2.5.1 Determination of taurine

In the lysate mix taurine was present in all groups that were used for the experiments. A statistically significant difference was found between the Fluvastatin Ad lib and CR group at both timepoints of measurement. In other groups no significant differences were measured (see figure 46).

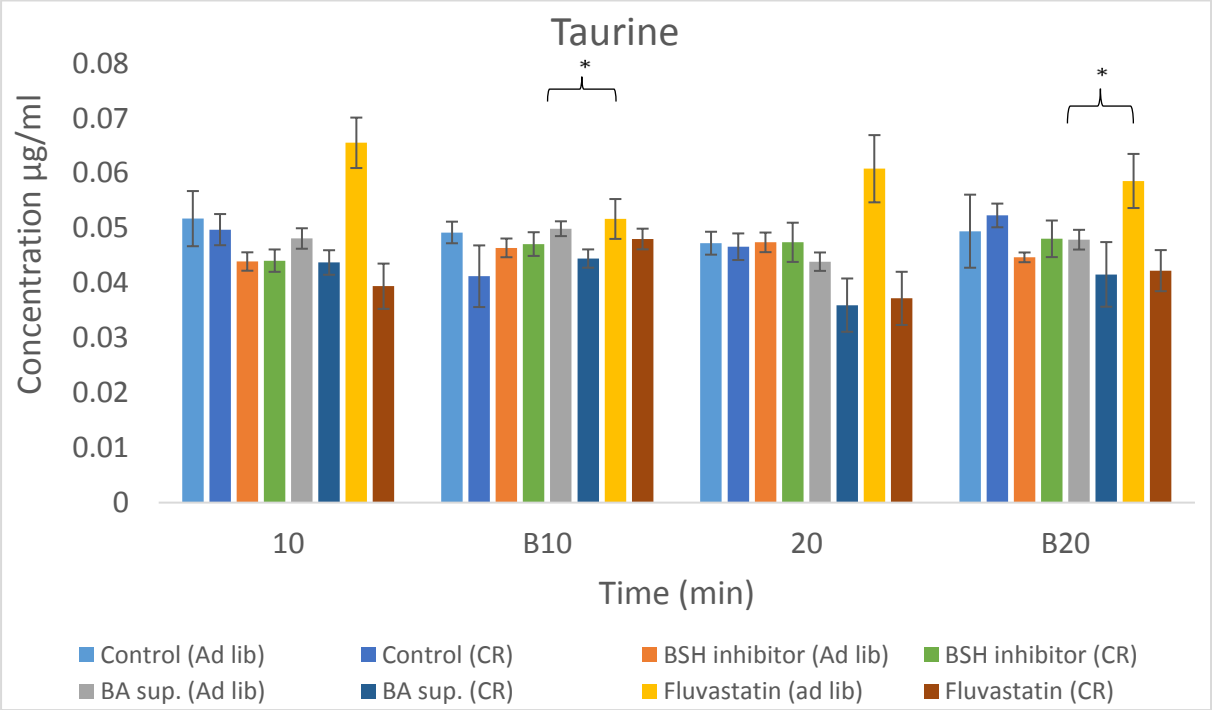

*Figure 46: Caloric restriction (CR) impacts on free taurine in the intestinal mucosa. Mice in the groups: BSH inhibitor, BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05.*

7.2.5.2 Determination of GSH

The important non-enzymatic antioxidant GSH was also measured in the lysate mix. There was a significance between the BSH group in ad libitum and CR. Within the ad libitum group a statistically significant difference was measured between the control and the Ad lib group at both time points. Within the CR group there was a significant difference between the control and the BSH inhibitor group (see figure 45).

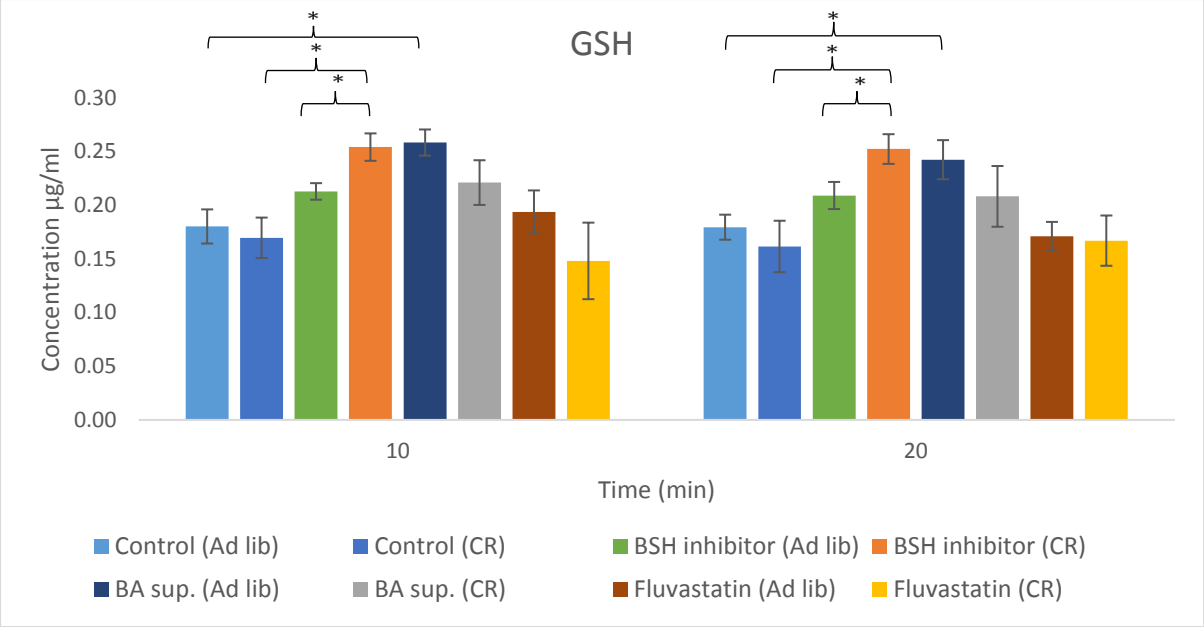

*Figure 47: Caloric restriction (CR) impacts on GSH in the intestinal mucosa. Mice in the groups: BSH inhibitor, BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05.*

7.2.5.3 Determination of Taurine-GSH conjugate

In the lysate mix also a taurine conjugate with a molecular mass of 431 g/mol was measured. It is a taurine-GSH coupled molecule.

At the first timepoint there was a statistically significant difference found between both control groups and Fluvastatin groups (Ad lib and CR respectively). Within the CR group there was also a statistically significant difference found between the control and the BSH inhibitor group. On the second time point there was no significant difference between the groups (see figure 48).

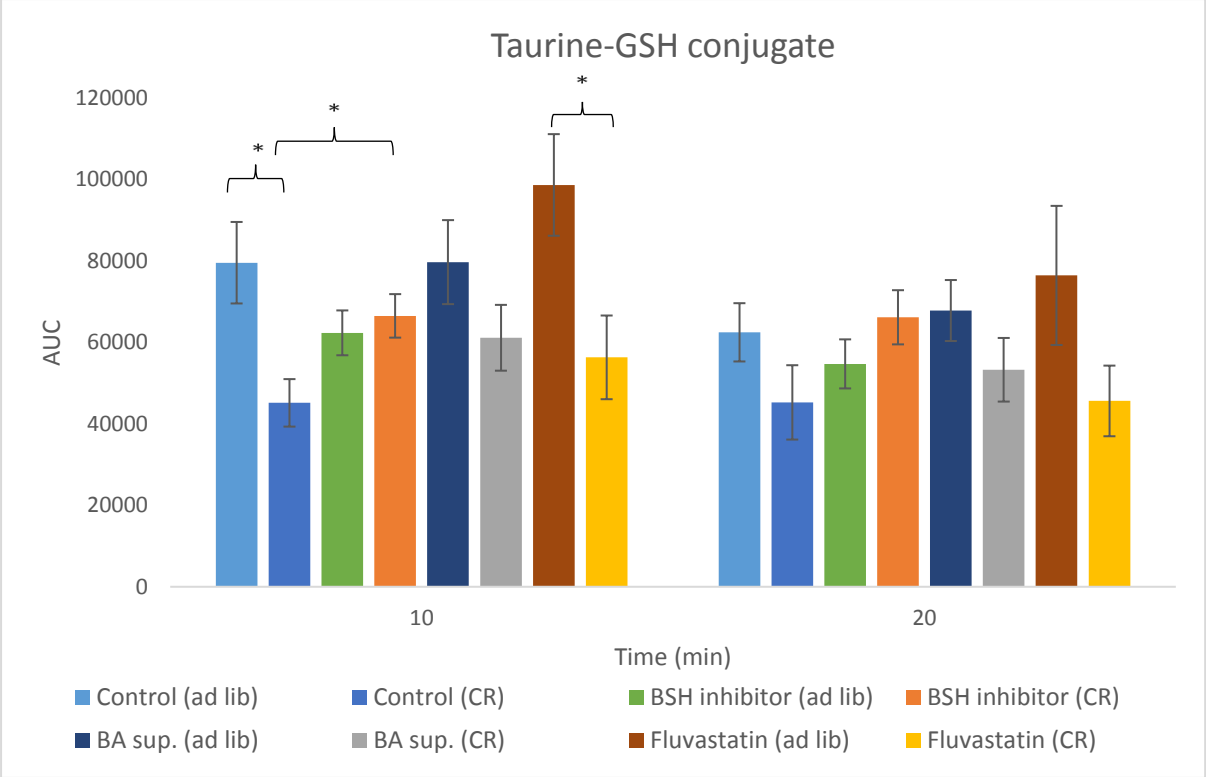

*Figure 48: Caloric restriction (CR) impacts on taurine conjugates in the intestinal mucosa. Mice in the groups: BSH inhibitor, BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05.*

7.2.6 Villi, Crypt and Goblet cells

7.2.6.1 Small Intestine

The aim of this study was to examine the impact of taurine and taurine conjugates on the tissue in the GIT. The section tissue was collected from all groups except the BSH inhibitor groups from ad lib and CR. From small intestine and proximal colon, the tissue was collected and pictures taken on 5x magnification. These pictures were used to measure the muscle thickness, which was measured with ImageJ software. The other two magnifications (10x and 20x) were used to measure the villi length and to count the goblet cells in the villi. The results show differences in the tissue samples.

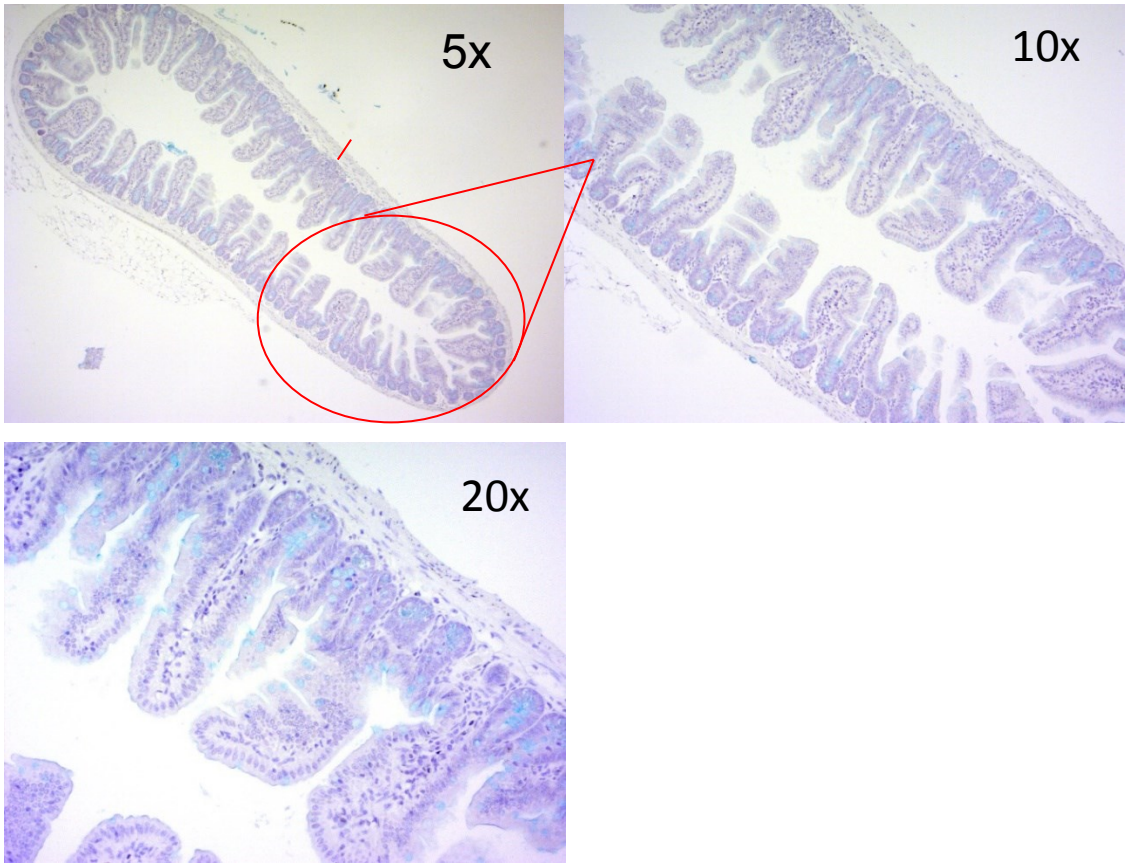

Figure 49: Small intestine: 5x, 10x and 20x magnification.

The measurement of muscle thickness showed statistically significant differences between the Ad lib and CR control group as shown in figure 50. Within the Ad lib group only a significant difference between the BA supplementation group and the control group was measured. Between the CR control and Fluvastatin group there was no significant difference in muscle thickness.

For the measurements of the villi leght there was found only a statistically significant difference within the Ad lib group between the control and the BA supplementation group.

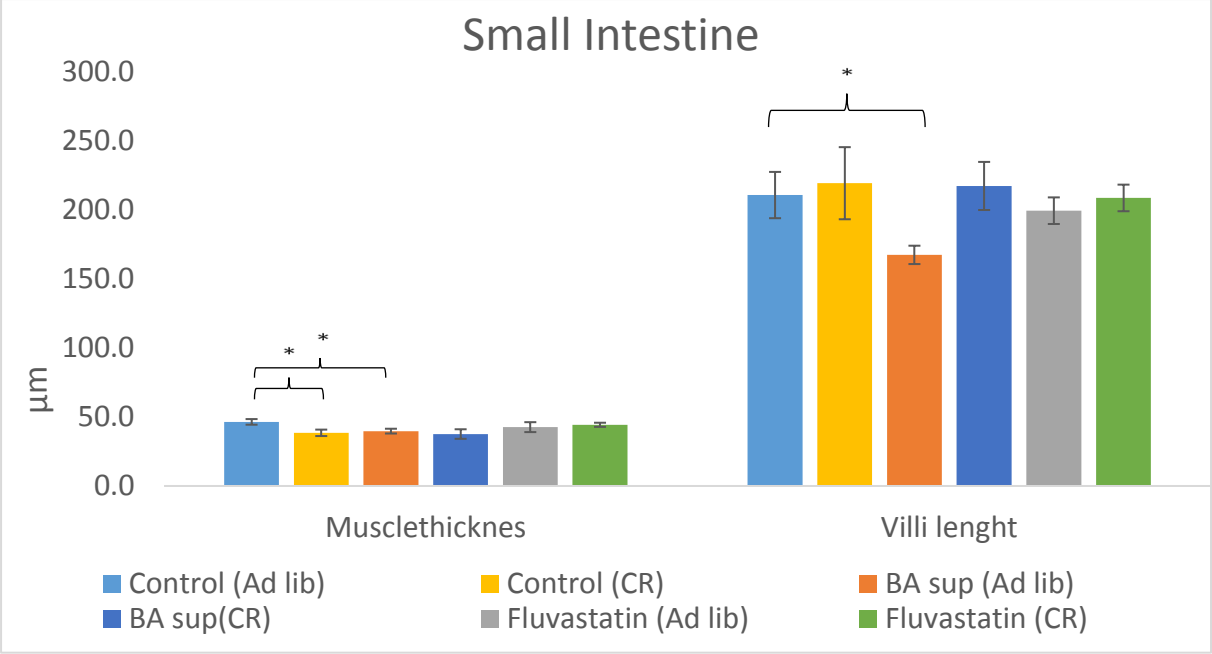

*Figure 50: Small intestine musclethicknes and villi lenght. Mice in the groups: BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05.*

Counting the goblet cells in the small intestine was challenging, as it was hard to separate them. Therefore all blue clearly defined cells were counted. Results showed that there was only a statistically significant difference between the Fluvastatin (Ad lib) and the corresponding control group (see figure 51).

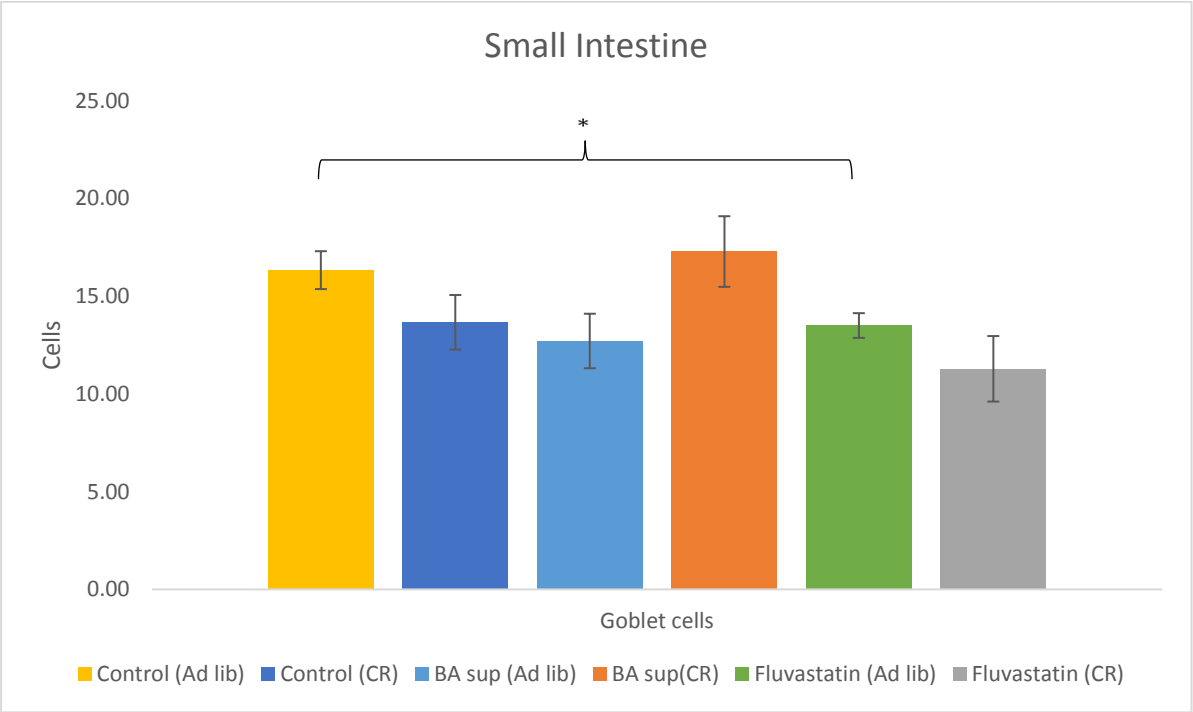

*Figure 51: Small intestine goblet cells. Mice in the groups: BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05.*

7.2.6.2 Proximal Colon

In the proximal colon the muscle thickness, crypt depth and the goblet cells were measured and counted. There are some differences between some groups, but no statistically significant differences were found, neither in muscle thickness nor in crypt depth (see figure 52).

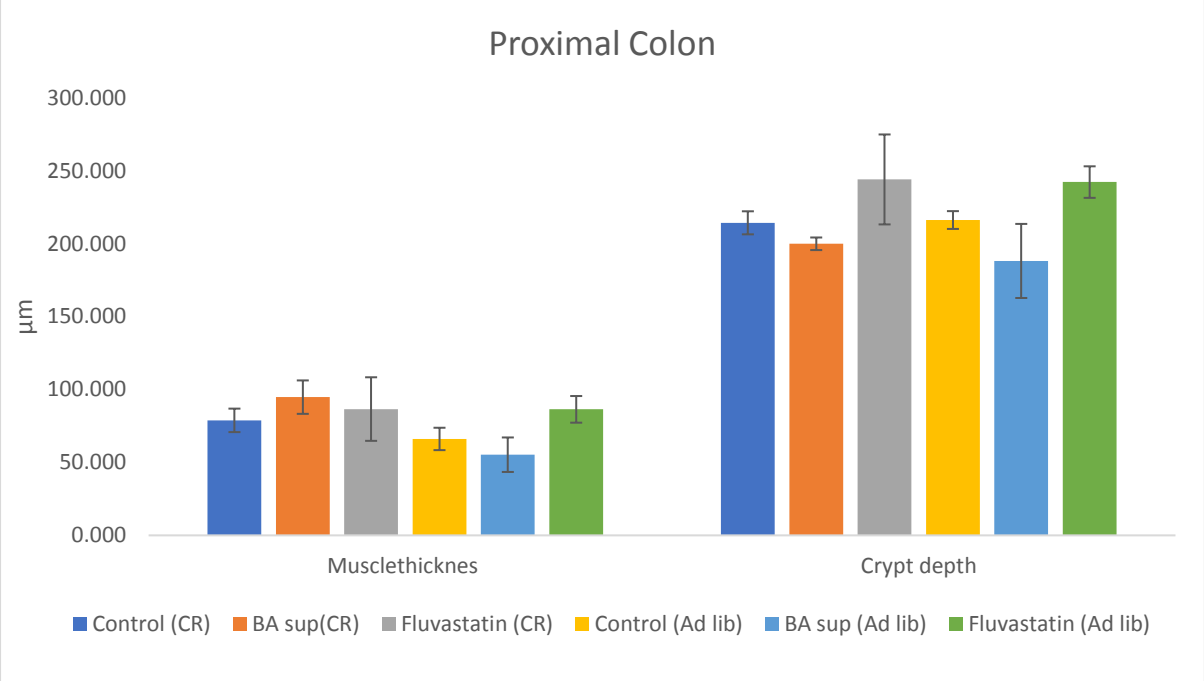

*Figure 52: Proximal colon musclethicknes and crypth depth. Mice in the groups: BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05.*

Counting the goblet cells in the proximal colon also did not show any statistically significant difference between the groups (see figure 53).

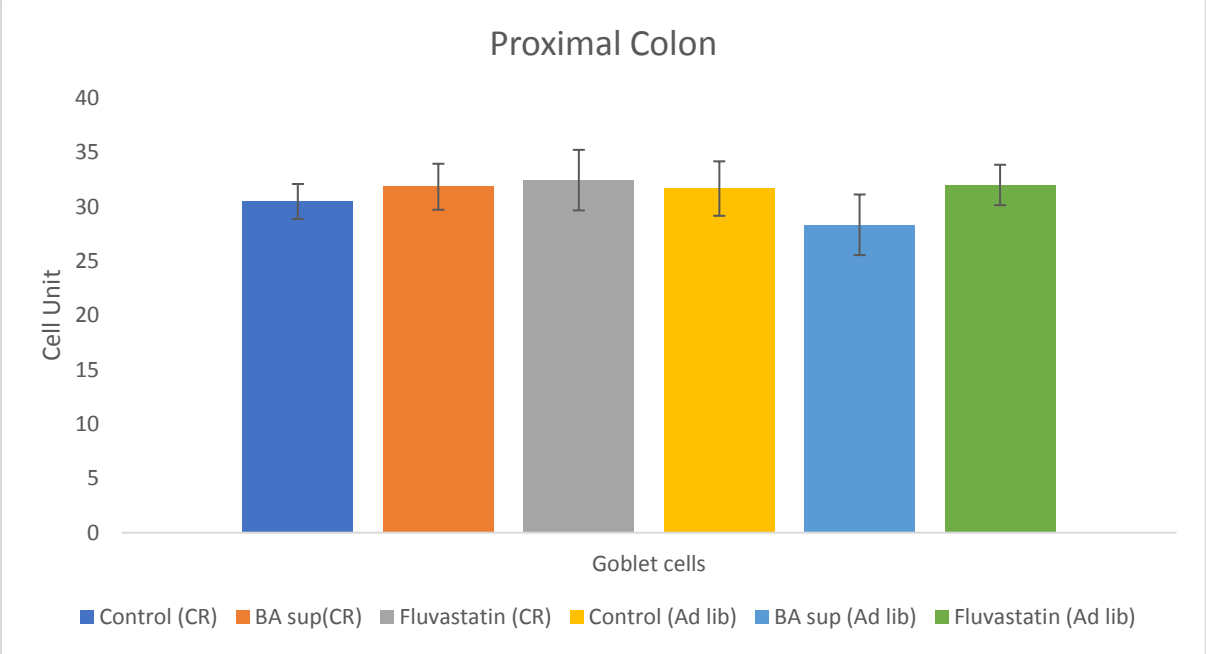

*Figure 53: Proximal colon goblet cells. Mice in the groups: BA sup., Fluvastatin and Control. * Statistically significant difference p < 0,05.*

8 Discussion

The number of old age individuals is rising worldwide together with the number of obese individuals. Many observations suggest that it has something to do with our lifestyle, moving and eating behavior. Obesity causes more than 70 % of the early deaths worldwide and is a major risk factor for non-communicable diseases. Chronical obesity is most accompanied with non-recallable diseases. The most popular individual mechanism against obesity is CR [1].

The effects of CR were observed on *C. elegans*, where it was shown that *C. elegans* have a higher lifespan under certain conditions [12]. In another study it was shown that taurine conjugated bile acids are increased under CR [43]. In our observation supported by our data we showed that *C. elegans* lived approximately 15 days. The results of the lifespan assay on *C. elegans* showed that the dose of 0.002 mg/ml or 0.0005 mg/ml taurine conjugated bile acids did not reduce or extend the lifespan compared to the control.

One of the beneficial effects of CR is the reduction of body weight [3]. Our data confirmed that after two weeks of observations every group of CR fed mice had a significant lower body weight than Ad lib fed mice. Furthermore, it has been assessed that mice under CR increased the bile acids production in liver and intestine and some substances enhances this increasing effect.

In liver it has been shown that CR has a higher impact on bile acids synthesis than the substances rapamycin or MHY1485. In contrast to that, in the Ileum MHY1485 had the highest impact on the concentration of bile acids.

Experiments with fluvastatin and BA supplementation showed that CR increases the bile acid concentration in the liver and in the Ileum. Generally, bile acid concentrations were significantly higher in CR than in Ad lib fed mice. An enhanced effect of higher bile acid concentrations in the liver and Ileum was shown in fluvastatin treated and bile acid supplemented groups.

The groups with BSH inhibitor showed that there are no significant differences between the BSH inhibitor Ad lib and CR groups in the liver and in ileum.

Furthermore, the levels of free taurine were increased under CR compared to Ad lib fed mice. The free taurine concentrations in intestine were measured at different time points. At each time point the concentration of taurine increases compared to the one before. The concentration of free taurine in the intestine was higher in CR group compared to Ad lib group. The effect was higher in the Ad lib BA supplemented group than in the BA supplemented CR group. The taurine concentration was significant higher in Fluvastatin CR compared to Ad lib Fluvastatin group as compared to the control CR group. There was no significant difference between the BSH inhibitor groups Ad lib and CR. We could assume that the concentration of free taurine in the intestine is bile acid dependent.

The reduced form of Glutathione (GSH) was also measured at different time points in the intestine. The concentration of GSH was in Fluvastatin Ad lib significantly lower compared to the Fluvastatin CR group. There is a statistically significant difference between BA supplemented Ad lib and BA supplemented CR group. Furthermore, there is a significant difference between the Fluvastatin CR and BA supplemented CR group compared to the control group.

In the lysate taurine concentration was higher in the Fluvastatin CR group compared to the Fluvastatin Ad lib group. The GSH was higher in the BSH inhibitor CR group compared to the BSH inhibitor Ad lib group and control group. BA supplementation Ad lib was higher than the control group. The taurine conjugate in the lysate was higher in the Fluvastatin and Ad lib control group compared to the CR counterpart.

The intestine morphology was also changed during the CR treatment. In one observational study it was shown that the gut villi during CR can enlarge the surface area by forming depressions to elevate the gut absorption. [69] In our observed tissue of the small intestine there was a statistically significant difference in muscle thickness. In Ad lib it was higher than in CR, whereas in the BA supplemented Ad lib group it was lower compared to the control group. The villi length was lower in BA supplemented Ad lib compared to the control group. The lowest amount of goblet cells was in the Fluvastatin Ad lib group compared to the control group. In the proximal colon there were no differences in the morphology between the groups.

9 Conclusion

The objective of this study was CR and the impact of bile acid on two different organisms. The observation that *C. elegans* supplemented with taurine conjugated bile acids tend to live longer is not supported by our data. It was shown that CR has its benefits in both organisms. The use of the taurine conjugated bile acid is probably beneficial but is not confirmed in our experiment with *C. elegans.*

In mice after two weeks of being on CR the parameter changed. The bile acids increased in the liver and the intestine in CR compared to Ad lib fed groups. Independently of the supplementation conjugated bile acids were higher in the CR group compared to Ad lib fed group. Bile acid supplementation combined with CR had the highest impact on conjugated bile acids in the liver.

Concentration of bile acids were highest in the CR control group. Some substances like fluvastatin enhanced the effect of CR and increased the bile acid concentration in the liver and ileum. It was shown that the BSH inhibitor and rapamycin have a low or no effect on the bile acid concentration.

The uptake of free taurine and GSH from the intestine increases at every measured time point in every group. It can be assumed that CR increase the levels of free taurine in the intestine. We detect increased levels of free taurine in the intestine in CR compared to ad libitum fed mice except BA supplementation where it was reverse. The same occur in the intestine with GSH.

In the mucosa we can assume that CR increases free taurine and its conjugates along the intestinal mucosa. Some substances like fluvastatin or BSH inhibitor enhance the effect.

CR has also an effect on the morphology in the small intestine and proximal colon. It was shown that BA supplementation decreased the muscle thickness and villi length in Ad libitum fed mice in the small intestine. The amount of goblet cells decreased. In the proximal colon there were no differences. Further studies are needed to determine if higher doses of conjugated bile acids and lower doses of fluvastatin have the same enhanced effect on increasing bile acids.

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