universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

The impact of macronutrient restriction on the gastrointestinal tract

verfasst von / submitted by

Valeriya Panteva, B.Sc.

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2023 / Vienna, 2023

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066 838
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Ernährungswissenschaften UG2002
Betreut von / Supervisor:	Dr. techn. Kalina Duszka

Acknowledgement

I am very thankful to have been provided the opportunity to work in Prof. J. König‘s lab.

First and foremost, I would like to express an extreme gratitude to my supervisor Dr. Kalina Duszka for letting me be part of the lab group and granting me this project. She motivated and trusted me throughout the whole process and always guided me in the right direction. Thank you for always taking the time to help and support me, as well as for being patient with me!

Next, I would like to thank Dr. András Gregor and our laboratory technician Sandra Auernigg-Haselmaier who spent a lot of time in teaching me how-to and always being available to help! Special thanks to my lab colleagues Kajs Hadžić, Stefan Bruckberger and Manuel Malleier. Working with all of you was a lot of fun, it motivated me and helped me with my research!

Dear parents, thank you for your endless support throughout my whole study life! Finally, thanks to my friends who stood by me!

Without all of you, this would not have been possible. Thank you!

Table of Contents

List of tables V

List of figures VI

List of abbreviations VIII

Abstract XI

Zusammenfassung XII

1 Introduction 13

1.1 Caloric restriction 13

1.1.1 Macronutrient restriction 14

1.1.2 Oxidative stress 14

1.1.3 Inflammation 16

1.1.4 Microbiome 17

1.1.5 Taurine and BAs 19

1.2 Objectives 22

2 Materials and methods 24

2.1 Animal experiments 24

2.2 Sample preparation 26

2.2 Real-time quantitative polymerase chain reaction (qRT-PCR) 26

2.3 Liquid Chromatography-Mass Spectrometry (LCMS) 26

2.4 Western blot (WB) 27

2.5 Histology 28

2.5.1 Sample preparation 28

2.5.2 Hematoxylin & eosin staining 28

2.5.3 Imaging 28

2.6 Statistics 28

3 Results 29

3.1 qRT-PCR 32

3.1.1 Jejunum 32

3.1.1.1 Antioxidative processes 32

3.1.1.2 Inflammation 34
3.1.1.3 Metabolism 35
3.1.1.4 Metabolism of taurine and BAs 37
3.1.2 Cecum 39
3.1.2.1 Microbiota 39
3.1.3 Liver 41
3.1.3.1 Metabolism of taurine and BAs 41
3.2 LCMS 43
3.2.1 Taurine 43
3.2.2 BAs 46
3.3 Western Blot 52
3.3.1 Jejunum 52
3.3.1.1 Restriction-related markers 52
3.4 Histology 56
3.4.1 Ileum 56
4 Discussion 64
4.1 Impact of CR on the GIT 64
4.2 Impact of macronutrient restriction on the GIT 65
4.2.1 Antioxidative processes 65
4.2.2 Inflammation 66
4.2.3 Metabolism 66
4.2.4 Metabolism of taurine and BAs 67
4.2.5 Microbiota 68
4.2.6 Restriction-related processes 70
4.2.7 Histological alterations of the gut 71
4.3 Limitations and outlook 71
5 Conclusion 73
References 75

List of tables

Table 1: Composition of the *ad libitum* low-macronutrient diets. 25

Table 2: Composition of the caloric restricted high-macronutrient diets. 25

List of figures

Figure 1: Regulation of the metabolism of taurine and bile acids (BAs). 21

Figure 2: Experimental setup of the *ad libitum* low-macronutrient groups. 24

Figure 3: Experimental setup of the caloric restricted high-macronutrient groups. 25

Figure 4: Body (A, B), liver (C, D) and cecum (E, F) weight measured during dissection of mice fed *ad libitum* diets (A, C, E) and CR diets (B, D, F). 30

Figure 5: Small intestine and colon length measured during dissection of mice fed *ad libitum* diets (A, C) and CR diets (B, C). 31

Figure 6: Relative mRNA expression of antioxidative genes in the jejunum mucosa of mice fed *ad libitum* diets (A) and CR diets (B). 33

Figure 7: Relative mRNA expression of inflammatory genes in the jejunum mucosa of mice fed *ad libitum* diets (A) and CR diets (B). 35

Figure 8: Relative mRNA expression of metabolic genes in the jejunum mucosa of mice fed *ad libitum* diets (A) and CR diets (B). 37

Figure 9: Relative mRNA expression of genes connected to taurine and BAs metabolism in the jejunum mucosa of mice fed *ad libitum* diets (A) and CR diets (B). 39

Figure 10: Relative bacterial abundance in the cecum of mice fed *ad libitum* diets (A) and CR diets (B). 41

Figure 11: Relative mRNA expression of genes connected to taurine and BAs metabolism in the liver of mice fed *ad libitum* diets (A) and CR diets (B). 42

Figure 12: Levels of free taurine (A, D), taurine conjugate with m/z 249 (B, E), and glutathione (GSH) (C, F) in the ileum mucosa (A-C) and liver (D-F) of mice fed *ad libitum* diets. 44

Figure 13: Levels of free taurine (A, D), taurine conjugate with m/z 249 (B, E), and GSH (C, F) in the ileum mucosa (A-C) and liver (D-F) of mice fed CR diets. 45

Figure 14: Content of primary (A, B), secondary (C, D, E) and taurine-conjugated (F, G, H, I) BAs measured in the ileum mucosa of mice fed *ad libitum* diets. 47

Figure 15: Content of primary (A, B), secondary (C, D, E) and taurine-conjugated (F, G, H, I) BAs measured in the liver of mice fed *ad libitum* diets. 48

Figure 16: Content of primary (A, B), secondary (C, D, E) and taurine-conjugated (F, G, H, I) BAs measured in the ileum mucosa of mice fed CR diets. 50

Figure 17: Content of primary (A, B), secondary (C, D, E) and taurine-conjugated (F, G, H, I) BAs measured in the liver of mice fed CR diets. 51

Figure 18: Protein levels of IκB (A, B), pAKT (A, C), ATG7 (A, D) and TOMM20 (A, E) in the jejunum mucosa of mice fed *ad libitum* diets. 53

Figure 19: Protein levels of IκB (A, B), pAKT (A, C), ATG7 (A, D) and TOMM20 (A, E) in the jejunum mucosa of mice fed CR diets. 55

Figure 20: Villi length of the ileum (A) of mice fed *ad libitum* diets, quantified with ImageJ. Representative histology sections of the ileum (B, C, D, E) at 10x resolution, stained with hematoxylin-eosin solutions..... 57

Figure 21: Histology sections of the ileum (A, B, C, D) at 5x resolution, stained with hematoxylin-eosin solutions..... 58

Figure 22: Histology sections of the ileum (A, B, C, D) at 20x resolution, stained with hematoxylin-eosin solutions..... 59

Figure 23: Thickness of *muscularis externa* of the colon (A) of mice fed *ad libitum* diets, quantified with ImageJ. Representative histology sections of the colon (B, C, D, E) at 10x resolution, stained with hematoxylin-eosin solutions. 61

Figure 24: Histology sections of the colon (A, B, C, D) at 5x resolution, stained with hematoxylin-eosin solutions..... 62

Figure 25: Histology sections of the colon (A, B, C, D) at 20x resolution, stained with hematoxylin-eosin solutions..... 63

List of abbreviations

Acot4	Acyl-CoA thioesterase 4
Acox2	Acyl-CoA oxidase 2
Acsl3	Acyl-CoA synthetase long chain family member 3
Ad lib	*Ad libitum* control chow
Ad lib LC	*Ad libitum* low-carbohydrates
Ad lib LF	*Ad libitum* low-fat
Ad lib LP	*Ad libitum* low-protein
AMPK	AMP-activated protein kinase
AUC	Area under the curve
BA	Bile acid
BSA	Bovine serum albumin
ATG7	Autophagy related 7
Bal	Bile acids CoA-ligase
Bat	Bile acids CoA:amino acid N-acyltransferase
Bsep	Bile salt export pump
Cdo	Cysteine dioxygenase
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CR	Caloric restriction
CR HC	Caloric restriction high-carbohydrates
CR HF	Caloric restriction high-fat
CR HP	Caloric restriction high-protein
Cyp7a1	Cholesterol 7 α-hydroxylase
DCA	Deoxycholic acid
DTT	Dithiothretiol
Fiaf	Fasting-induced adipose factor
Fxr	Farnesoid X receptor
GIT	Gastrointestinal tract
GSH	Glutathione
GST	Glutathione S-transferases
Gsta3	Glutathione S-transferase α 3
Ibat	Ileal bile acid transporter
IBD	Irritable bowel disease
IκB	Inhibitor of nuclear factor kappa β
IL	Interleukin

Irf1	Interferon regulatory factor 1
LCA	Lithocholic acid
LCMS	Liquid chromatography-mass spectrometry
Mgst1	Microsomal glutathione S-transferase 1
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor kappa of activated B-cells
Ntcp	Na^+-taurocholate cotransporting polypeptide
Nos2	Nitric oxid synthase 2
Nrf2	Nuclear factor erythroid-derived 2-like 2
Osta	Organic solute transporter alpha
Ostb	Organic solute transporter beta
pAKT	Phosphorylated protein kinase B
PBS	Phosphate buffered saline
PPAR	Peroxisome proliferator-activated receptor
qRT-PCR	Real-time quantitative polymerase chain reaction
Reg3g	Regenerating family member 3 gamma
ROS	Reactive oxygen species
RT	Room temperature
Rsad2	Radical S-adenosyl methionine domain containing 2
Scd1	Stearoyl-coenzyme desaturase 1
SCFA	Short-chain fatty acids
SEM	Standard error of mean
Shp	Small heterodimer partner
Sirt1	Sirtuin 1
SPF	Specific-pathogen-free
Stat1	Signal transducer and activator of transcription 1
TauT	Taurine transporter
TBST	Tris-buffered saline with Tween20
TCA	Taurochloric acid
TDCA	Taurodeoxycholic acid
Tgr5	Takeda G-protein-coupled bile acid receptor 5
TLCA	Taurolithocholic acid
TNFa	Tumor necrosis factor alpha
Tlr3	Toll like receptor 3
TOMM20	Translocase of outer mitochondrial membrane 20
Trx2	Thioredoxin 2

TUDCA	Tauroursodeoxycholic acid
UDCA	Ursodeoxycholic acid
WB	Western blot

Abstract

The impact of caloric restriction (CR) on life- and health-span is largely known, but its influence on the gastrointestinal tract (GIT) remains undetermined. Macronutrient restriction has been investigated in terms of ageing, but to the best of the current knowledge, this is a first study to address its influence on the development of inflammation in the GIT. This project aims at defining a nutritional approach in the prevention of GIT diseases.

Low-macronutrient *ad libitum* diets were designed to identify if their restriction could induce a CR phenotype. Macronutrient increase during CR aims at recognizing if a certain macronutrient would neutralize the benefits of CR. The investigation included markers of antioxidation, inflammation, CR-related parameters in the intestine; microbiota; taurine and bile acids (BAs) metabolites in intestine and liver and gut morphology.

The *ad libitum* low-carbohydrates diet mimicked a CR diet in terms of glutathione regulation, longevity and fatty acid metabolism. It positively influenced taurine and BAs metabolism-related genes and partly increased BAs levels in the ileum and liver, but did not reduce inflammation markers in the gut or achieve a similar microbiota profile as CR in the cecum. The high-macronutrient CR diets could not neutralize the CR phenotype, but influenced BAs metabolism. The CR high-carbohydrates and CR high-fat diets reduced BAs content in ileum and liver, whereas the CR high-protein increased taurine-conjugated BAs.

This investigation presents an illustration of the impact of macronutrient restriction on the gut and sets the potential for further research to improve gut health.

Zusammenfassung

Die Wirkung von kalorischer Restriktion (CR) auf die Gesundheit und die Lebenserwartung ist weitgehend bekannt, aber deren Einfluss auf den Gastrointestinaltrakt (GIT) bleibt unbestimmt. Makronährstoffrestriktion wurde in Bezug auf den Alterungsprozess untersucht. Nach bestem Wissen, ist diese die erste Studie, die Makronährstoffrestriktion auf der Entwicklung von Entzündung im GIT untersucht. Mit dem Projekt wurde versucht, einen ernährungsphysiologischen Ansatz zur Prävention von Erkrankungen des GIT zu definieren.

Makronährstoffarme *ad libitum* Diäten wurden entwickelt, um zu identifizieren, ob Makronährstoffrestriktion einen CR-Phänotyp verursachen könnte. Eine Makronährstofferhöhung während CR zielt darauf ab zu erkennen, ob ein Makronährstoff die positive Wirkung von CR rückgängig machen könnte. Die Untersuchung berücksichtigt Antioxidations-, Entzündungs- und CR-bezogenen Markern im Darm; Mikrobiota; Taurin- und Gallensäuren (BAs)-Metabolite im Darm und in der Leber und Darmmorphologie.

Die *ad libitum* kohlenhydratarme Diät imitierte CR in Bezug auf Glutathionregulation, Lebensdauer und Fettsäurenmetabolismus. Sie hat die Taurin- und BAs-Metabolismus-bezogene Gene positiv beeinflusst und teilweise zu erhöhnten BAs-Niveaus im Ileum und in der Leber geführt, aber konnte nicht die Entzündungsmarkern im Darm reduzieren oder ein CR-änliches Mikrobiotaprofil im Blinddarm erzeugen. Die CR makronährstoffreiche Diäten waren nicht in der Lage einen CR-Phänotyp rückgängig zu machen, aber haben den BAs-Metabolismus beeinflusst. Die CR kohlenhydratreiche und CR fettreiche Diäten reduzierten den BAs-Spiegel, wohingegen die CR proteinreiche Diät die Taurin-konjugierte BAs in dem Ileum und in der Leber erhöhte.

Diese Untersuchung stellt die Auswirkungen einer Makronährstoffrestriktion auf den Darm dar und legt das Potenzial für weitere Forschung zur Verbesserung der Darmgesundheit fest.

1 Introduction

1.1 Caloric restriction

Caloric restriction (CR) is a dietary approach that limits energy intake without resulting in malnutrition. It is one of the main interventions for health promotion and weight management [2–4]. The lowering of calories has been connected to a decrease in inflammation and the prevalence of cancer and obesity, reduction of blood glucose, as well as an increase in insulin sensitivity [5]. The caloric intake limitation can vary between 10-50 % and the duration can be set at several weeks to a lifelong intervention [6]. The first experiments to show that reduction of food intake leads to prolongation of lifespan stem from the beginning of the 20th century and were done on rats [7, 8]. Ever since then, numerous studies have been conducted in many different animal species, which confirmed the life-extending, health-promoting and anti-ageing effect of CR [9–11]. An increased lifespan during CR has also been linked to a reduction in the development rate of age-associated and metabolic diseases, such as cardiovascular diseases, neurodegeneration, cancer, obesity and type 2 diabetes [4, 6, 12, 13]. Although it cannot entirely be confirmed if CR in humans can increase lifespan, several investigations have shown a positive effect on metabolic factors [12, 14].

The exact mechanism behind the beneficial impact of CR on the body is not known. However, it is assumed that the health benefits of CR come from metabolic adaptation, which leads to a decreased inflammatory state, reduction in metabolic rate and body temperature, as well as changes in fat oxidation and insulin sensitivity [3]. Moreover, the reduction of energy intake is probably responsible for the life-extending effect of CR [15]. The main factors discussed about the way that CR influences the body are dependent on nutrient and energy availability and include sirtuin 1 (Sirt1), mammalian target of rapamycin (mTOR), peroxisome proliferator-activated receptors (PPARs), AMP-activated protein kinase (AMPK) and insulin-signaling pathway. Correspondingly, an interaction with these mechanisms during CR leads to a reduction of oxidative stress and inflammation, as well as an improved autophagy and mitochondrial function [6, 16]. Autophagy and mitochondrial metabolism are some of the most important physiological processes in the cell and therefore participate in the regulation of longevity. A decline in their function contributes to inflammation and disruption of cellular homeostasis [17]. As previously published, during CR an increase in the levels of taurine and bile acids (BAs) have been observed, which could also contribute to the anti-inflammatory effects of CR [3, 16]. In addition, an interaction of these metabolites with the gut microbiota has been recognised, but the exact mechanism remains unknown [18].

Coming from this background, a few of the proposed benefits and biological pathways involved with CR, including antioxidation, inflammation, metabolism, as well as the emerging field of the

gut microbiome and the metabolism of taurine and BAs, will be discussed in detail in the following paragraphs.

1.1.1 Macronutrient restriction

To date, restriction of selected macronutrients or single amino acids has been primarily studied in the concept of ageing and life-span [19–22]. The effect that their reduction or exclusion would have on other processes in the body has not really been investigated. An older study indicated that a low-protein diet can lead to protein malnutrition, inflammation and oxidative stress in the liver. This was observed in decreased weight, an increase in plasma levels of inflammatory markers and a decrease in glutathione (GSH) content in the liver [23]. A further investigation of the same working group showed that only reducing calories is not enough to trigger an inflammatory response, suggesting a role of protein on inflammation [24]. However, the impact of macronutrient limitation on inflammation of the gastrointestinal tract (GIT) has not yet been investigated. To the best of the current knowledge, this is a first study to examine the influence of the restriction of each macronutrient on gut health in mice and compare it to CR.

1.1.2 Oxidative stress

Ageing is a process connected to an increased production of reactive oxygen species (ROS), which leads to oxidative damage in the body [6]. Accumulation of ROS could negatively impact molecules such as DNA, proteins and lipids, which, in reverse, makes oxidative stress the main cause of ageing processes and the diseases related to them [6, 15]. Some of the major positive effects of CR are the reduction of ROS levels and oxidative stress and, as a result, the slowing-down of ageing [25]. There are three main mechanisms proposed for these outcomes of CR: The first one implies that CR reduces metabolic rate which on its own has been connected to the generation of ROS that causes oxidative damage. The second one is the inactivation/detoxification of ROS and the third one proposed is the amelioration of ROS-caused damage through stimulation of repair mechanisms [4, 6]. One of the primary non-enzymatic factors to play a role in these mechanisms is reduced GSH, which is plentiful in the GIT. On the one hand, its production is controlled by the nuclear factor erythroid-derived 2-like 2 (Nrf2) which is also one of the main transcription factors activated in response to oxidative stress [3]. On the other hand, the glutathione transferases GSH S-transferase α 3 (Gsta3) and microsomal GSH S-transferase 1 (Mgst1) are responsible for the conjugation of its reduced state.

Another factor to consider when thinking about oxidative stress is mitochondria. Mitochondria's constant production of free radicals, oxygen and the related to it ROS makes them the main

player in ageing and studies about mitochondria led to the development of the mitochondrial hypothesis of ageing. In addition, defects in mitochondrial DNA and its function increase with age and have been proposed to cause inflammation. The reason for this phenomenon is that the dysfunctional mitochondria can still produce mitochondrial ROS and lead to their accumulation, which on their terms activate the nuclear factor kappa of activated B-cells (NF-κB) pathway that is responsible for the development of many inflammatory diseases [4, 6, 15, 26, 27]. There is also an increasing evidence connecting damaged mitochondria to intestinal diseases such as irritable bowel disease (IBD) and colorectal cancer, which is probably based on an interaction between the gut microbiota and the mitochondria. It has been indicated that the mitochondrium is the most sensible organelle to bacteria's signaling [28]. Investigations on mitochondria of caloric restricted animals have shown a reduced mitochondrial ROS production. This might be a consequence of reduced potential and better proton transport along the mitochondrial inner membrane, resulting in the reduction of ROS generation and a healthy mitochondria during CR [4].

Autophagy is another process to take into account when investigating mitochondria, since there is a proven interplay between the two. They are crucial in the regulation of longevity and therefore are tightly connected to CR. The main activators of autophagy are stress conditions such as food deprivation and oxidative stress [29]. Autophagy is a process that is responsible for the degradation of protein and faulty organelles, including mitochondria, through transport to the lysosomes where they are being destroyed. As follows, a disturbance in autophagy would lead to the accumulation of defective mitochondria [17]. Therefore, this destruction of damaged particles is an important mechanism for cell survival and homeostasis. The proper functionality of this process has been connected to the prevention of numerous illnesses like cancer and diabetes; cardiovascular, liver, neurodegenerative and autoimmune diseases [29]. Autophagy is also linked to the functionality of the GIT through sustaining a healthy gut barrier and mucosal immune function. Therefore it is considered to be a participant in the pathogenesis of IBD [30]. In addition, investigations on autophagy related 7 (ATG7) knockout mice in the intestine have indicated a disruption in Paneth cells and elevated expression of inflammatory factors [31].

To summarize, CR acts positively on oxidative stress to a large extent through the promotion of mitochondria's function and the stimulation of autophagy and can be used as a potential treatment of various diseases, including those of the GIT [17]. To further investigate the influence of CR and macronutrient restriction on antioxidation processes, NF-κB pathway, mitochondrial function and autophagy, this investigation looked into the expression of several genes connected to antioxidation (see Paragraph 3.1.1.1) and into the protein content of inhibitor of nuclear factor kappa β (IκB), ATG7 and translocase of outer mitochondrial membrane 20 (TOMM20) in the jejunum (see Paragraph 3.3.1.1).

1.1.3 Inflammation

The increase of inflammation during ageing is a condition also known as “inflammageing” and led to the development of the “Inflammation hypothesis of ageing” [6, 27, 32]. The connected to ageing upregulated inflammatory factors such as NF-κB, interleukin (IL)-ß, IL-6, tumor necrosis factor alpha (TNFa) and others can induce a pathological state and increase the risk of numerous diseases. The reason for this shift in favor of inflammatory cytokines production is very often, as already discussed in Paragraph 1.1.2, an elevated ROS production. Other criteria like genetics, as well as environmental factors like obesity, chronic immune infections, increased gut permeability and microbiota changes can also contribute to inflammageing [27]. The reduction in calories intake is not only one of the most successful interventions in increasing life-span in animal models (as already mentioned in Paragraph 1.1), but it also helps reduce levels of inflammatory cytokines and with this exerts a positive effect on inflammation in the body and the associated morbidities, including those in the gut [27, 32].

The prevalence of intestinal inflammatory diseases and dysfunctions is generally growing in the human population [2, 33]. Since the available information about the effects of nutritional compounds on a present intestinal disease is scarce, the main treatment used to this point is a mixture of drugs, which reduce inflammation but are not enough to provoke mucosal healing. However, the existing research on animal models points out, that dietary protein might play a very important role in the course of gastrointestinal diseases, depending on its quality and quantity [34]. For example, a high-protein diet was found to decrease cytokine’s gene expression in the ileum compared to a normal protein diet. Interestingly, the same diet did not cause significant changes in the expression in the colon, suggesting that the impact of dietary protein might be different depending on the organ [35]. Its consumption might also be able to influence microbiota’s activity, which, on the other hand, can be dependent on the type of protein digested. However, more studies are needed to define what contribution protein might have to the development and treatment of IBD [34]. Although the cause of the disease is still not quite clear, the proposed mechanism for the increasing prevalence of IBD is mostly a Western diet, which is high in fat and sugar and low in fruits and vegetables. This typical modern nutrition can negatively influence the gut’s immune system and cause changes in its barrier function, as well as its microbiome. In contrast, it has been implicated that certain restrictive diets can actually contribute to the improvement of inflammation and are the most common and effective way to treat disease. Considering the influence of CR on the GIT, studies have shown that it diminishes cell proliferation in the duodenum and rectum’s crypt cells, as well as that it influences the immunity of the gut and prevents reduction in nutrient absorption during ageing [2].

It has been previously observed that CR reduces the expression of inflammatory factors and increases the one of metabolic genes along the GIT. In detail, in the small intestine of caloric restricted mice, the expression of signal transducer and activator of transcription 1 (Stat1), toll like receptor 3 (Tlr3), interferon regulatory factor 1 (Irf1) and antimicrobial peptide regenerating family member 3 gamma (Reg3g) was statistically significantly downregulated with the strongest effect observed in the jejunum. Contrariwise, the acyl-CoA thioesterase 4 (Acot4), stearoyl-coenzyme A desaturase 1 (Scd1) and acyl-CoA oxidase 2 (Acox2) were upregulated compared to *ad libitum* mice (meaning animals are fed without restriction at all times) [16]. This is in line with previous observations where CR had an impact on fat oxidation, although the pathway was not entirely clear [36]. A more recent publication about other popular restrictive diets also illustrated similar effects as CR on inflammatory genes in the jejunum mucosa, as well as several other tissues [2]. However, there are still limited investigations concerning the impact of CR and macronutrient restriction on the GIT. The work here further investigated the effects of CR and how the specific macronutrients might influence the development of inflammation in the gut. The research was specifically focused on mRNA expression profile of inflammatory and metabolic genes in the jejunum mucosa (see Paragraphs 3.1.1.2 and 3.1.1.3).

The influence of nutrition on the histological anatomy of the intestine has been a point of interest in several investigations during the last few decades. Former studies have already proven the importance of diet on the morphology and physiology of the gut in different life stages [37]. The restriction of nutrients may exert its positive effects on the gut through an increase in the ratio of villi‘s length to crypth‘s depth. Villi are a significant part of the small intestine and are responsible for the absorption of nutrients. This elongation of the villi during CR can therefore result in a possible enhanced nutrient intake due to enlargement of the surface area [2, 38]. The large intestine, on the other hand, is the last part of the GIT and is important for the resorption of water and electrolytes, as well as forming and excretion of feces. Previous studies have indicated that the different structures of the colon change during food restriction. However, investigations have shown conflicting results concerning the development of the outer muscle layer of the colon during restriction [37, 39]. Considering this information, the presented experiment looks into how the decrease of either protein, fat or carbohydrates during an *ad libitum* diet might affect the length of the ileum’s villi, as well as the thickness of the *muscularis externa* of the colon (see Paragraph 3.4).

1.1.4 Microbiome

The GIT is responsible for macronutrient and micronutrient digestion and absorption. It also hosts around 100 trillion microorganisms including bacteria, fungi, viruses and protozoa called

the microbiota [40]. The GIT, together with food intake, plays a major role in the microbial shaping. Correspondingly, the gut microbiota and its metabolites influence the host‘s health, suggesting a strong symbiotic relationship [16, 41]. As follows, through the regulation of satiety and hunger hormones, as well as through the production of neurotransmitters and other signaling molecules, bacteria impact the host’s intestinal motility, absorption and functionality [1, 2, 42]. Bacteria are also responsible for the fermentation of starches and dietary fibers, which leads to the production of short-chain fatty acids (SCFA), that on their own contribute to the maintenance of the intestinal barrier [27]. In addition, the microbiota plays a major role in the metabolism of taurine and BAs, which will be further discussed in Paragraph 1.1.5. This relationship makes microbiota essential to the host‘s health and immune system. A disruption in the bacterial population is connected to numerous diseases like IBD, coeliac disease, immune cells imbalance, obesity, liver diseases, type 1 and 2 diabetes, neurological diseases and others [1, 43]. There is also a rising evidence that links changes in the microbiota and gut permeability with ageing [27].

The interest in microbiota research has risen drastically in the last few decades. The accumulated studies have shown that the microbiome is versatile and that it is different depending on the host. A numerous amount of aspects influences its development such as child delivery, age, gender, lifestyle, and host‘s immune system [18, 42]. A human study from the year 2018 also showed that environmental factors like diet, drugs and anthropometry are responsible for over 20 % of the inter-person microbiome variability [44]. Namely, the diet has a great capability of changing the gut microbiota. A mice study illustrated that food can be held responsible for 57 % of changes in the microbiome, whereas genetics for only 12 % [45]. This suggests that external factors play a bigger role than genetics and led to the rising of many investigations focusing on the influence of nutrition on microbiota [41]. Accordingly, CR has been proven to have a positive impact on microbiota‘s composition compared to an *ad libitum* feeding. As already discussed, CR has also been connected to healthy ageing, which probably results from the fact, that CR causes changes in the microbiome, decreases inflammation and enhances gut barrier function [27]. Observations on caloric restricted mice have shown an increase in bacteria correlated to life extension such as *Lactobacillus* and a decrease in negatively correlated ones like *Clostridium* [46]. Although not entirely understood, the microbiota vice versa participates in the influence of diet and therefore of CR on the body. It has been reported that germ-free mice do not become obese in comparison to mice with gut microbiota after being fed a Western-style high-fat-sugar-rich diet, suggesting that the microbiome plays a major role in obesity’s development [47]. In recent publications, it has also been discussed that gut bacteria take part in the reduction of body weight and that they mediate metabolism and hormonal changes typically observed during CR [6, 18]. These findings imply that the microbiome influences energy balance in both directions: it takes part in the gaining of

energy from food, as well as that it exerts power in energy expenditure. Interestingly, in a previous study from 2021, Gregor et al. observed that an antibiotic treatment of CR mice reduces or completely neutralizes the effects of CR, whereas microbiota transplant of CR to *ad libitum* fed mice only partly induced a typical CR phenotype. These results supported the hypothesis that gut bacteria are crucial during CR but that they are not the only important factor for achieving the beneficial effects of CR [18]. This information could be used for the development of different microbiota-dependent dietary strategies to improve energy balance and enhance host's health and with this that of the GIT. Reflecting on the proven major role of the microbiota in health, this work investigates the impact of macronutrient restriction and CR on bacteria composition in the cecum (see Paragraph 3.1.2).

1.1.5 Taurine and BAs

Taurine is one of the most concentrated free essential amino acids in the mammalian body. It is not used for protein synthesis, but it rather supports the development of the central nervous system and the retina and plays a role in calcium metabolism, membrane stabilization, reproduction and immunity. It is also known for its antioxidative and osmoprotective abilities [1, 48]. Namely, it upregulates the expression of thioredoxin (Trx) in the macrophages and inhibits ROS through Nrf2 modulation. It also supports the mitochondrial stability and function. Taurine works anti-inflammatory through action on the myeloid differentiation primary response 88 (MyD88)-NF-κB pathway [1]. It can also induce positive changes in the gut's microbiome composition and promote SCFA production [49]. The main source of taurine is dietary protein, however, it can be internally obtained in the liver (main synthesis site) from cysteine with the help of cysteine dioxygenase (Cdo) enzyme or through deconjugation of taurine of BAs by the gut microbiota. Taurine's mechanisms are concentration-dependant and are mainly regulated by the taurine transporter (TauT). After deconjugation from BAs, taurine creates several secondary conjugates, which roles are still unclear [3, 18, 48]. A previous study observed increased levels of taurine and its conjugates after CR along the different parts of the small intestine. In the liver this was not reproduced. However, there was an elevated expression of Cdo enzyme, indicating an increased internal synthesis [3].

Taurine interacts with GSH in different ways. On one hand, the two of them need cysteine to be synthesized, which may result in a competition for the substrate. On the other hand, both of them have antioxidative properties and stabilize mitochondria, and taurine has been found to assert these effects partly through acting on GSH [1]. Interestingly, former results indicated that during CR an increase in the building of GSH-taurine conjugates takes place, which supports taurine reuptake from the intestine [3]. In a following study, it was demonstrated that

this mechanism is microbiota-dependant [18]. This information confirmed and intensified the strong influence of the gut microbiome on the organism.

Taurine is tightly connected to BAs. After formation, BAs are being conjugated to either glycine or taurine to increase their solubility [1, 18]. Correspondingly, a CR-induced increase in taurine levels resulted in observed elevated content of taurine-conjugated BAs in the duodenum [16] and plasma [3] of mice. Interestingly, their levels were not that strongly influenced in the liver. However, the expression of genes connected to the synthesis and metabolism of BAs in the liver of the CR mice was elevated [3].

The primary pathway for the synthesis of BAs includes the enzyme cholesterol 7 α-hydroxylase (Cyp7a1) that converts cholesterol to 7α-hydrocholesterol which is then being converted to either chenodeoxycholic acid (CDCA) or cholic acid (CA) (both primary BAs). These are then modified by bile acids CoA:amino acid N-acyltransferase (Bat) and bile acids CoA-ligase (Bal) and bound to either taurine or glycine to create their conjugates [50]. After conjugation, BAs are transported with the help of the bile salt export protein (Bsep) from the hepatocytes into the bile and after ingestion, they are being secreted into the gut [1, 51]. After being released into the small intestine via the ileal bile acid transporter (Ibat), BAs are responsible for lipid digestion, as well as fat-soluble vitamins‘ absorption and cholesterol metabolism. After that ~95 % are being reabsorbed. They enter the blood with the help of organic solute transporters alpha and beta (Osta and Ostb) and are transported into the hepatic circulation by the Na^+-taurocholate cotransporting polypeptide (Ntcp). The little amount of BAs left in the gut is being modified by enteric bacteria. It is either converted into secondary BAs (lithocholic acid (LCA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA)) or deconjugated, which leads to increased levels of free taurine and unconjugated BAs [1, 3, 18, 51].

Different conjugation state and bacterial changes define the function of BAs in the body and contribute to their diversity. With that being said, BAs can have a versatile effect on microbiota and health. They can differ in their solubility, toxicity and ability to activate or inhibit BAs receptors [1]. The main receptors being induced by BAs are farnesoid X receptor (Fxr) and Takeda G protein-coupled receptor (Tgr5) which can regulate metabolic profiles [51–53]. The first one raises the expression of the small heterodimer partner (Shp), which on its own inhibits the transcription of the Cyp7a1 enzyme and therefore reduces BAs’ synthesis [51]. It has also been associated with the promotion of diet-induced obesity through interaction with the microbiota [42, 52]. The activation of the ladder has been linked to an anti-inflammatory and a metabolic role through the regulation of NF-κB and protein kinase B (Akt) pathways [51, 54]. Accordingly, problems with BAs’ metabolism can lead to diseases such as cholestasis, gallstones, inflammation, lipid malabsorption, bacterial infections in the gut and others [51]. Figure 1 represents an overview of the metabolism of taurine and BAs and the included factors.

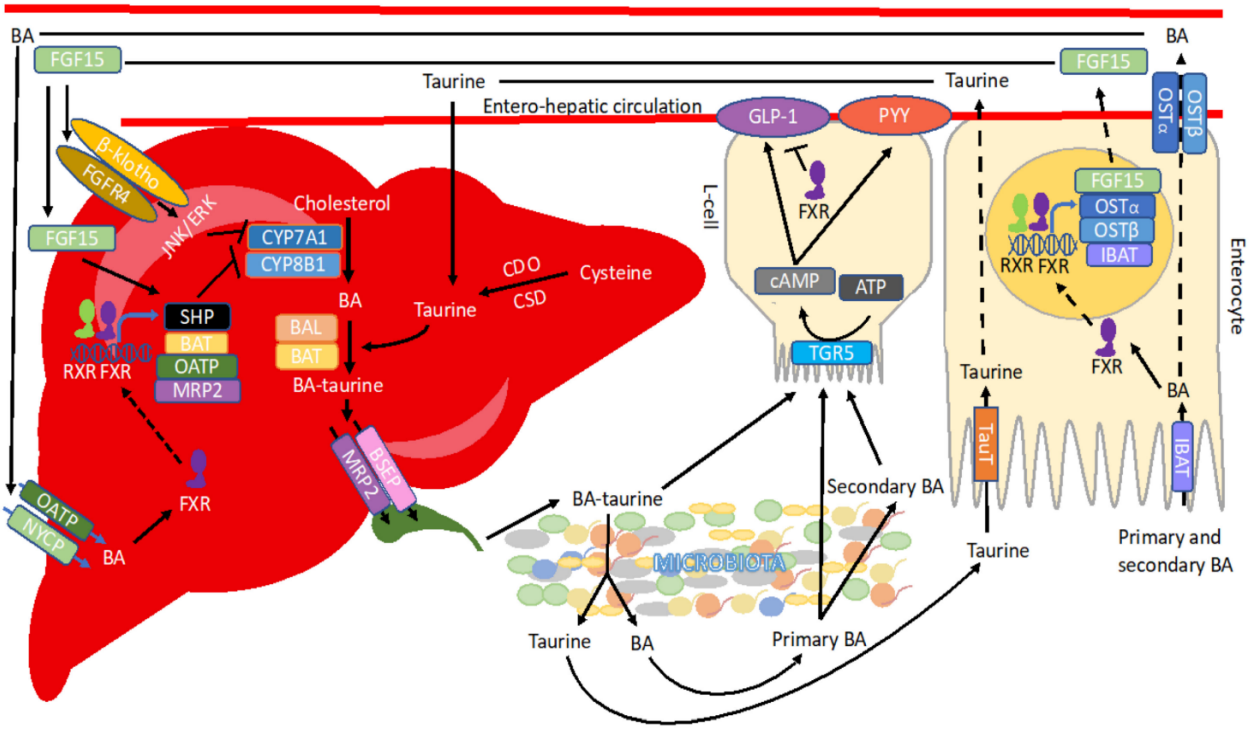


Figure 1: Regulation of the metabolism of taurine and bile acids (BAs). Source: [1]

As already mentioned above, intestinal microbiota and BAs are tightly connected. Interestingly, BAs (especially secondary BAs) have an antimicrobial property through their ability to decompose bacterial membranes. As follows, they exert a major function in the gut metabolism and microbiota's shaping and serve as protection against pathogens. The amount of BAs in the body is strongly influenced by the diet. For example, a high-fat diet, which was previously found to raise BAs' production and induce obesity, can therefore influence microbiota and lead to inflammation. However, a reduction in BAs' levels has also been linked to bacterial imbalance and inflamed mucosa [1].

Microbial impact on BAs is also as equally important [1, 50]. Gut bacteria are primarily responsible for the deconjugation of BAs of taurine and glycine, as well as their conversion from primary to secondary BAs. The microbiome can also transform secondary BAs DCA and LCA back to CA and CDCA. Gut bacteria can influence Fxr and Tgr5 expression, since depending on their conjugation state, BAs can either be agonists or antagonists of the named receptors [1]. Host's microbiota also impacts the expression of genes connected to BAs' synthesis and metabolism [42]. These microbial modifications of BAs not only result in a diversity in BAs' pool but can also have an impact on the host's health. Namely, a reduction in deconjugation has been linked to gut diseases like ulcerative colitis, Crohn's disease and irritable bowel syndrome [55]. A study showed that LCA and UDCA, which are a result of

bacterial modifications of primary BAs, can protect against colonic inflammation in mice [56]. A better intestinal barrier function and blocking of bacterial invasion has been observed to be induced by UDCA. However, a long-term high dosage of UDCA can increase the risk of cancer in colitis patients [1]. The conjugation of BAs generally makes them less toxic than unconjugated BAs, which results in a few positive effects. Namely, tauroursodeoxycholic acid (TUDCA) is more effective in the treatment of liver cirrhosis than the unconjugated UDCA [57]. It also positively influences metabolic problems and inflammation, as well as the functionality of the intestine and its barrier function during a high-fat diet [58]. Moreover, further research on TUDCA has proven its protective function on the central nervous system and the related diseases. These observations only amplify the importance and benefits of taurine supplementation since it can contribute to an increase in the pool of taurine-conjugated BAs [1, 59]. A few epidemiological studies in animals and humans also recognized LCA and DCA as possible inducers of cancer. DCA has also been linked to cholesterol gallstone disease and imbalance in the gut [1, 50]. With that being said, BAs have been a centre of numerous investigations for a few centuries now. However, there is still a lot of room left for research concerning the exact effects of BAs on general health, as well as their impact on the GIT. Their interactions with taurine and microbiota and their role during CR still remain to a large extent undetermined.

1.2 Objectives

Numerous investigations have proven that CR has a strong influence in promoting health and increasing life-span. One of the most important parts of our body is the GIT since it is essential for digestion, absorption of micronutrients and microbiota shaping. As already described, Duszka et al. have previously reported a reduction in inflammatory factors along the GIT with an increased expression of metabolic genes in mice after CR [16]. However, the results concerning the exact impact of CR and its mechanisms on the GIT are contradictory and still need to be further investigated.

The reduction of calories impacts the quality of life and can be hard to maintain long-term, making it often an unsuitable therapy choice for patients with GIT problems. Moreover, CR leads to a total decrease in the intake of all nutrients. This makes it difficult to define if CR or a certain macronutrient influences the host's health. A few studies have explored the impact of limiting macronutrients on life-span, but the investigations on other outcomes of macronutrient restriction are scarce. Accordingly, finding an alternative diet which mimics the health-promoting and life-extending benefits of CR, but without a reduction in energy intake, could lead to significant improvement in the way diseases are cured, especially those of the GIT. For this reason, the current experiment was designed to compare chosen restrictive dietary protocols (reduction in either protein/fat/carbohydrates) during an *ad libitum* diet to

identify if the lowering of a single macronutrient could play a role in mimicking the effects of CR. In addition, an increase of the previously mentioned macronutrients was applied during CR. The aim is to find out if a general decrease in energy intake is the deciding factor for positive changes in the gut or if the increase in one of the macronutrients could negatively influence the pathways during a CR diet. The described experiment was conducted on healthy animals and its goal was to identify which of the designed diets has the strongest effect on maintaining a healthy intestinal function and can prevent the development of diseases of the GIT. The project presented in this thesis introduces a deeper look into the molecular impact of macronutrient restriction and CR on different factors in the GIT and liver. The expression of genes connected to antioxidation, inflammation and metabolism in the intestine was measured. The investigation further looked into the influence of the proposed diets on microbiota, markers of autophagy and mitochondrial function, as well as on histology of the small intestine and colon. It also puts focus on the impact of the different restrictions on taurine and BAs' production and metabolism not only in the gut but also in the liver with the goal to develop a better view of the connection between the three and to understand the impact of intestinal microbiota on this relationship.

The objective of the designed investigation is to examine the molecular impact of macronutrient restriction and CR on the immune system of the gut. The study aims at finding out if and what macronutrient plays a major role in the typical pathways connected to CR. On the one hand, by decreasing the chosen macronutrients during an *ad libitum* feeding, a possible CR-mimic diet without the inconveniences of energy reduction could be recognised. An increase in the amount of the selected macronutrients during CR, on the other hand, would allow to assess if any of the macronutrients would neutralize the effects of CR. The current project aims at identifying a possible nutritional, drug-free approach in the prevention of diseases of the GIT.

2 Materials and methods

2.1 Animal experiments

For the experiments C57BL/6 male 4-weeks-old mice were purchased from Janvier Inc. Labs (Le Genest, France). The animals were housed in standard specific-pathogen-free (SPF) conditions, using Tecniplast open cages, under a 12h light/12h dark cycle, in the animal facility of the Department of Nutritional Sciences and Division of Clinical Pharmacy and Diagnostics, University of Vienna for 6 weeks before the interventions to adapt to the environment. At 10 weeks of age, the mice were randomly divided into experimental groups of eight animals with one *ad libitum* control group (Ad lib) and three *ad libitum* groups low in the selected macronutrients (low-protein (Ad lib LP), low-fat (Ad lib LF), low-carbohydrates (Ad lib LC)), as well as one CR control group and three CR groups high in the selected macronutrients (high-protein (HP), high-fat (HF), high-carbohydrates (CR HC)) (see Figure 2 and Figure 3). The used diets were custom-made, produced by SSNIFF Spezialdiäten GmbH (Germany). All of the diets were given for a total of 4 weeks. The CR groups received 80 % of their regular food intake and were fed once a day. In the high-macronutrient diets, the selected macronutrient is increased so that the 20 % restriction does not lead to a restriction of the given macronutrient. The composition of the experimental diets is presented in Table 1 and Table 2. The animals were sacrificed at 14 weeks and the collected tissues were snap frozen in liquid nitrogen and stored at -80 °C.

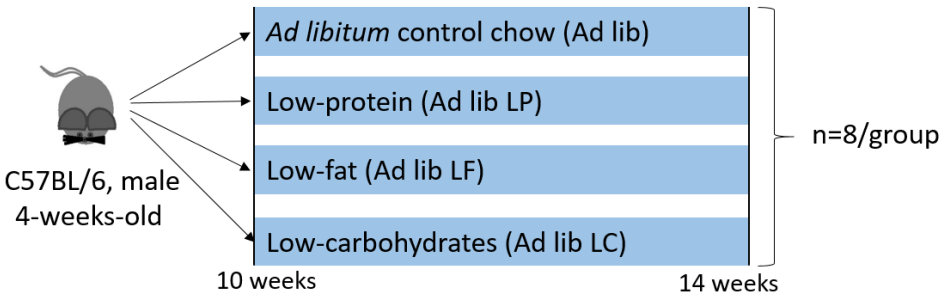


Figure 2: Experimental setup of the *ad libitum* low-macronutrient groups.

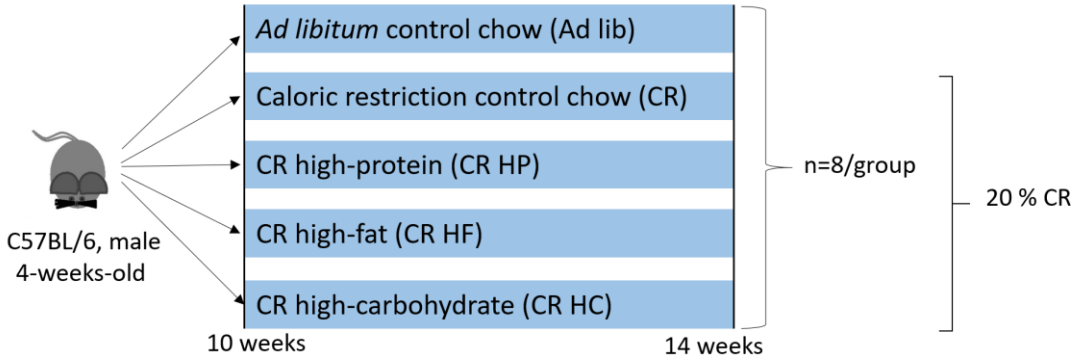


Figure 3: Experimental setup of the caloric restricted high-macronutrient groups.

Table 1: Composition of the *ad libitum* low-macronutrient diets.

Diet / Ingredients and energy	Ad lib	Ad lib LP	Ad lib LF	Ad lib LC
Carbohydrates (gm%)	65	79	74	**36**
Protein (gm%)	20	**5**	25	44
Fat (gm%)	7	7	**35**	11
Fibre (gm%)	5	5	5	5
Vitamins and minerals (gm%)	1	1	1	1
Choline	3	3	3	3
Total energy (kcal/g)	4	4.0	3.7	4.2
% energy from carbohydrates	65	80	74	**35**
% energy from protein	20	**5**	25	42
% energy from fat	15	15	**1**	23

Table 2: Composition of the caloric restricted high-macronutrient diets.

Diet / Ingredients and energy	CR	CR HP	CR HF	CR HC
Carbohydrates (gm%)	65	29	40	**85**
Protein (gm%)	20	**50**	13	5
Fat (gm%)	7	4	**35**	2
Fiber (gm%)	5	5	5	5
Vitamins and minerals (gm%)	1	1	1	1
Choline	3	3	3	3
Total energy (kcal/g)	4	3.9	5.3	3.8
% energy from carbohydrates	65	30	30	**90**
% energy from protein	20	**60**	10	5
% energy from fat	15	10	**60**	5

2.2 Sample preparation

For the mentioned experiments below (Paragraphs 2.2-2.4), the desired amount of tissue was cut on dry ice with a scalpel. After that, it was further processed according to the different methods protocol.

2.2 Real-time quantitative polymerase chain reaction (qRT-PCR)

Intestinal jejunum scrapings (≤15 mg) were dissolved in lysis buffer using a syringe and a needle. Liver samples (≤15 mg) were transferred into Precellys homogenizing tubes with 1.4 mm ceramic beads and disrupted using Precellys®24 Tissue Homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France). RNA from both tissues was extracted using the PeqGOLD Total RNA Kit (VWR International GmbH, Germany) following the manufacturer's protocol. SuperScript® II Reverse Transcriptase (Quanta Biosciences, MA, USA) was used for cDNA synthesis. The used steps for reverse transcription were: 22 ºC for 5 min; 42 ºC for 30 min; 85 ºC for 5 min. The cDNA samples were then stored at -20 ºC until further use.
For the qRT-PCR measrements, 8 µl cDNA sample and 2 µl SYBR Green PCR Master Mix were pipetted on ice on a qPCR plate in biological triplicates for each sample. The qRT-PCR measurement was conducted using the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Life Technologies, CA, USA). The results are presented as averaged ΔΔCt values normalized to Eef1a1.
Bacterial DNA was extracted from cecum samples (~30 mg) according to manufacturer's protocol using the InnuPREP Stool DNA kit (Analytik Jena, Germany). The qRT-PCR measurements were done in the same way as described above. The relative bacterial DNA content was calculated using 16S RNA Ct value as a reference.

2.3 Liquid Chromatography-Mass Spectrometry (LCMS)

The ileum (7-10 mg) and liver (10-20 mg) tissues were put into Precellys homogenizing tubes with 1.4 mm ceramic beads and 9x methanol (kept at at -20 ºC) of the weight of the sample was added. The tissues were homogenized twice at Precellys®24 Tissue Homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) for 30 seconds at 4000 rpm. The samples were transferred to 1.5 ml Eppendorf tubes and centrifuged for 5 min at 3500 xg, 4 ºC. The supenatant was collected into new 1.5 ml Eppendorf tubes, cetrifuged again at 15000 xg, 4 ºC for 5 min and transferred into HPLC vials (2 ml, Sigma-Aldrich Chemie GmbH). The analysis was done using a LCMS-8040 Liquid Chromatograph Mass Spectrometer (Shimadzu Corporation, Kyoto, Japan). The prepared samples were first measured for taurine and GSH content in a negative modus, afterwards the same were analysed for the content of BAs in a positive modus using an Atlantis T3 3 µm column (2.1×150 mm, Waters, Milford, MA, USA)

with a set temperature at 30 ºC. The mobile phase A consisted of 0.1 % formic acid and 20 mM ammonium acetate solved in water. The mobile phase B contained 0.1 % formic acid and acetonitrile:methanol (3:1) in water. Standard dilution series were prepared and included in the measurement for all of the targeted metabolites (for taurine, taurine-conjugates, GSH a total of 9 standards and for BAs a total of 11 standards was measured). The observed peaks of the standards and samples were the measured retention times and presented as the area under the curve (AUC) by the LabSolutions Software (Shimadzu Corporation, Kyoto, Japan). These were integrated and the values of the samples were further analysed with Microsoft Excel® according to the standard measurements using the y = mx + b equation.

2.4 Western blot (WB)

Jejunum mucosal tissue (7-10 mg) was dissolved using a syringe in RIPA buffer (10x, EMD Millipore Billerica, MA, USA) with Pierce Phosphatase Inhibitor (Mini Tablets, Thermo Scientific, Rockford, IL, SA) and Protease Inhibitor Cocktail (cOmplete Mini, EDTA-free, Roche, Mannheim, Germany). For protein extraction the samples were incubated for 15 min on ice, centrifuged for 10 min at 10.000 xg, 4 ºC and then the supernatant was collected. The protein concentration in the samples was determined using the Pierce™ BCA protein assay kit (Thermo Scientific). The used protein concentration was 1 µg/µl for β-Actin and 3 µg/µl for IκB, phosphorylated protein kinase B (pAKT), ATG7 and TOMM20. The SDS-PAGE gels consisted of 10 % separating gel and 5 % stacking gel and were prepared one day prior to electrophoresis. For each protein a new set of gels was made. For protein separation 10 µl sample mixture (sample + RIPA-buffer + loading buffer + dithiothretiol (DTT)) was loaded into the gel, as well as 2 µl of Precision Plus Protein ladder (Bio Rad, Carlsbad, CA, USA) on each side of the gel and served as a molecular weight standard. The electrophoresis was run for 1.5-2h (depending on targeted protein) at 90 V in a cold room. Subsequently, the samples were blotted onto PVDF membranes using the Trans-Blot Turbo Transfer System (Bio Rad). The membranes were blocked in bovine serum albumin (BSA) for 1h at room temperature (RT) and afterwards incubated with primary antibody at 4 ºC overnight (β-Actin: 1:1000 in 2.5 % BSA tris-buffered saline with Tween20 (TBST), IκB: 1:1000 in 5 % BSA TBST, pAKT: 1:1000 in 5 % BSA TBST, ATG7: 1:1000 in 5 % BSA TBST and TOMM20: 1:5000 in 5 % BSA TBST) on a shaker. After 3 washing steps in TBST, the membranes were further incubated with secondary antibody (anti-rabbit IgG, 1:3000) for 1h at RT. The membranes were afterwards washed again 3x in TBST. Subsequently, they were covered with Pierce ECL Western Blotting Substrate in dilution 1:1 (1 ml of total volume used on one whole membrane) in preparation for the development. The detection of the proteins was conducted using the ChemiDoc XRS+ System (Bio Rad) and analysed with the ImageJ software (version 1.53) using β-Actin as a reference.

2.5 Histology

2.5.1 Sample preparation

During dissection, ileum and colon tissues were fixed in 4 % phosphate buffer saline (PBS)-buffered formalin for 48 h. Following steps: washing for 15 min under running tap water, ≥ 1h 75 % ethanol, ≥ 1h 85 % ethanol and 95 % ethanol + 5 % methanol overnight. After that the samples were embedded in paraffin and let to harden overnight. Next, they were cut into 5 µm thick tissue sections using a microtome and placed on a microscope slide. The samples were left to dry for 72h at 37 ºC.

2.5.2 Hematoxylin & eosin staining

In preparation for staining, the sections were dewaxed with xylene, dehydrated using decreasing concentration of ethanol and rinsed in double-distilled water in 10 min steps. The tissue sections were then stained for 1 min in hematoxylin, shortly rinsed in tap water, dehydrated with ethanol for 8 min and counterstained in eosin for 2 seconds. In the end, the samples were dehydrated in ethanol (10-20 sec) and cleaned with xylene for 10 min. A cover glass was fixed with a drop of Entellan and the samples left to dry for 24h. All reagents used were purchased from Sigma Aldrich.

2.5.3 Imaging

Axiolab 5 microscope, Axiocam ERc 5s camera and ZEN Microscopy Software (ZEISS, Oberkochen, Germany) were used for the capturing of the images. Only the *ad libitum* groups were included in the histology examination. For each structure 5 images/sample were captured, adding up to 40 images/structure for each diet group. The length of the ileum villi and the thickness of the colon wall were measured using the ImageJ software (version 1.53) and the mean was calculated. The villi length was measured as the distance between the end of the crypt and the tip of the villi. The thickness of the colon wall (*muscularis externa*) was defined as the outer layer observed in the colon sections.

2.6 Statistics

Results are presented as mean ± standard error of mean (SEM). One-way ANOVA and post-hoc analysis were used for the evaluation of the statistical significances. Differences between two experimental groups were assessed using Excel Microsoft ® and Student's t-test. Each of the groups included 7-8 biological replicates. A p-value ≤ 0.05 was considered statistically significant and a p-value ≤ 0.01 was considered highly statistically significant.

3 Results

To assess the impact of macronutrient restriction on the GIT during an *ad libitum* and a CR diet, male 10-weeks-old C57BL/6 mice were randomly submitted to two types of dietary interventions: *ad libitum* diets, restricted in selected macronutrients and 20 % CR diets, high in the selected macronutrients for 4 weeks. A statistically significant reduction in body and liver weight was observed in the CR group compared to Ad lib. The weight of the cecum was not different between Ad lib and CR but was statistically significantly reduced after an Ad lib LF and Ad lib LC diet. The length of the small intestine, as well as that of the colon did not change significantly after the interventions. The only exception was the CR HF group which had statistically significantly longer small intestine compared to CR (see Figure 4 and Figure 5).

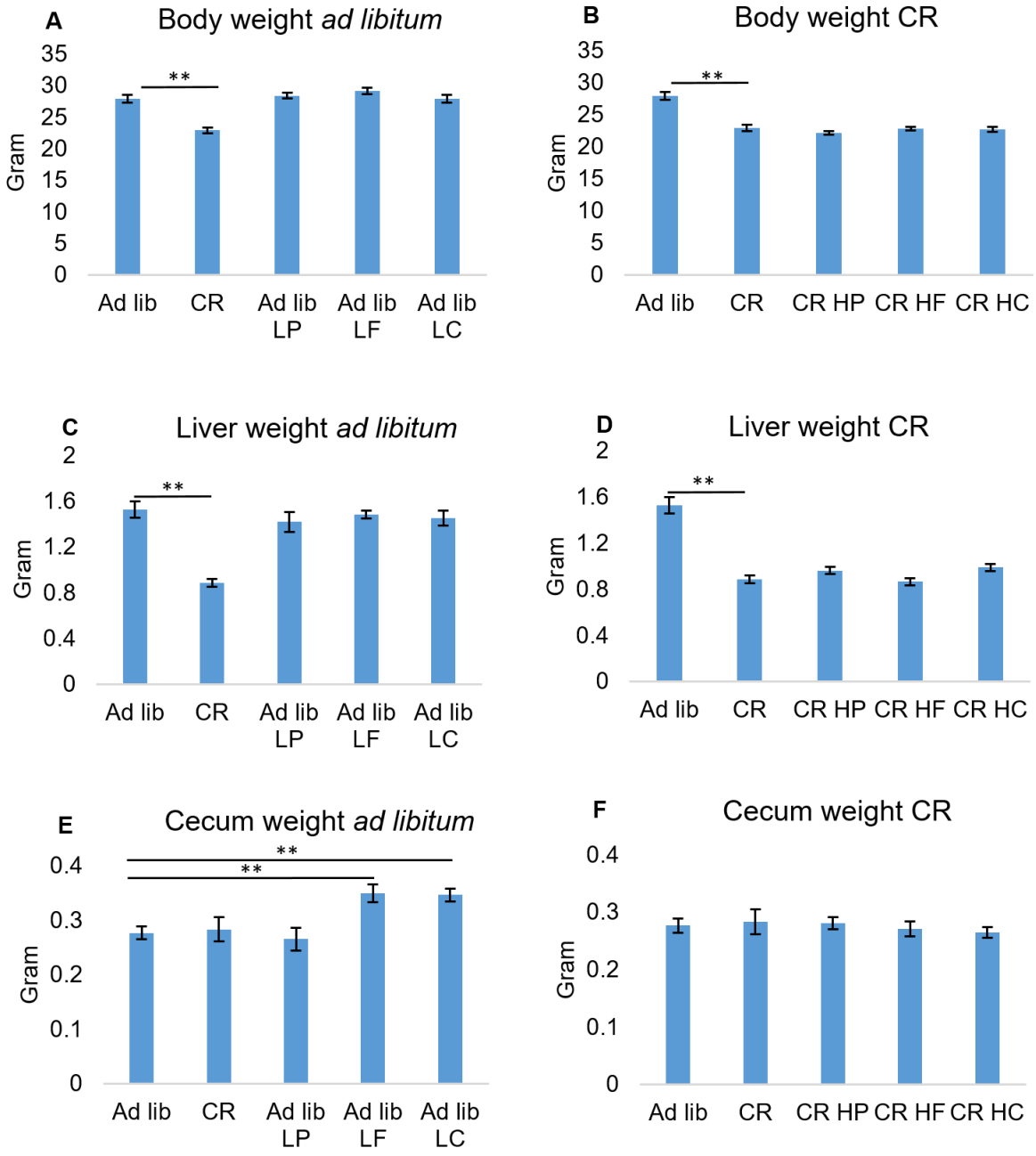


Figure 4: Body (A, B), liver (C, D) and cecum (E, F) weight measured during dissection of mice fed *ad libitum* diets (A, C, E) and CR diets (B, D, F).
One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM. *Ad libitium* control chow (Ad lib), Low-Protein (LP), Low-Fat (LF), Low-Carbohydrates (LC), Caloric Restriction (CR), High-Protein (HP), High-Fat (HF), High-Carbohydrates (HC).

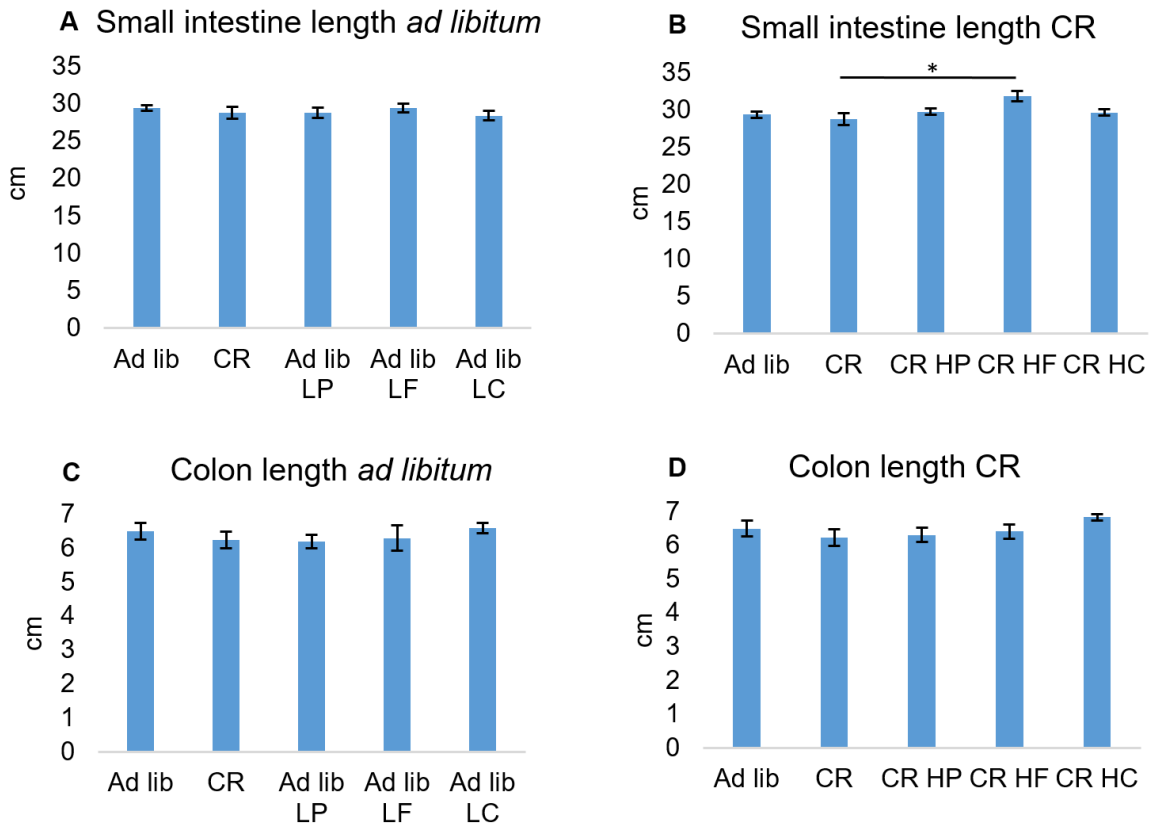


Figure 5: Small intestine and colon length measured during dissection of mice fed *ad libitum* diets (A, C) and CR diets (B, C).
One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM.

3.1 qRT-PCR

3.1.1 Jejunum

3.1.1.1 Antioxidative processes

Since one of the main impacts of CR is reduction of oxidative stress (see Paragraph 1.1.2), the expression of genes connected with antioxidative processes was measured in the jejunum mucosa of the mice. There was a decreased expression of thioredoxin 2 (Trx2), catalase, nitric oxid synthase 2 (Nos2) and Nrf2 in the gut of the CR mice compared to Ad lib with a statistical significance observed only for Trx2. On the other hand, the GSH transferases Gsta3 and Mgst1 were statistically significantly upregulated in the CR group compared to Ad lib, which coincides well with the previously observed increased glutathione S-transferases (GST) activity measured during CR [3]. The Ad lib LC diet led to the highest measurements of the antioxidative enzymes Trx2 and Catalase and a reduced gene expression of Nrf2. However, compared to the Ad lib group, it increased Gsta3 and Mgst1 expression, even with a statistical difference detected for Gsta3, which was the same effect observed in the CR group (see Figure 6 A).

None of the high-macronutrient diets was able to erase the antioxidative effect of CR. The Nrf2 gene was even statistically significantly downregulated in all of the high-macronutrient groups, when compared to CR. In the CR HP, the effect of CR on the increased expression of the GST enzymes seemed to be strengthened, since the Gsta3 gene even had a statistically significantly higher expression compared to CR. An increase of carbohydrates during CR led to a statistically significantly lower expression of Catalase and, as already mentioned, of Nrf2. Out of the CR groups, the CR HC caused the strongest downregulation of Gsta3 and Mgst1, although the difference was not statistically significant (see Figure 6 B).

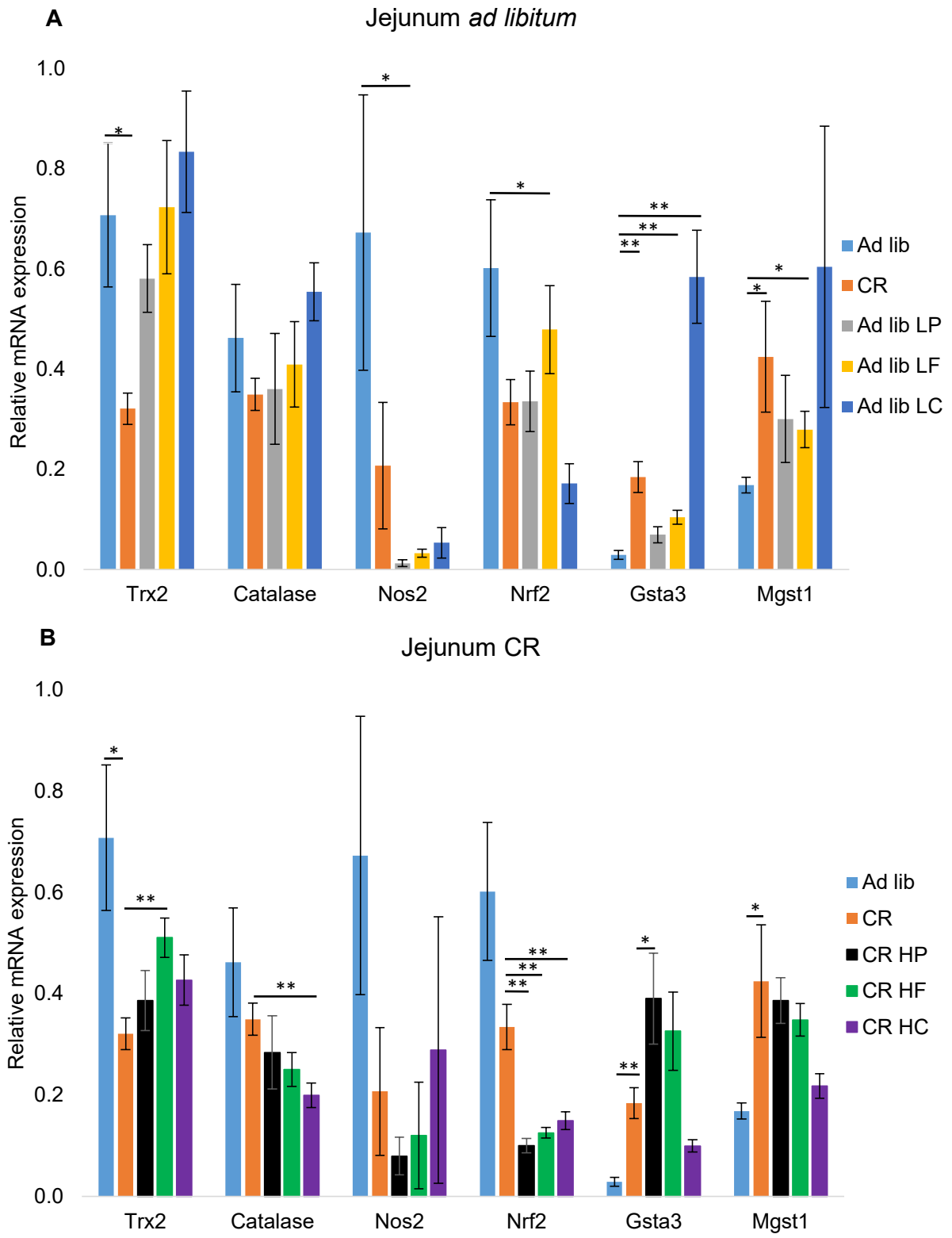
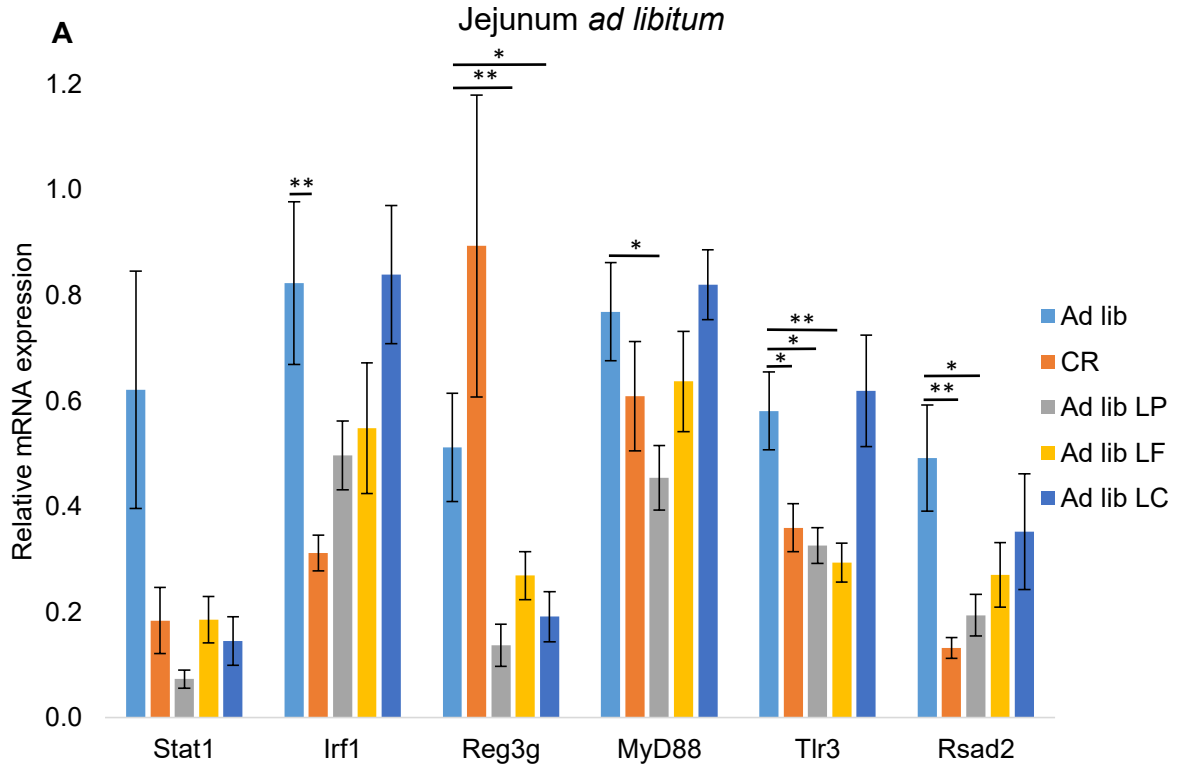


Figure 6: Relative mRNA expression of antioxidative genes in the jejunum mucosa of mice fed *ad libitum* diets (A) and CR diets (B).
One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM. Thioredoxin 2 (Trx2), Nitric oxid synthase 2 (Nos2), Nuclear factor erythroid-derived 2-like 2 (Nrf2), Glutathione S-transferase α 3 (Gsta3), Microsomal glutathione S-transferase 1 (Mgst1).

3.1.1.2 Inflammation

As already described, previous investigation on CR mice have shown a decrease in inflammatory factors along the GIT [16]. This research further investigates inflammation in the jejunum mucosa in response to macronutrient restriction and CR. All of the measured inflammatory genes were downregulated in the CR group compared to Ad lib. The Irf1, Tlr3 and radical S-adenosyl methionine domain containing 2 (Rsad2) showed a statistically significant difference. The only exception was the antimicrobial peptide Reg3g, which was highly upregulated after a standard CR diet, but this measurement was with a big SEM and no statistical difference was found between the two control groups. Out of the low-macronutrient groups, the Ad lib LP had the lowest expression for the measured inflammatory genes with Reg3g, MyD88, Tlr3 and Rsad2 making a statistically significant difference compared to control. The Ad lib LC, on the other hand, was with a slightly higher expression than Ad lib for Irf1, MyD88 and Tl3, but this difference was not statistically significant (see Figure 7 A).
Generally, the high-macronutrient CR diets did not have a strong impact on the measured inflammatory parameters. However, after administrating a CR HC diet, a slight upregulation of Stat1, Irf1 and Rsad2 was observed, but the differences were not statistically significant compared to control (see Figure 7 B).



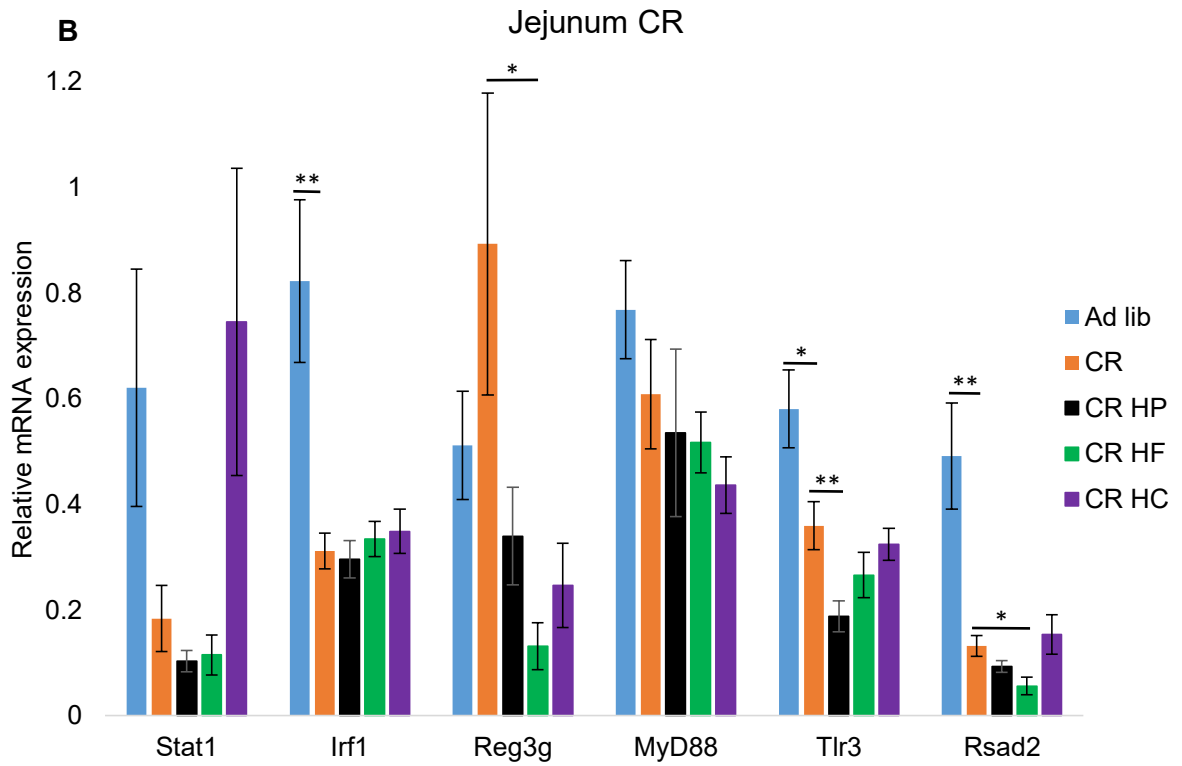
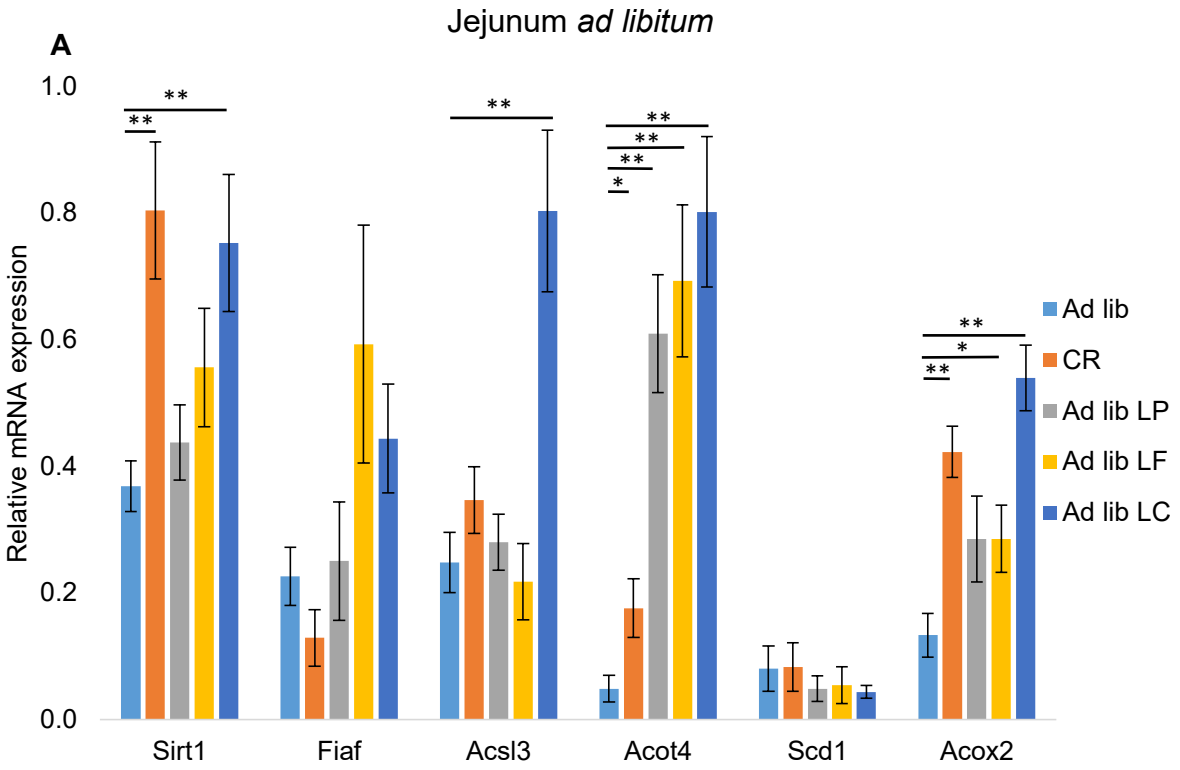


Figure 7: Relative mRNA expression of inflammatory genes in the jejunum mucosa of mice fed *ad libitum* diets (A) and CR diets (B).
One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM. Signal transducer and activator of transcription 1 (Stat1), Interferon regulatory factor 1 (Irf1), Regenerating family member 3 gamma (Reg3g), Myeloid differentiation primary response 88 (MyD88), Toll like receptor 3 (Tlr3), Radical S-adenosyl methionine domain containing 2 (Rsad2).

3.1.1.3 Metabolism

Sirtuins are a landmark of CR and a mediator of longevity [60]. The gene expression of Sirt1 in the jejunum mucosa of CR mice was highly statistically significantly upregulated compared to Ad lib ($p \leq 0.01$). The Ad lib LC showed a similar effect as CR on this parameter. As already mentioned, it has been previously shown that CR leads to an increase in metabolic genes in different parts of the GIT [16]. This investigation further studies how the different restrictions would influence genes linked to lipid metabolism in the jejunum mucosa. In the CR group there was an increased expression of acyl-CoA synthetase long chain family member 3 (Acsl3), Acot4, Scd1 and Acox2 with a statistical difference for Acot4 and Acox2 compared to Ad lib. Out of the *ad libitum* diets, the Ad lib LC led to the highest measurement for Acsl3, Acot4 and Acox2. These genes were statistically significantly upregulated in the Ad lib LC in comparison to Ad lib (see Figure 8 A).

The high-macronutrient CR diets sustained the positive effect of CR on the Sirt1 gene expression. The CR HF showed the highest expression of Acsl3, Acot4 and Scd1 with the last two genes being statistically significantly different compared to CR (see Figure 8 B).



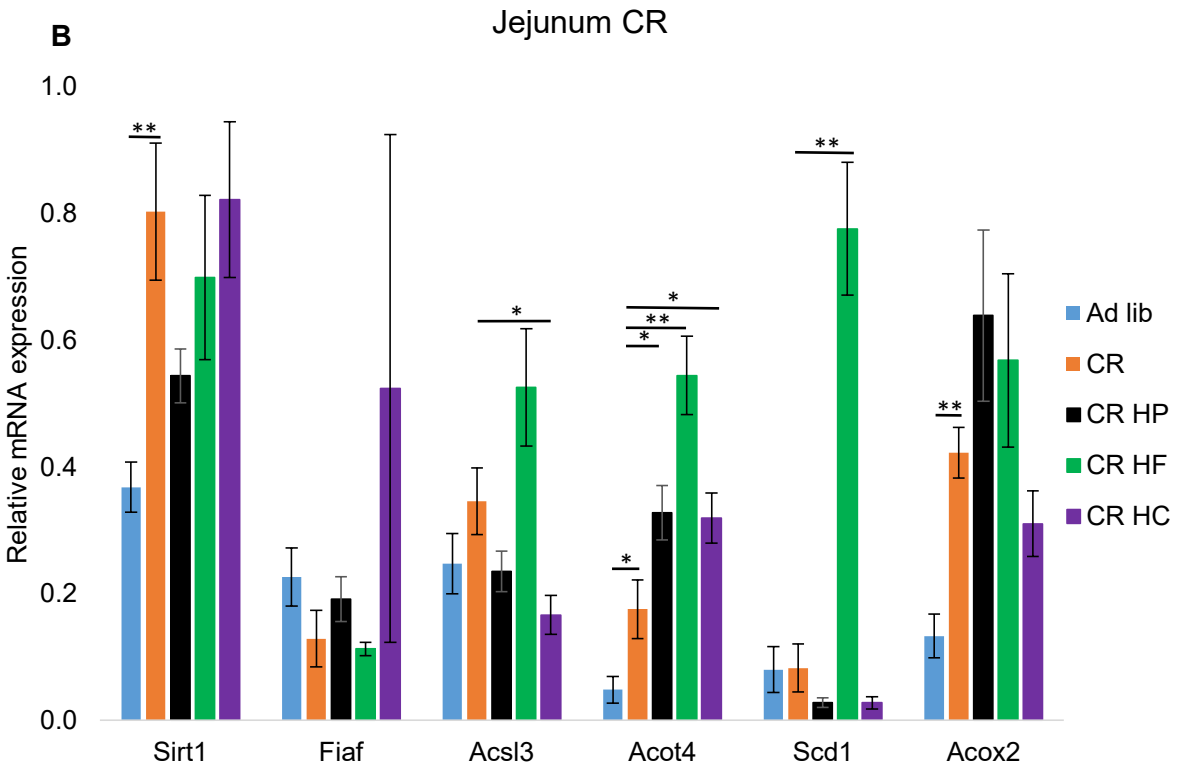
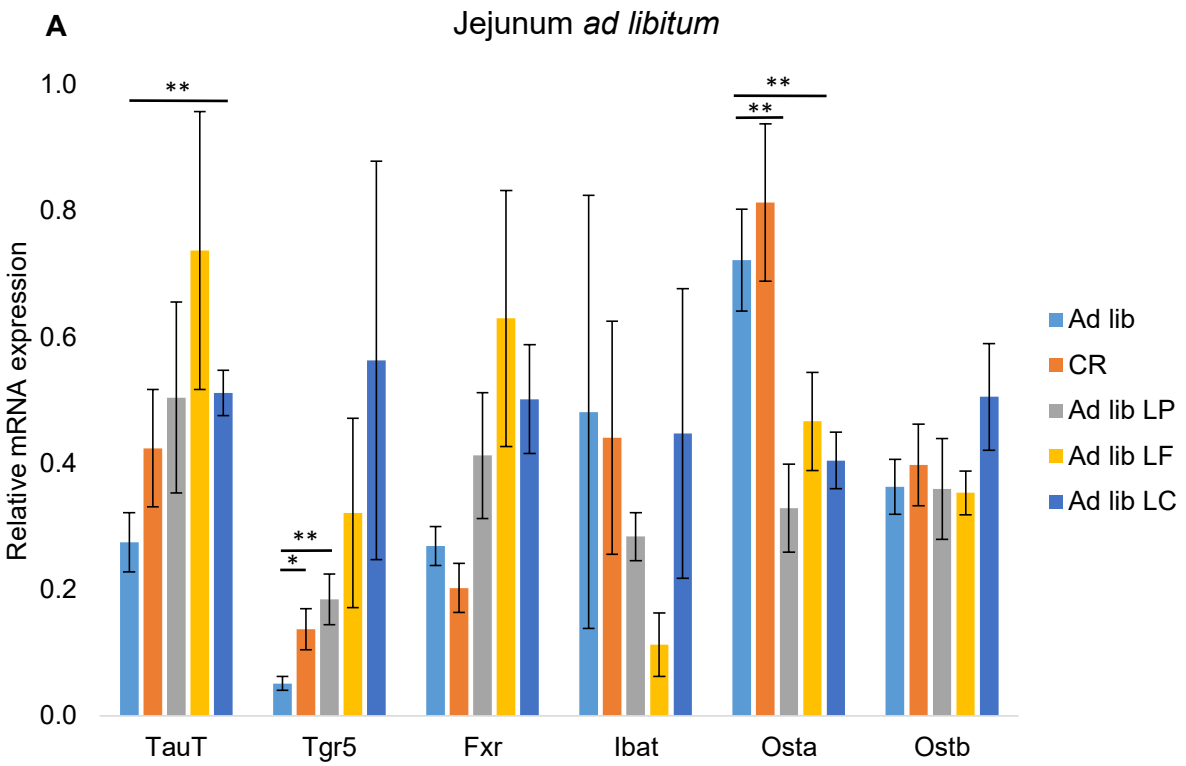


Figure 8: Relative mRNA expression of metabolic genes in the jejunum mucosa of mice fed *ad libitum* diets (A) and CR diets (B).
One-way ANOVA was used to examine the statistical significance; n = 7-8;*p ≤ 0.05,**$p \leq 0.01$. Data are presented as mean ± SEM. Sirtuin 1 (Sirt1), Fasting-induced adipose factor (Fiaf), Acyl-CoA synthetase long chain family member 3 (Acsl3), Acyl-CoA thioesterase 4 (Acot4), Stearoyl-coenzyme A desaturase 1 (Scd1), Acyl-CoA oxidase 2 (Acox2).

3.1.1.4 Metabolism of taurine and BAs

The last qRT-PCR results in the jejunum mucosa present a measurement of the expression of genes connected to taurine and BAs metabolism. As previously published, CR positively regulates the expression of TauT in the mucosa of the ileum [3]. The same effect was confirmed here in the jejunum mucosa, although the difference between CR and Ad lib was not statistically significant. The Ad lib LC group had a similar expression as CR of TauT and a statistical difference compared to Ad lib was identified ($p \leq 0.01$). This diet also induced the highest expression of Tgr5, but with a big SEM observed. Moreover, the gene expression of Tgr5 was statistically significantly higher in the CR and Ad lib LP groups compared to Ad lib. The Fxr gene expression in the CR was lowered compared to Ad lib but with no statistical difference observed. The bidirectional BAs transporters Osta and Ostb were both slightly upregulated in the CR group and Ostb in the Ad lib LC group but there was no statistical difference to Ad lib (see Figure 9 A).

In the high-macronutrient CR groups the CR HP had the highest TauT gene expression, but the impact was not statistically significant. A CR HF led to a statistically significantly higher expression of Fxr in comparison to CR. In all of the the high-macronutrient diets the BAs transporter Osta was statistically significantly downregulated in comparison to CR (see Figure 9 B).



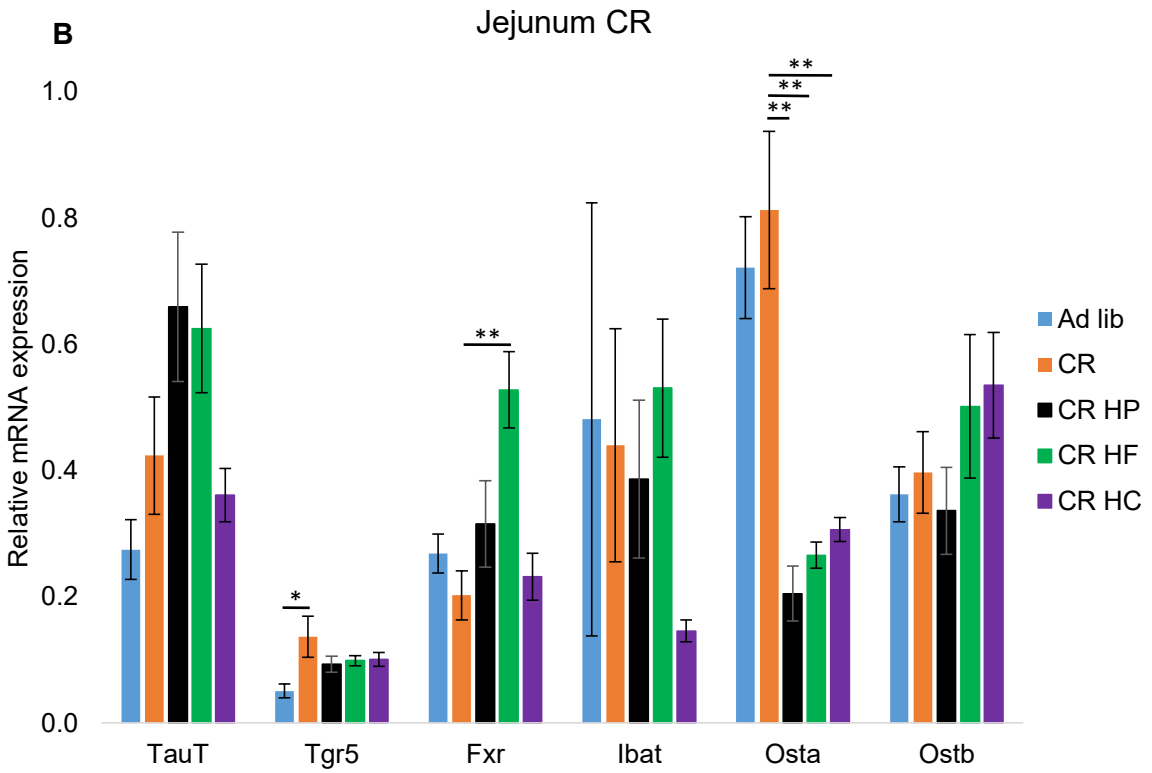


Figure 9: Relative mRNA expression of genes connected to taurine and BAs metabolism in the jejunum mucosa of mice fed *ad libitum* diets (A) and CR diets (B).
One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM. Taurine transporter (TauT), G-protein-coupled bile acid receptor 5 (Tgr5), Farnesoid X receptor (Fxr), Ileal bile acid transporter (Ibat), Organic solute transporter alpha (Osta), Organic solute transporter beta (Ostb).

3.1.2 Cecum

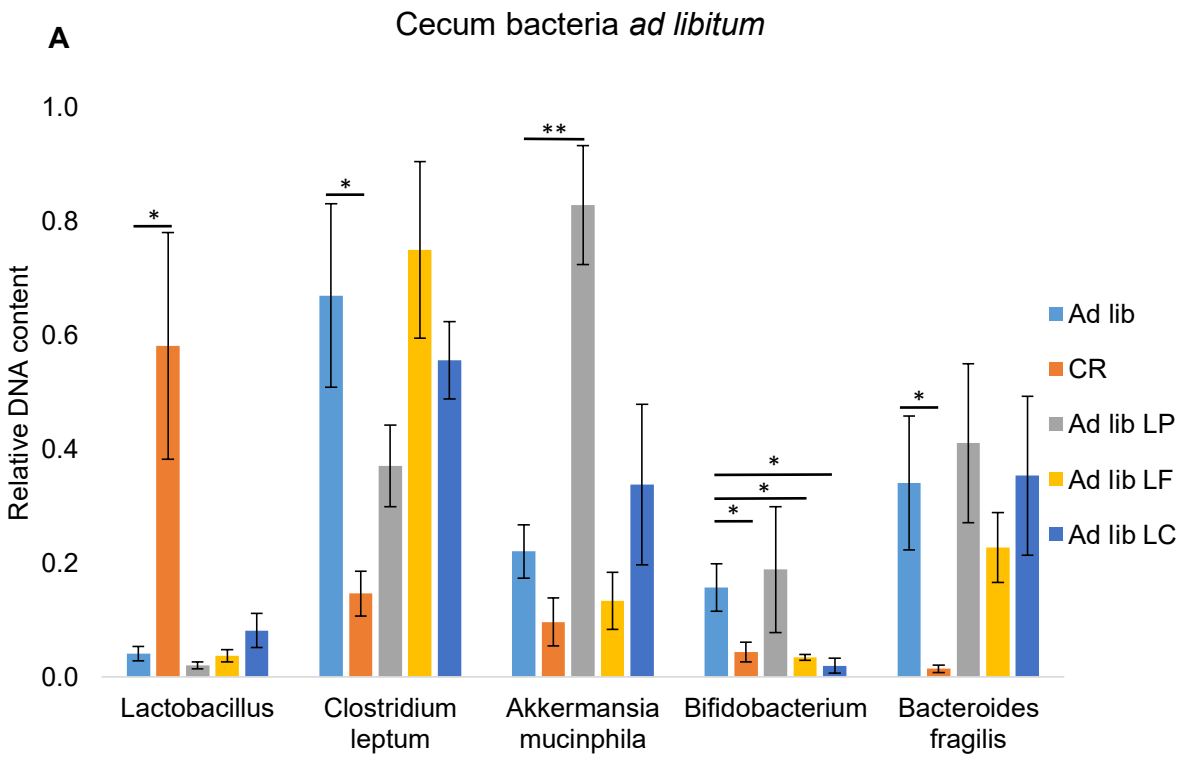
3.1.2.1 Microbiota

Considering the rising information about the importance of gut microbiota to human's health (see Paragraph 1.1.4), as well as well its role in the metabolism of taurine and BAs (see Paragraph 1.1.5), cecal bacterial content was investigated next. As already described, the cecum's weight was not statistically significantly different between the Ad lib and CR group. However, the Ad lib LF and Ad lib LC diets caused a statistically significant increase in the cecum's weight in comparison to Ad lib. In the CR groups, there was no difference observed (see Figure 4 E and 4 F).

The highest content of *Lactobacillus* was found in the cecum of the CR mice with a statistical difference compared to Ad lib. None of the low-macronutrient diets was able to cause a significant increase in this bacterium's content. All of the other investigated bacteria were with a lower relative DNA content in the CR group in comparison to Ad lib with almost all of them reaching a statistical significance (exception was *Akkermansia mucinphila*). In contrast, the Ad

lib LP had the highest content of *Akkermansia mucinphila, Bifidobacterium* and *Bacteroides fragilis* with the first bacterium's amount being statistically significantly elevated compared to Ad lib (see Figure 10 A).

On the other hand, none of the caloric restricted high-macronutrient diets was able to keep up the increase in *Lactobacillus* content after a standard CR diet. The CR HC even led to a statistically significant downregulation compared to CR. The CR HP, similar as CR, was with the lowest content of *Akkermansia mucinphila*, *Bifidobacterium* and *Bacteroides fragilis*, whereas the CR HF had the highest content of these three bacteria with the last two being statistically significantly upregulated compared to CR (see Figure 10 B).



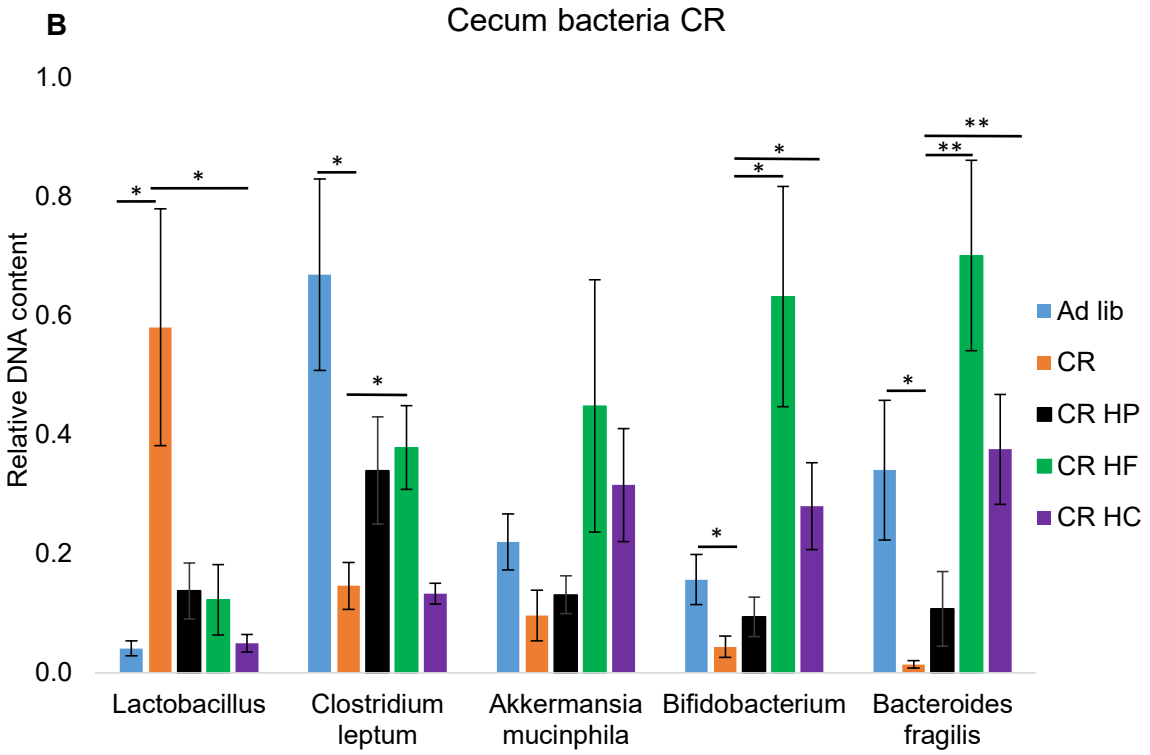


Figure 10: Relative bacterial abundance in the cecum of mice fed *ad libitum* diets (A) and CR diets (B). One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM.

3.1.3 Liver

3.1.3.1 Metabolism of taurine and BAs

To finalize the qRT-PCR investigations, the expression of genes associated with taurine and BAs metabolism and transport was examined in the liver. In the CR group, there was a statistically significant upregulation compared to Ad lib of Cdo and Ntcp. The same was observed for Cyp7a1, Bat, Bal and Bsep, but this difference was not statistically significant. The Shp was the only gene being with a lower expression in the CR group compared to Ad lib, but this observation was not statistically different. The Ad lib LF showed a statistically significant higher expression of Ntcp, Bat, Bal and Bsep compared to Ad lib. The Ad lib LC diet led to a statistically significant upregulation of Cdo, Ntcp, Bat, Bal, Shp and Bsep compared to Ad lib (see Figure 11 A).

The high-macronutrient diets did not seem to have as strong an effect as CR on the taurine and BAs metabolic genes in the liver. The Cyp7a1 gene was statistically significantly higher expressed after a CR HP diet in comparison to CR. However, the Cdo and Ntcp gene expressions were statistically significantly downregulated in the CR HP and CR HC groups

compared to CR, whereas the CR HF reached a statistically significant decreased expression only for Ntcp (see Figure 11 B).

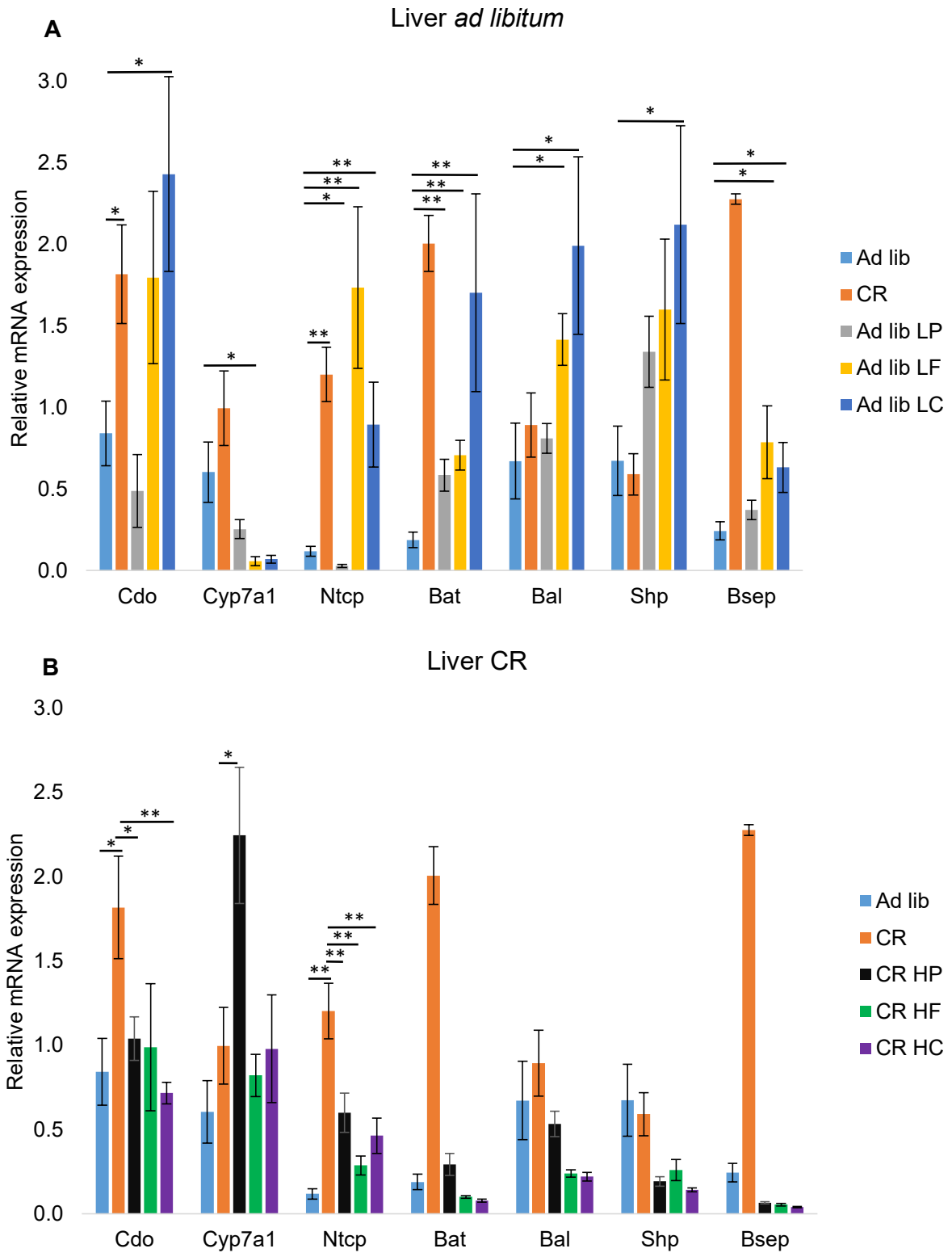
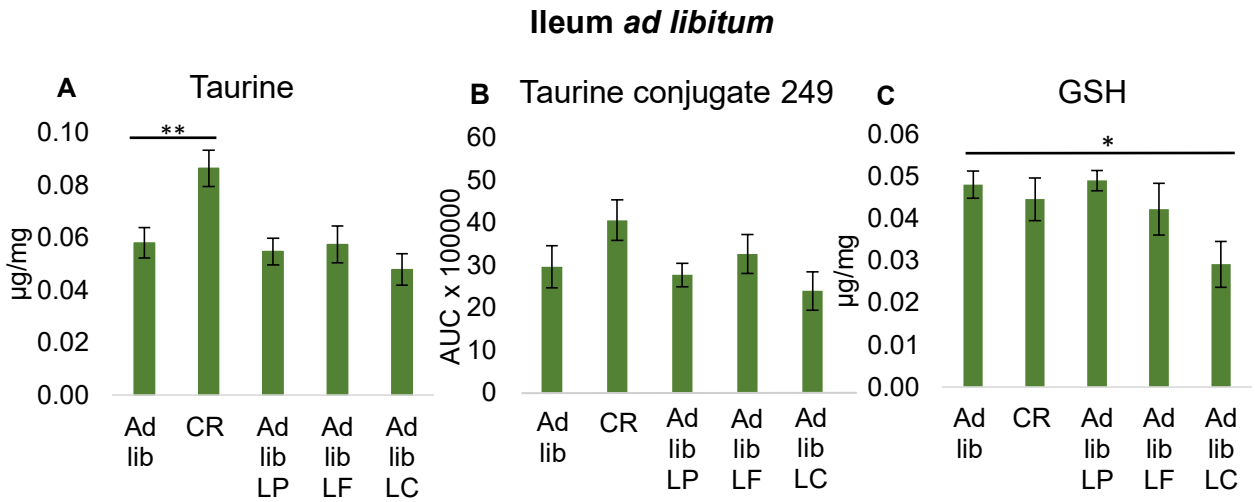


Figure 11: Relative mRNA expression of genes connected to taurine and BAs metabolism in the liver of mice fed *ad libitum* diets (A) and CR diets (B).
One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM. Cysteine dioxygenase (Cdo), Cholesterol 7 α-hydroxylase (Cyp7a1), Na^+-taurocholate cotransporting polypeptide (Ntcp), Bile acids CoA:amino acid N-acyltransferase (Bat), Bile acids CoA-ligase (Bal), Small heterodimer partner (Shp), Bile salt export pump (Bsep).

3.2 LCMS

3.2.1 Taurine

As already described, free taurine levels, as well those of its conjugates have been previously reported to be increased in different parts of the gut during CR [3, 18]. The current results present a statistically significantly higher amount of taurine in the ileum mucosa of mice fed a CR diet when compared to Ad lib (see Figure 12 A). The macronutrient restriction during an *ad libitum* diet did not have any significant impact on taurine levels in the ileum. Numerous taurine conjugates (conjugate 249 is displayed) were also elevated in the ileum of the CR group but the difference was not statistically significant (see Figure 12 B). Statistically significantly lower GSH levels were observed in the ileum of Ad lib LC compared to Ad lib (see Figure 12 C).
In the liver, the taurine content was again increased after CR, but this observation was not statistically significant (see Figure 12 D). The Ad lib LC had statistically significantly lower taurine content, as well as that of its 249 conjugate compared to Ad lib (see Figure 12 D and 12 E). The Ad lib LF led to statistically significantly decreased levels of taurine conjugate 249, as well as those of GSH compared to Ad lib (see Figure 12 E and 12 F).



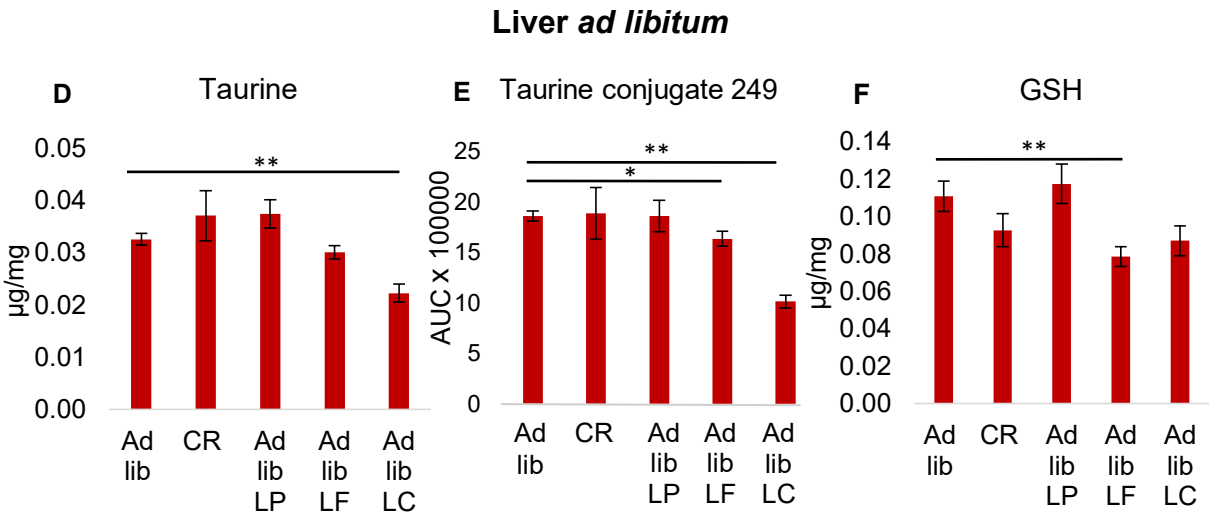
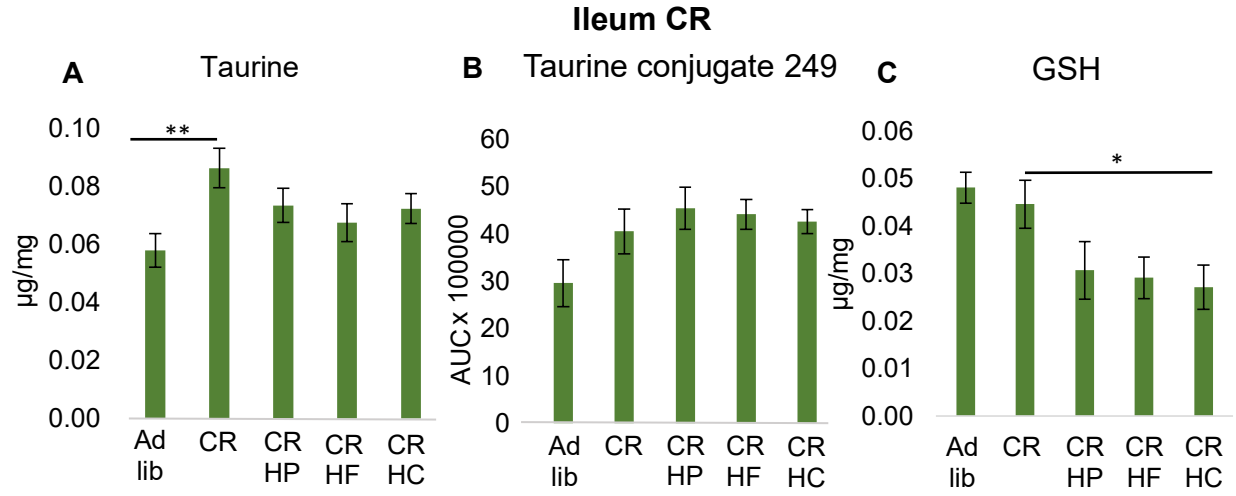


Figure 12: Levels of free taurine (A, D), taurine conjugate with m/z 249 (B, E), and glutathione (GSH) (C, F) in the ileum mucosa (A-C) and liver (D-F) of mice fed *ad libitum* diets.
One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM.

The measurements in the high-macronutrient CR groups showed no statistically significant different levels of taurine and its 249 conjugate in the ileum mucosa when compared to CR (see Figure 13 A and 13 B). A CR HC diet led to statistically significantly lower levels of GSH in the ileum in comparison to CR (see Figure 13 C).

In the liver, on the other hand, the CR HP group manifested a statistically significantly lower concentration to CR of free taurine and its presented 249 conjugate (see Figure 13 D and 13 E). There were no significant changes in the GSH levels (see Figure 13 F).



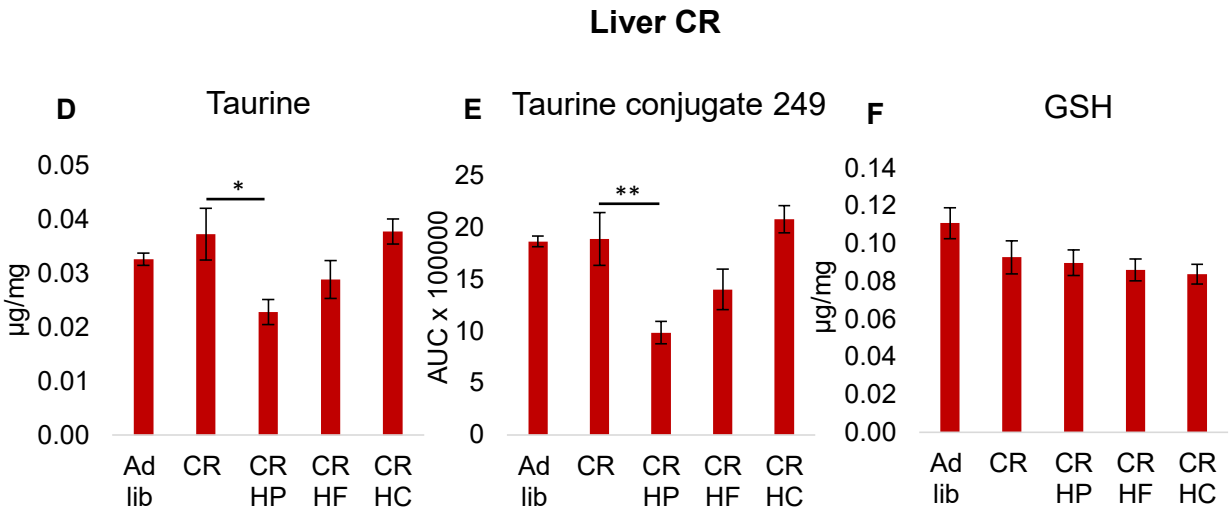


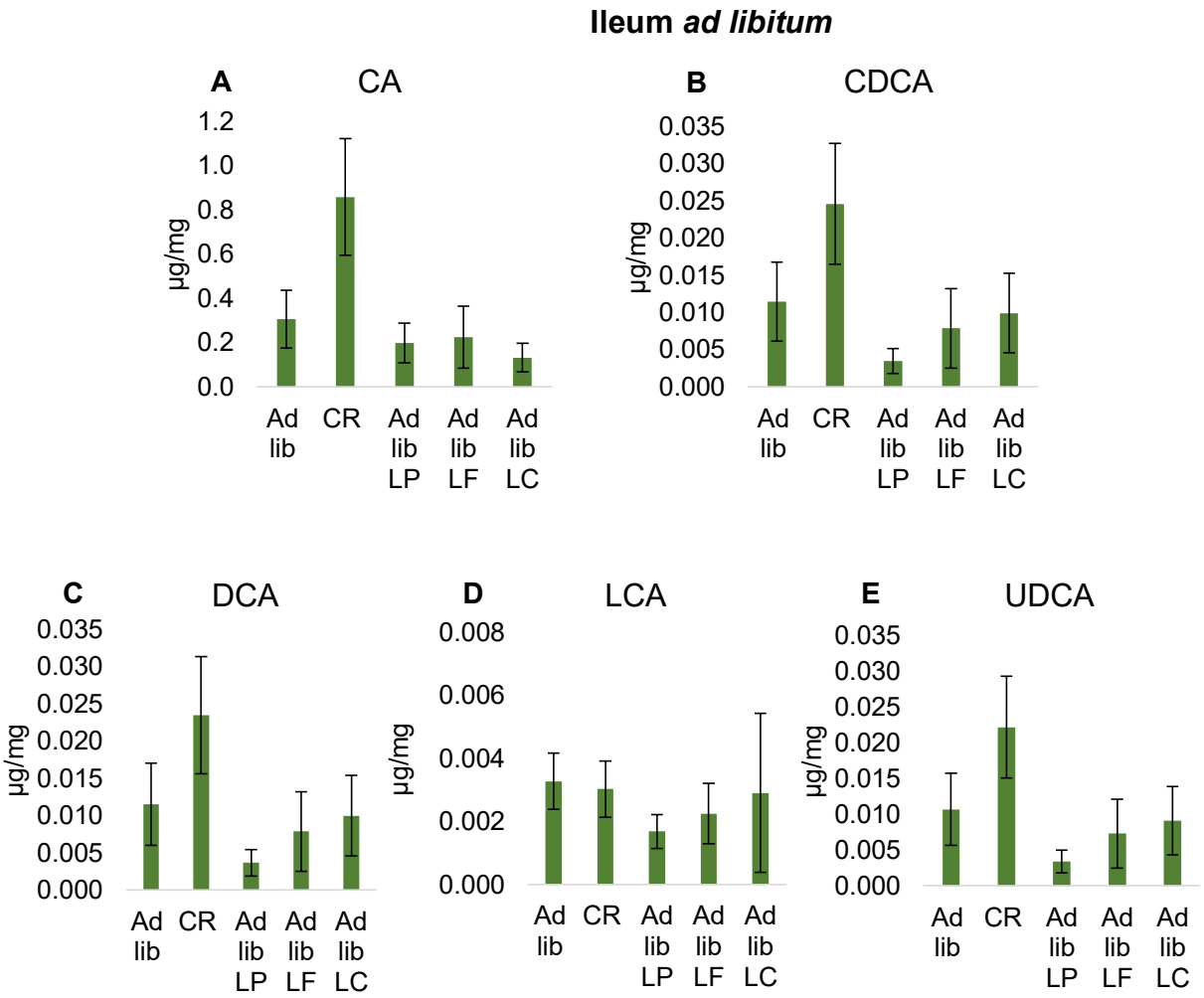
Figure 13: Levels of free taurine (A, D), taurine conjugate with m/z 249 (B, E), and GSH (C, F) in the ileum mucosa (A-C) and liver (D-F) of mice fed CR diets.
One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM.

3.2.2 BAs

Following previous results, where an upregulation during CR in the BAs levels was observed [2, 3, 16, 18], the content of BAs in the ileum mucosa and liver of the mice was measured.

In the ileum of CR mice there were higher amounts of primary and secondary BAs compared to Ad lib (see Figure 14 A-C and 14 E), whereas the taurine-conjugated BAs were slightly decreased (see Figure 14 F-I). However, the differences were not statistically significant. Generally, the lowest BAs content was measured in the Ad lib LP group, while the Ad lib LF and Ad lib LC groups had similar amounts of almost all the BAs, but with no statistical differences detected in comparison to control (see Figure 14 A-I).

In the liver a statistically significantly higher content of almost all the BAs, including taurine-conjugated ones, was observed in the CR group compared to Ad lib (see Figure 15 A-I). The Ad lib LC was the only low-macronutrient diet that led to a statistical difference compared to Ad lib. Namely, there were statistically significantly higher amounts of CDCA, DCA, and UDCA in comparison to Ad lib (p = 0.01) (see Figure 15 B, 15 C and 15 E).



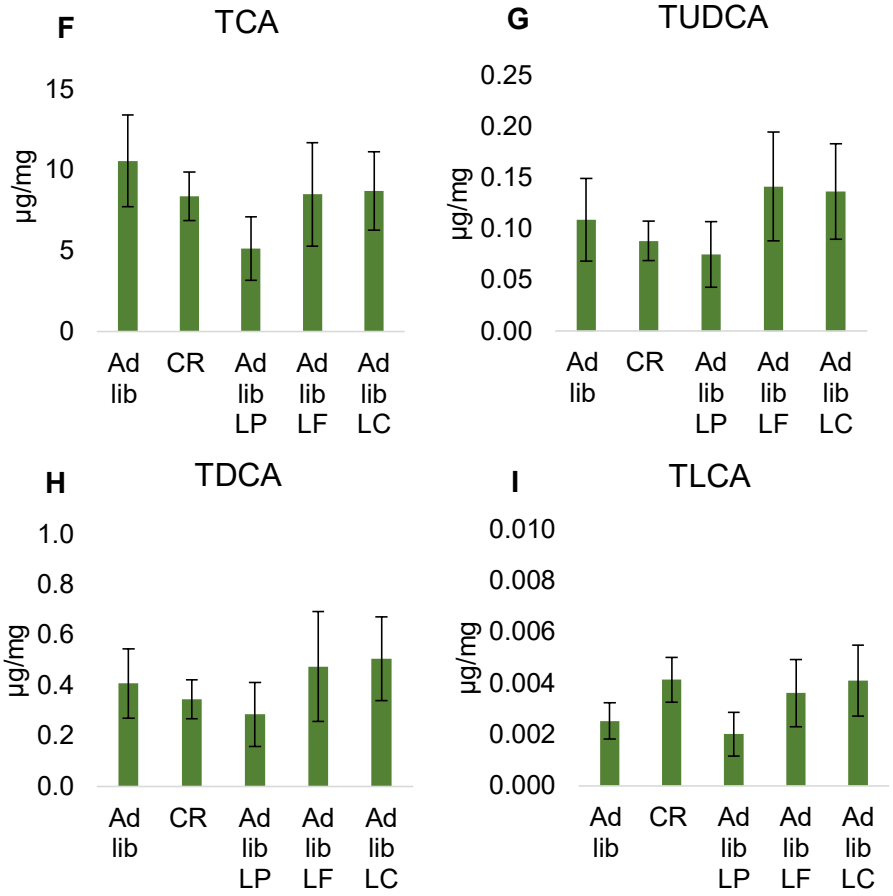


Figure 14: Content of primary (A, B), secondary (C, D, E) and taurine-conjugated (F, G, H, I) BAs measured in the ileum mucosa of mice fed *ad libitum* diets.
One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM. Cholic acid (CA), Chenodeoxycholic acid (CDCA), Deoxycholic acid (DCA), Lithocholic acid (LCA), Ursodeoxycholic acid (UDCA), Taurochloric acid (TCA), Tauroursodeoxycholic acid (TUDCA), Taurodeoxycholic acid (TDCA), Taurolithocholic acid (TLCA).

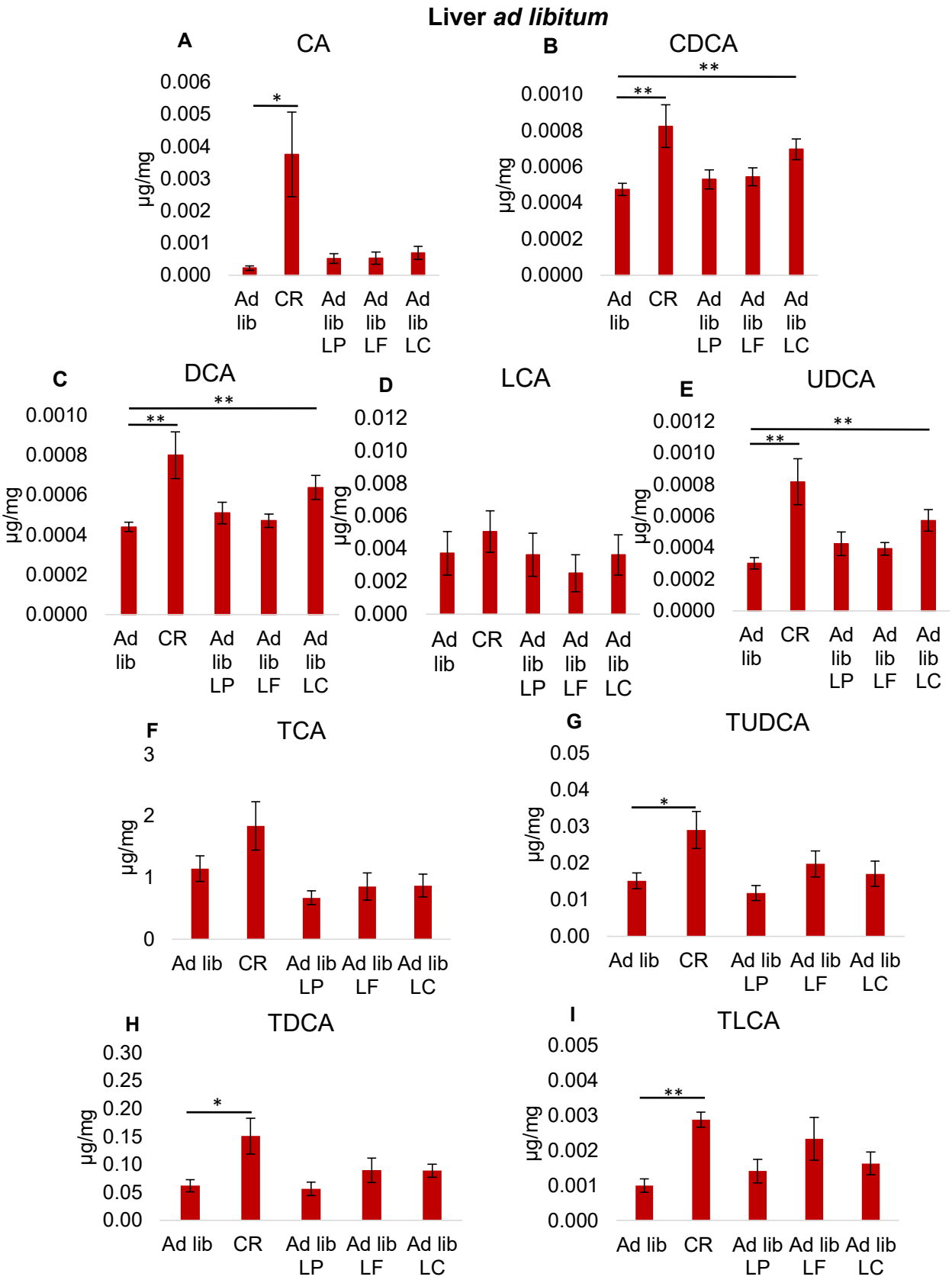
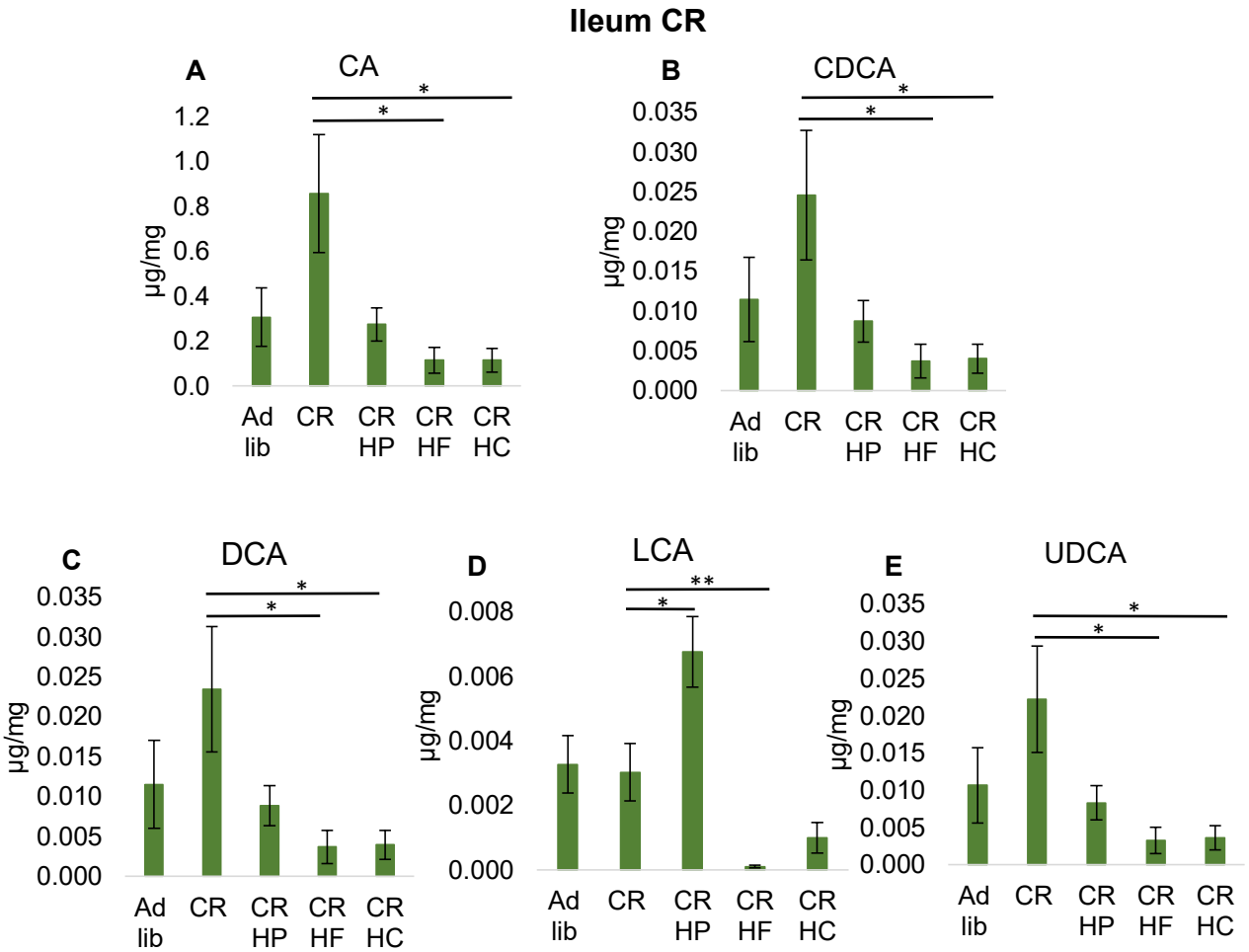


Figure 15: Content of primary (A, B), secondary (C, D, E) and taurine-conjugated (F, G, H, I) BAs measured in the liver of mice fed *ad libitum* diets.
One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM.

The high-macronutrient diets were able to induce changes in the BAs content when compared to CR. Namely, in the ileum mucosa, the CR HF and CR HC diets led to a statistically significantly lower concentration of almost all the BAs when compared to CR (see Figure 16 A-I). The only exception was LCA – the CR HP group had the highest content of this BA even with a statistical difference to CR (see Figure 16 D). Moreover, after a CR HP diet the highest concentration of taurine-conjugated BAs in the ileum was observed but it did not reach a statistical significance when compared to control (see Figure 16 F-I).

In the liver a statistically significantly decreased amount of primary, secondary and taurine-conjugated BAs in the CR HF and CR HC groups was again observed in comparison to CR (see Figure 17 A-I). Same as in the ileum mucosa, the CR HP diet led to the highest content of taurine-conjugated BAs in the liver. However, this difference was again not statistically significant (see Figure 17 F-I).



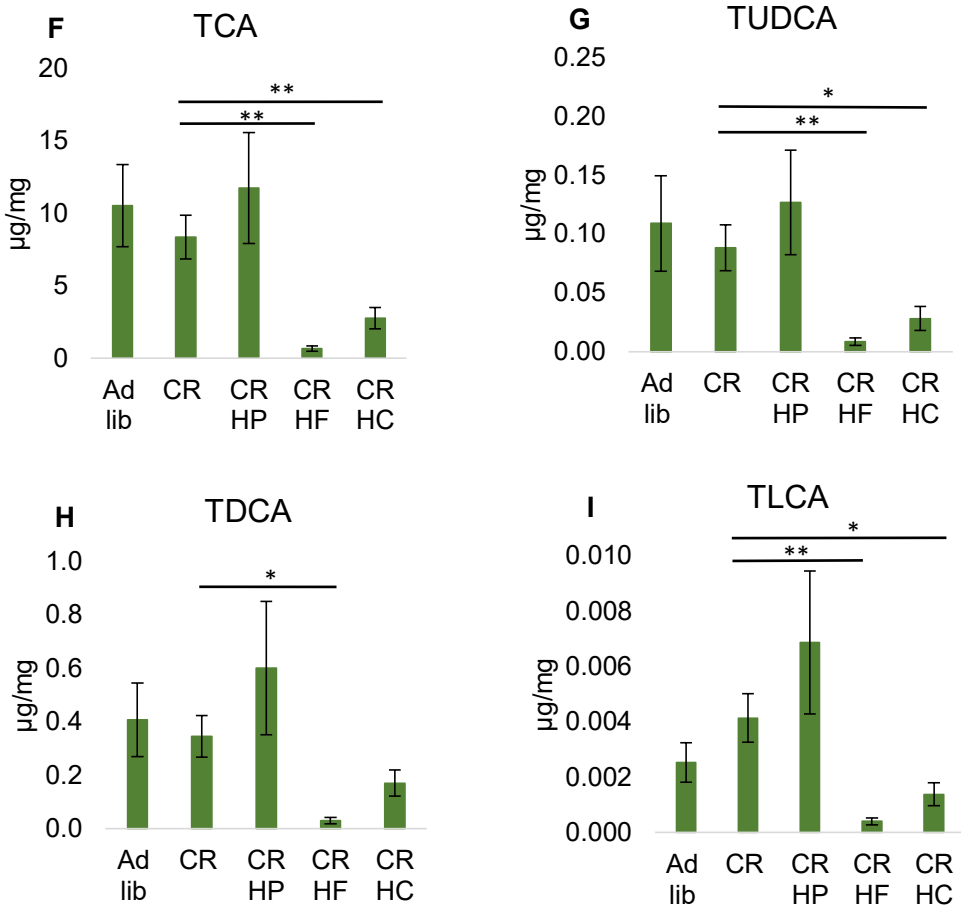
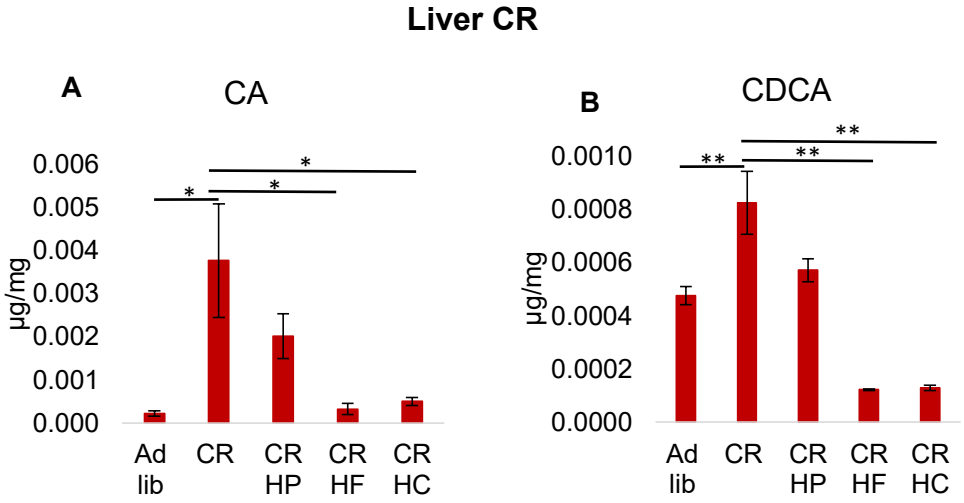


Figure 16: Content of primary (A, B), secondary (C, D, E) and taurine-conjugated (F, G, H, I) BAs measured in the ileum mucosa of mice fed CR diets.
One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM.



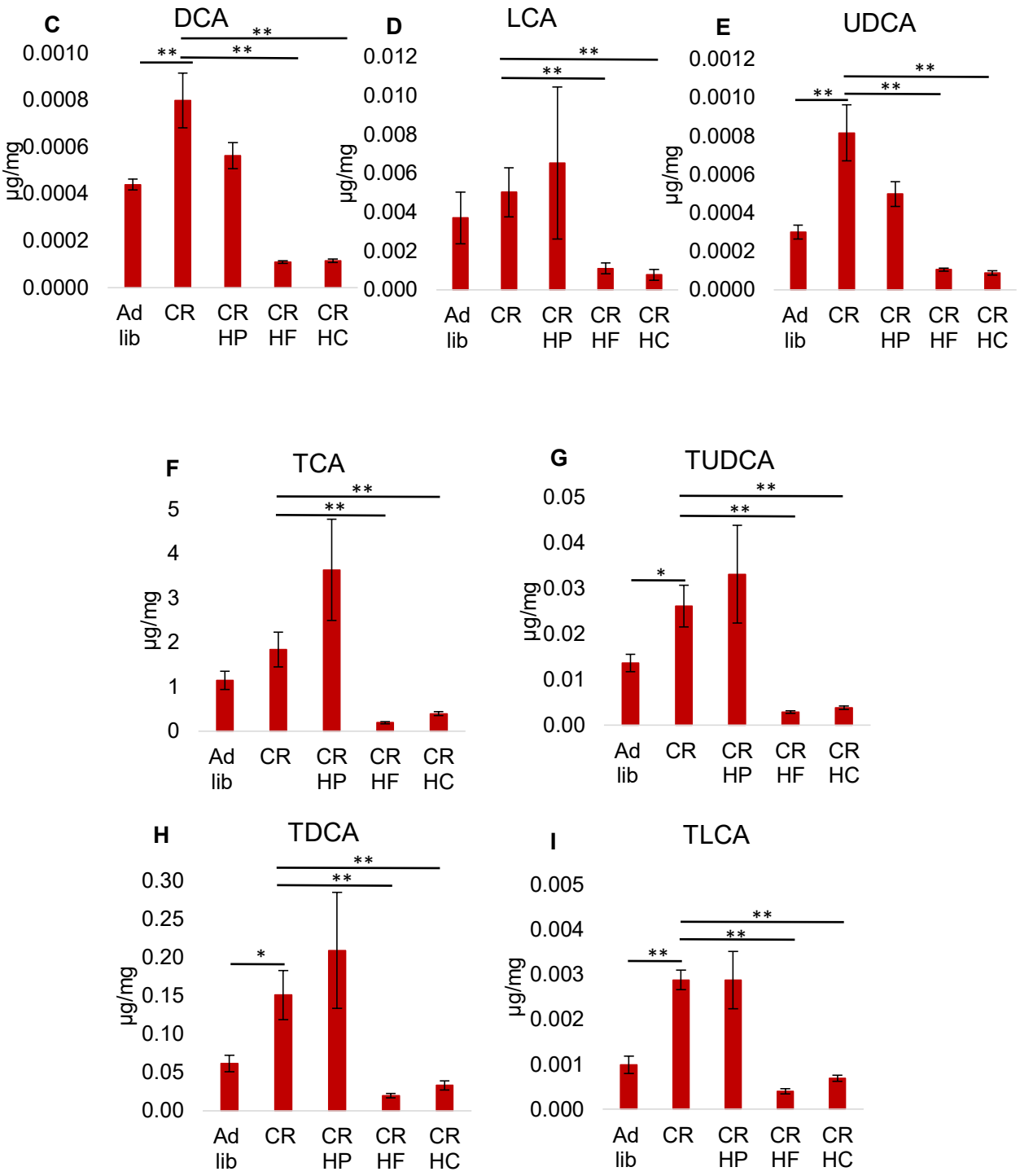


Figure 17: Content of primary (A, B), secondary (C, D, E) and taurine-conjugated (F, G, H, I) BAs measured in the liver of mice fed CR diets.
One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM.

3.3 Western Blot

3.3.1 Jejunum

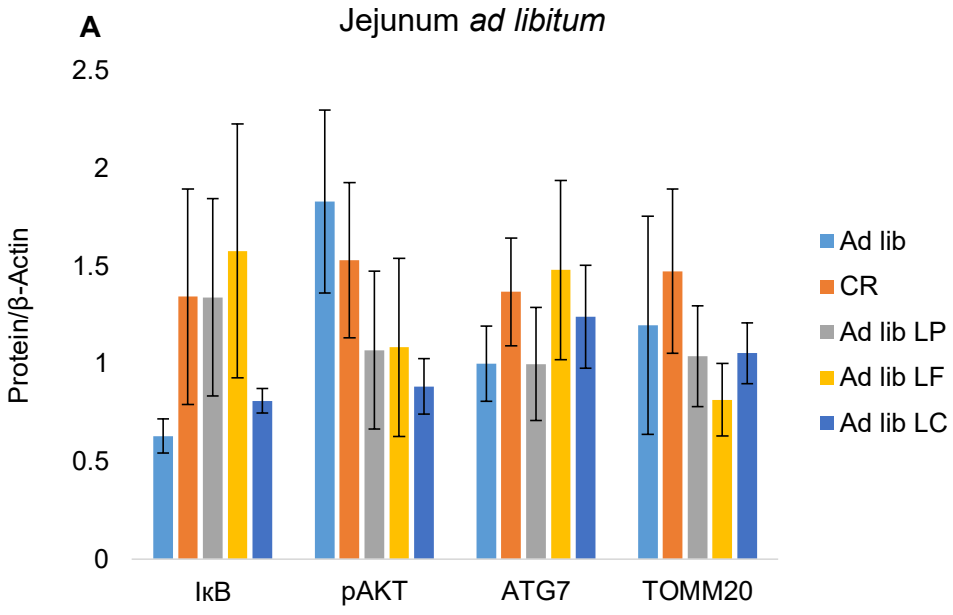
3.3.1.1 Restriction-related markers

To further examine the effect of the different diets on inflammation in the gut, the amount of IκB in the jejunum mucosa was measured. In the CR, Ad lib LP and Ad lib LF groups there were elevated amounts of this protein, whereas the Ad lib LC had similar measurements as Ad lib. However, none of these differences were statistically significant (see Figure 18 A and 18 B).

An improved glucose metabolism and insulin sensitivity are both connected to CR [12]. The levels of pAKT, which plays an important role in the insulin-signaling pathway, were downregulated in the CR group compared to Ad lib. The amount of this protein was not statistically significantly affected by any of the low-macronutrient diets, but it needs to be noted that the Ad lib LC showed the lowest amount of pAKT (see Figure 18 A and 18 C).

Since one of the benefits of CR is promotion of autophagy the amount of the ATG7, that is a key player in cell recycling, was measured [29, 31]. The protein was elevated after a CR diet compared to Ad lib, although with no statistical significance. The Ad lib LF and Ad lib LC seemed to have a similar upregulating effect as CR on ATG7, but no statistical difference was observed when compared to control (see Figure 18 A and 18 D).

The amounts of TOMM20 were higher in the CR compared to Ad lib, which indicated a better proton transport along the inner mitochondrial membrane and an improved mitochondrial function. However, neither the CR diet, nor the low-macronutrient diets had a statistically significant impact on this protein (see Figure 18 A and 18 E).



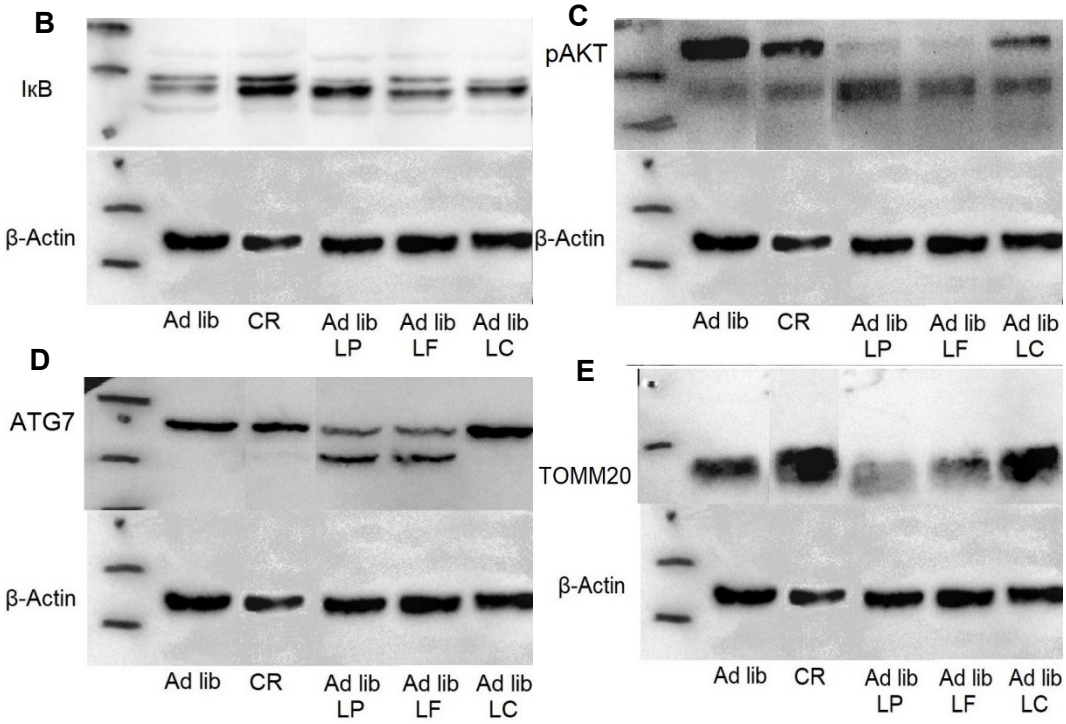
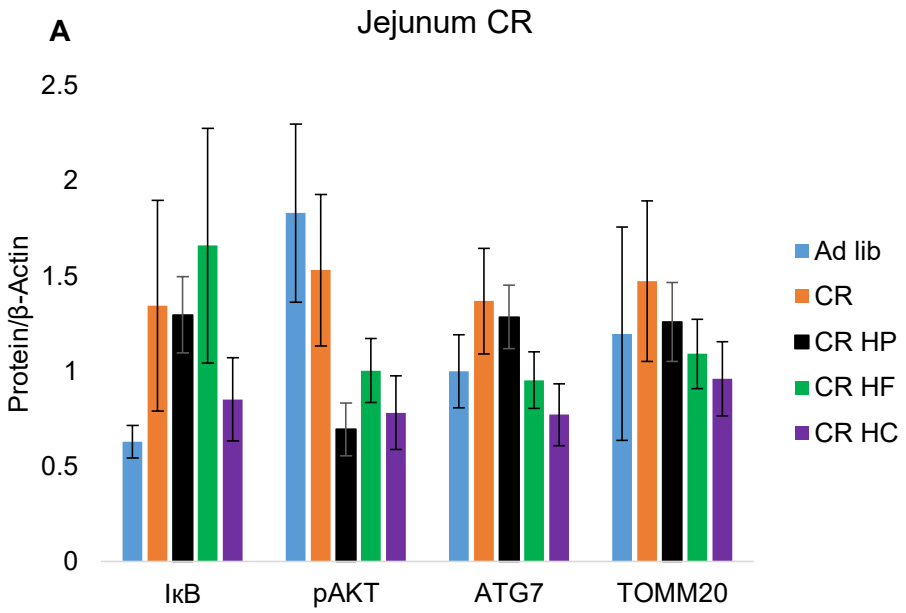


Figure 18: Protein levels of IκB (A, B), pAKT (A, C), ATG7 (A, D) and TOMM20 (A, E) in the jejunum mucosa of mice fed *ad libitum* diets.
One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM. Inhibitor of nuclear factor kappa β (IκB), Phosphorylated protein kinase B (pAKT), Autophagy related 7 (ATG7), Translocase of outer mitochondrial membrane 20 (TOMM20).

The CR HP and CR HF diets seemed to maintain the anti-inflammatory effect of CR which was displayed in an increase of IκB, whereas the CR HC had similar levels of this protein as an Ad lib diet (see Figure 19 A and 19 B). The pAKT content was decreased in all of the high-macronutrient groups compared to CR with the CR HP having the lowest levels of the protein (see Figure 19 A and 19 C). The quantification of ATG7 and TOMM20 showed a similar pattern in the high-macronutrient groups. Namely, the CR HP diet seemed to keep up best with the effect of a CR diet on autophagy and mitochondrial function, whereas an increase in fat or carbohydrates slightly reduced these observations (see Figure 19 A, 19 D and 19 E).

In summary, none of the high-macronutrient diets was able to completely erase the effects of CR on the measured parameters, but the CR HC seemed to be the one intervention that would have an adverse effect on IκB, ATG7 and TOMM20. However, none of the observed differences were statistically significant when compared to CR (see Figure 19 A).



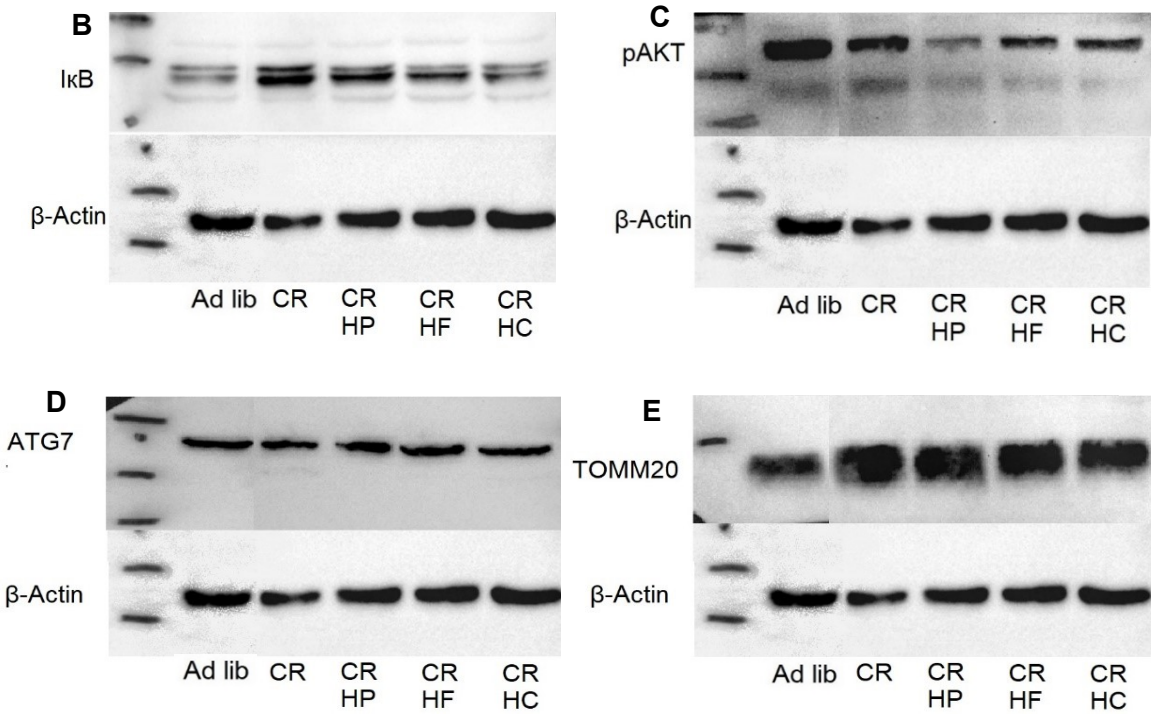


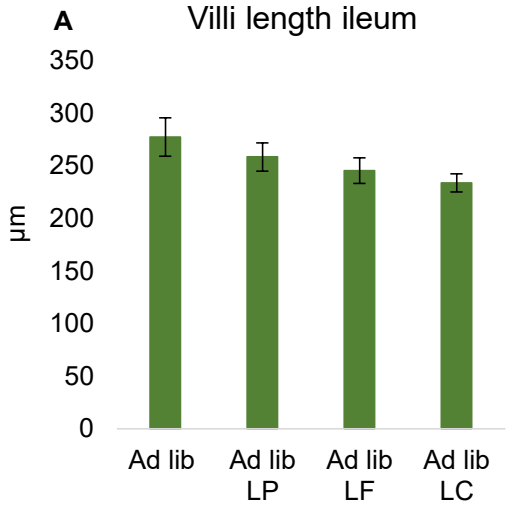
Figure 19: Protein levels of IκB (A, B), pAKT (A, C), ATG7 (A, D) and TOMM20 (A, E) in the jejunum mucosa of mice fed CR diets.
One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM.

3.4 Histology

3.4.1 Ileum

Finally, the possible morphological alterations on the gut after the *ad libitum* diets were analyzed.

As mentioned, the low-macronutrient diets induced no significant changes on the length of the small intestine (see Figure 5 A). In the ileum was a trend towards slight shortening of the villi's length in the low-macronutrient groups compared to Ad lib with the Ad lib LC group having the shortest villi. However, none of these observations were statistically significant (see Figure 20 A). Images of the histology sections of the ileum are presented below (see Figure 20 B-E, Figure 21 A-D and Figure 22 A-D).



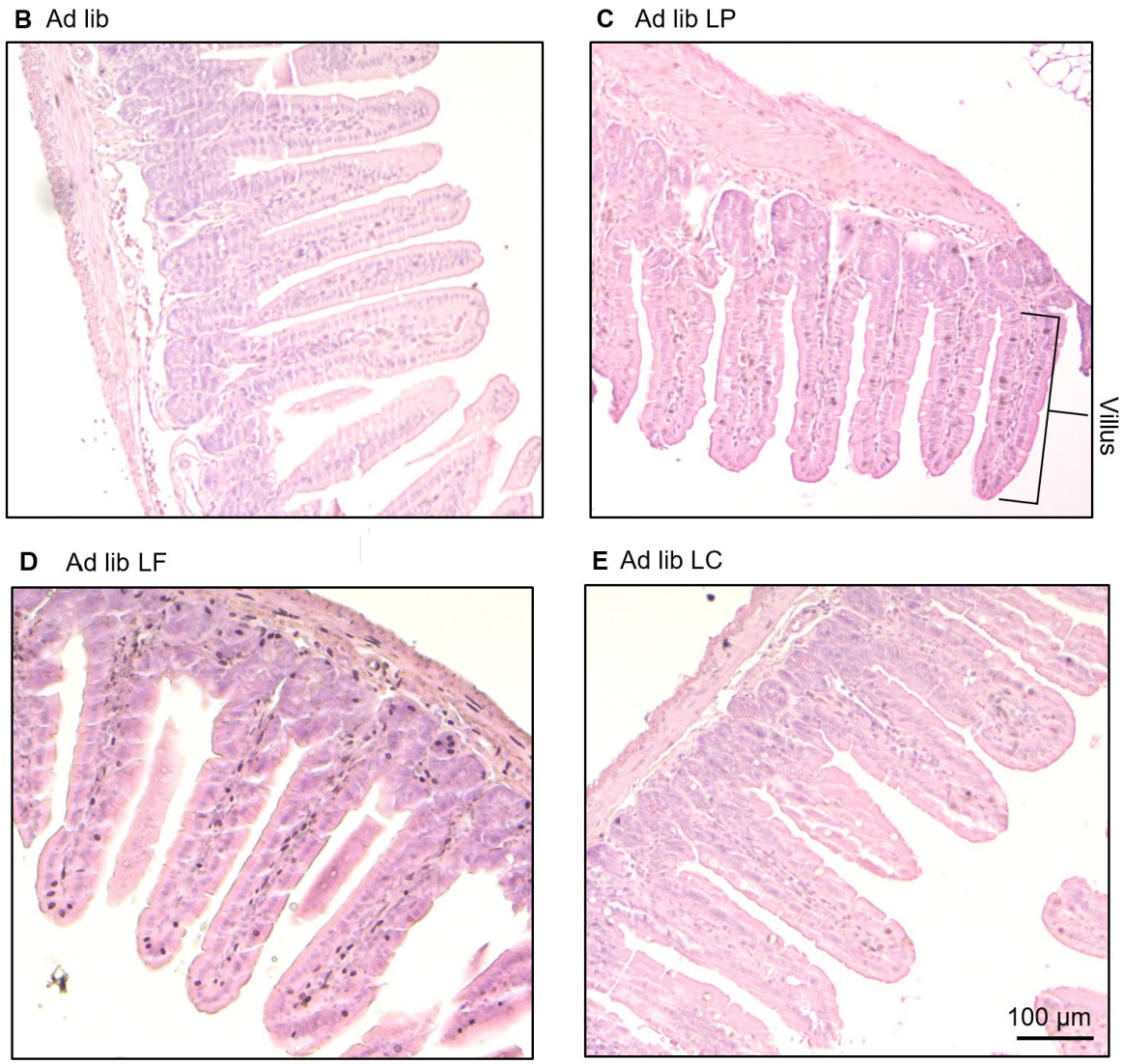


Figure 20: Villi length of the ileum (A) of mice fed *ad libitum* diets, quantified with ImageJ. Representative histology sections of the ileum (B, C, D, E) at 10x resolution, stained with hematoxylin-eosin solutions. Microscopy images are taken with Zeiss Axio Lab 5x HSF 22 microscope, Axiocam ERc 5s camera and ZEN software (Zeiss efficient Navigation). One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM.

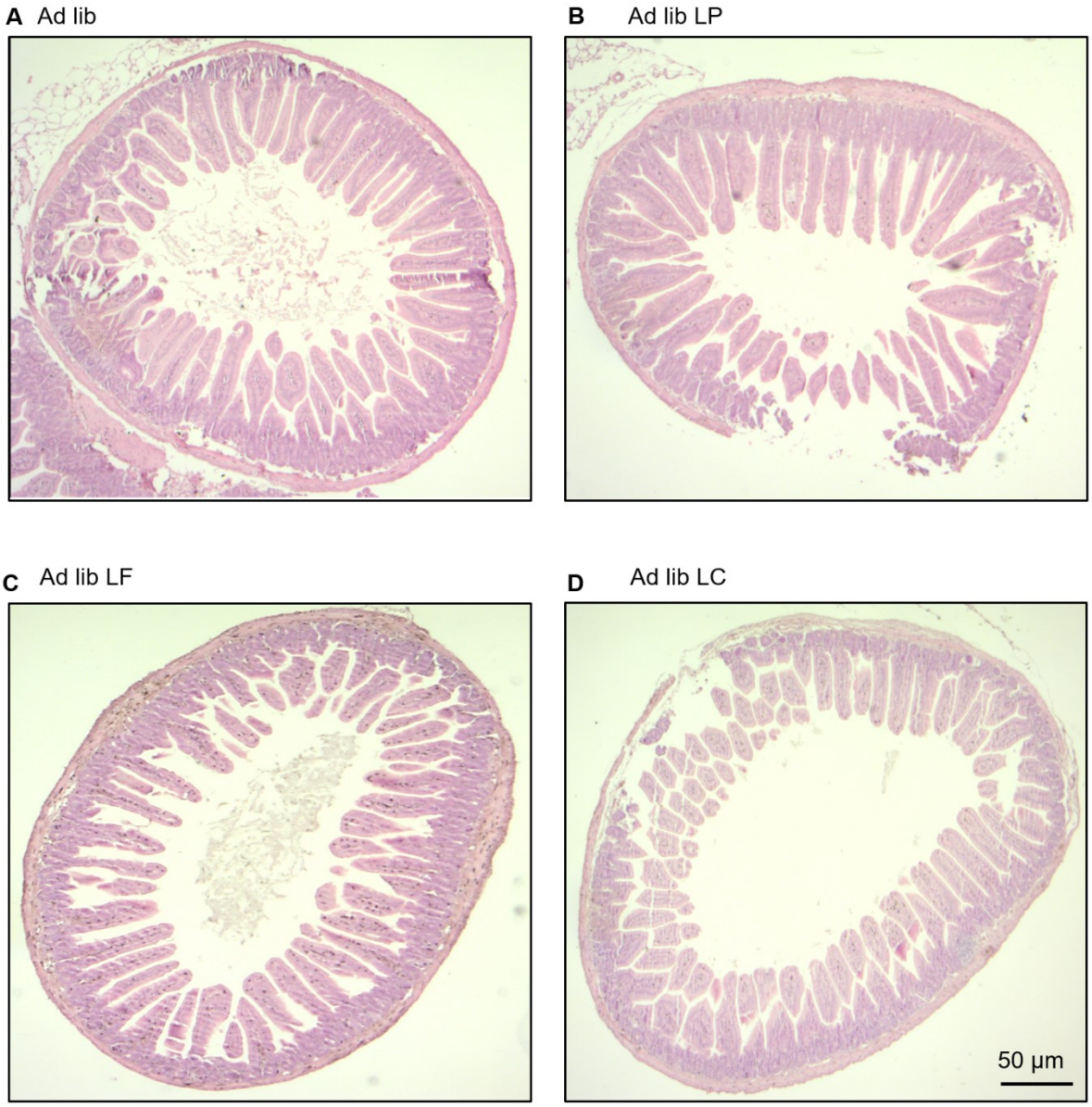


Figure 21: Histology sections of the ileum (A, B, C, D) at 5x resolution, stained with hematoxylin-eosin solutions.
Microscopy images are taken with Zeiss Axio Lab 5x HSF 22 microscope, Axiocam ERc 5s camera and ZEN software (Zeiss efficient Navigation).

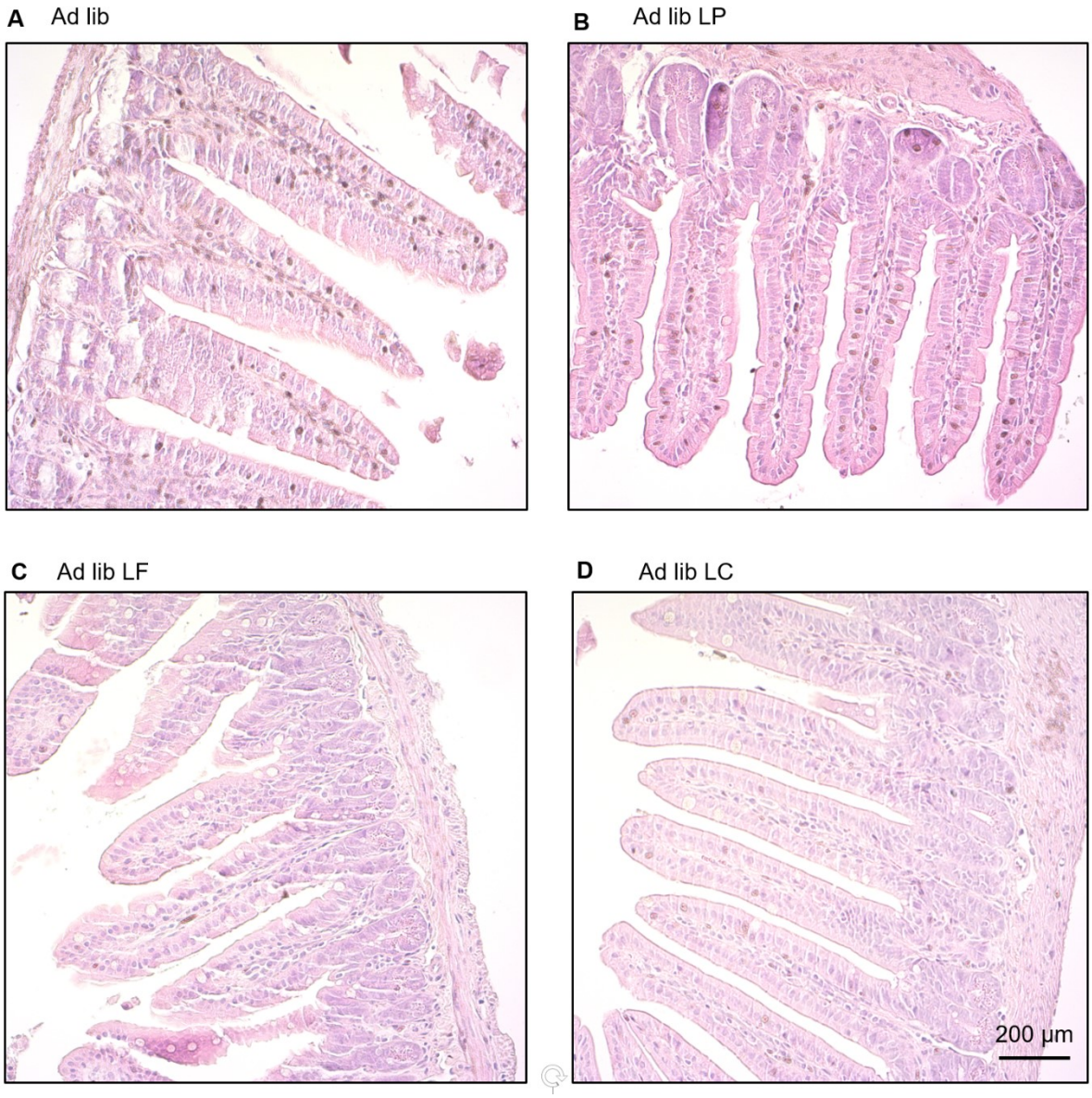
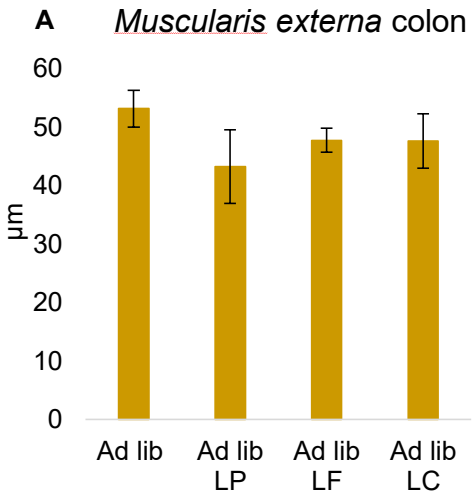


Figure 22: Histology sections of the ileum (A, B, C, D) at 20x resolution, stained with hematoxylin-eosin solutions.
Microscopy images are taken with Zeiss Axio Lab 5x HSF 22 microscope, Axiocam ERc 5s camera and ZEN software (Zeiss efficient Navigation).

3.4.2 Colon

The different diets did not have any significant effect on the length of the colon (see Figure 5 C). The measurement of the thickness of the *muscularis externa* indicated a shrinkage of the colon wall in the Ad lib LP. However, no statistically significant differences were observed neither in this group, nor in any of the other low-macronutrient groups when compared to Ad lib (see Figure 23 A). Images of the histology sections of the colon can be observed below (see Figure 23 B-E; Figure 24 A-D and Figure 25 A-D).



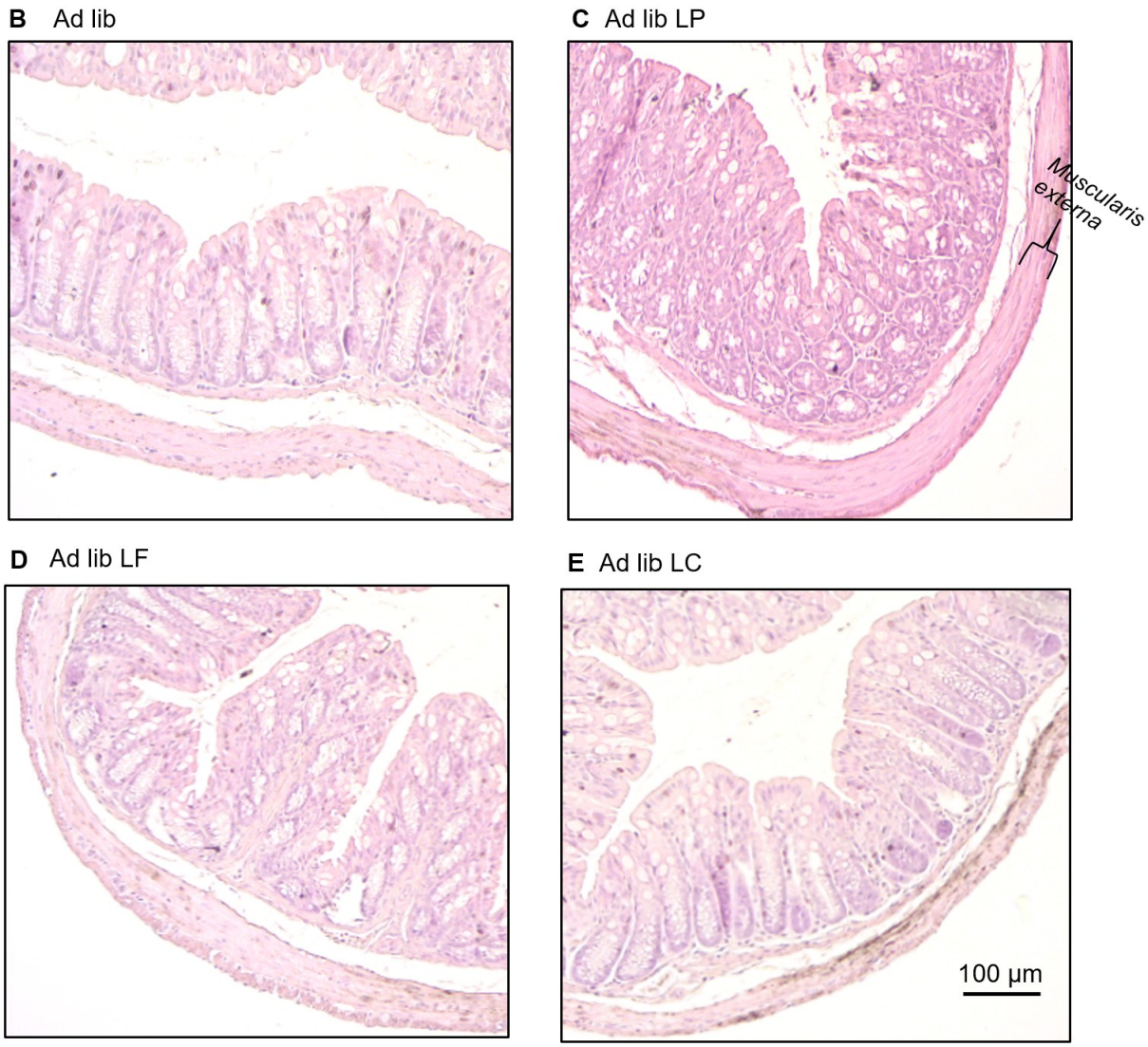


Figure 23: Thickness of *muscularis externa* of the colon (A) of mice fed *ad libitum* diets, quantified with ImageJ. Representative histology sections of the colon (B, C, D, E) at 10x resolution, stained with hematoxylin-eosin solutions.
Microscopy images are taken with Zeiss Axio Lab 5x HSF 22 microscope, Axiocam ERc 5s camera and ZEN software (Zeiss efficient Navigation). One-way ANOVA was used to examine the statistical significance; n = 7-8;*$p \leq 0.05$,**$p \leq 0.01$. Data are presented as mean ± SEM.

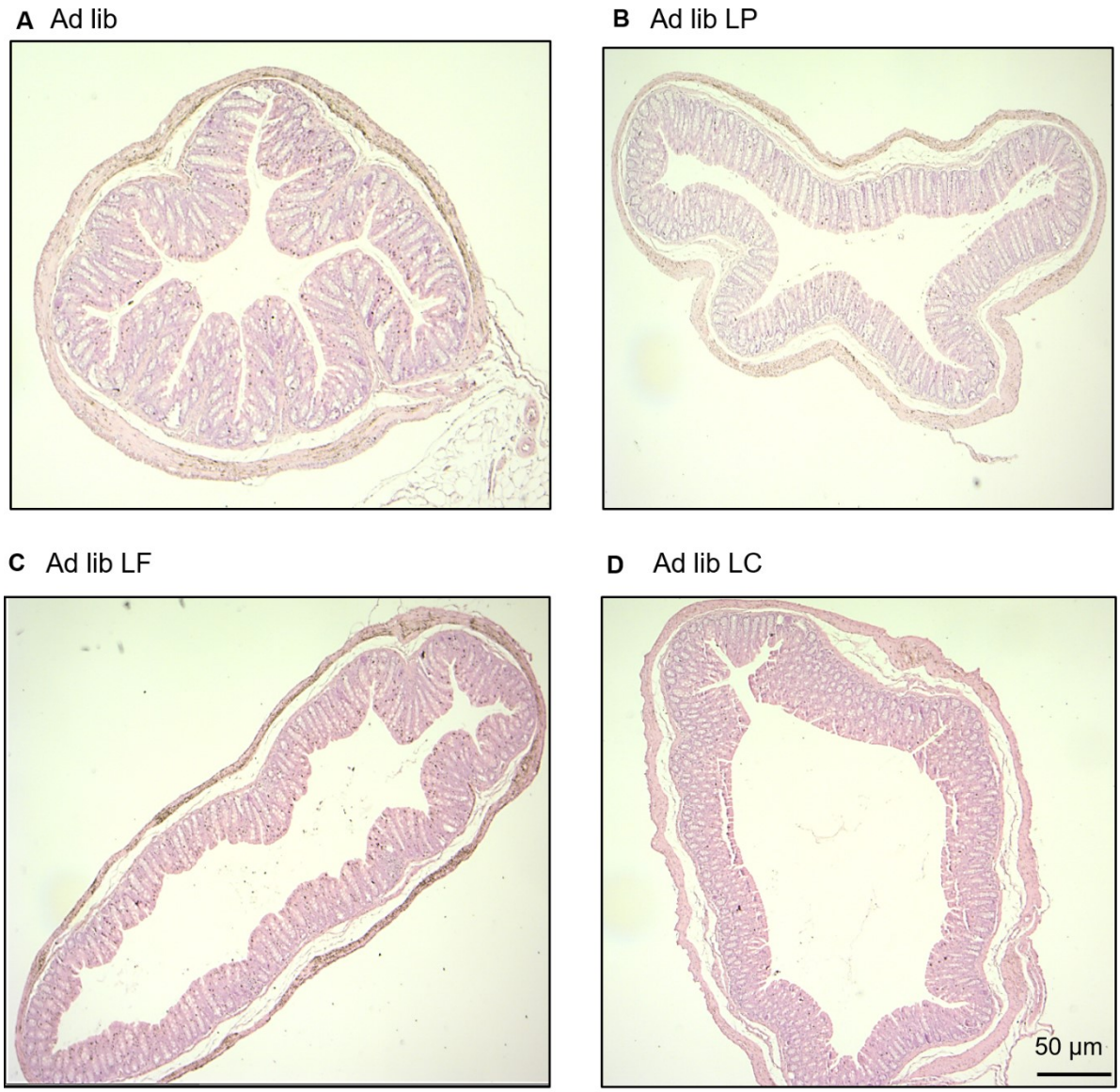


Figure 24: Histology sections of the colon (A, B, C, D) at 5x resolution, stained with hematoxylin-eosin solutions
Microscopy images are taken with Zeiss Axio Lab 5x HSF 22 microscope, Axiocam ERc 5s camera and ZEN software (Zeiss efficient Navigation).

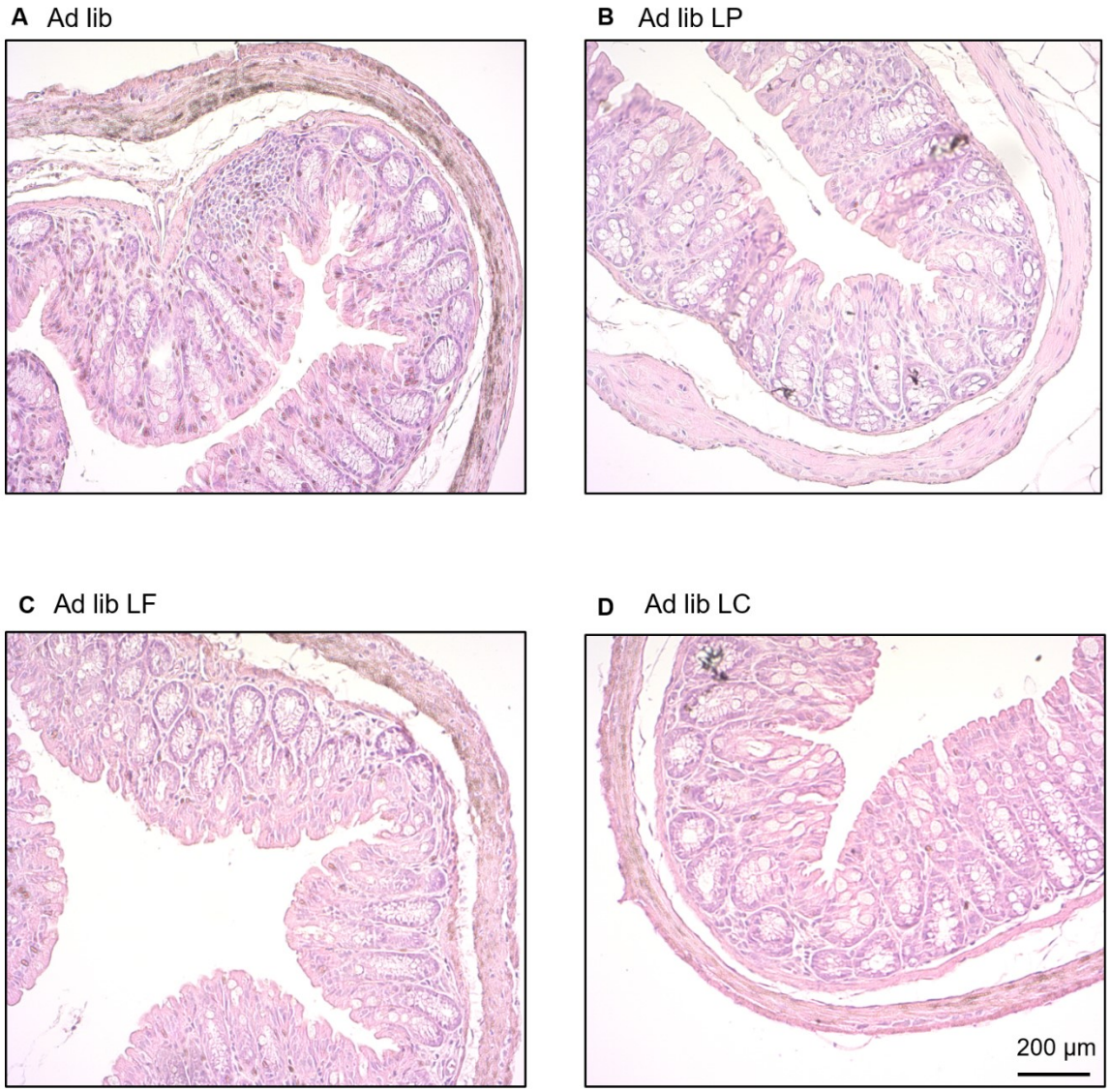


Figure 25: Histology sections of the colon (A, B, C, D) at 20x resolution, stained with hematoxylin-eosin solutions
Microscopy images are taken with Zeiss Axio Lab 5x HSF 22 microscope, Axiocam ERc 5s camera and ZEN software (Zeiss efficient Navigation).

4 Discussion

This work provides an insight into the influence of macronutrient restriction and CR on the GIT. It has been previously shown that the gene expression of inflammation markers after several restrictive diets was comparable in the small intestine's mucosa and colon, as well as in the Peyer's patches. On the other hand, in the lymph nodes these observations were less pronounced, indicating that restriction of nutrients has a stronger effect on the gut [2]. For this reason, this study puts further focus on the impact that macronutrient restriction and CR might have on the GIT. It presents a detailed gene expression profiling of antioxidative, inflammatory, CR-related and metabolic factors in the jejunum mucosa after the applied restrictive diets. It also investigates the effect that these restrictions have on the trilogy of microbiota composition and taurine and BAs' metabolism. The gut was further studied for markers of autophagy and mitochondrial function and changes in its morphology.

4.1 Impact of CR on the GIT

The current investigation validates the ability of CR to reduce oxidative stress and inflammation, while simultaneously increasing metabolism in the small intestine. This was reflected in the decreased expression of genes responsible for protecting the cell from oxidative damage. Furthermore, the observed upregulation of Gsta3 and Mgst1 in the CR group supports a previous theory that the conjugation ability of GSH is relevant during CR rather than its antioxidative capacity (see Figure 6 A) [3]. This assumption was strengthened by the lower content of GSH in the ileum and liver of the CR group, although the difference did not have any statistically significant impact (see Figure 12 C and 12 F). Moreover, the results indicate that the reduction of inflammation in the gut during CR is probably regulated through two of the main players mediating inflammatory reactions and innate immune response, namely the Irf and NF-κB pathways. This was mirrored by a lower gene expression of Stat1 and Irf1, as well as that of MyD88 and Tlr3 in the jejunum of the CR group compared to Ad lib (see Figure 7 A). In addition, the protein content of IκB was higher in the CR group in comparison to Ad lib, although not statistically significant (see Figure 18 A). The results also confirmed an increasing effect of CR on longevity and fatty acid metabolism, which was mirrored in the higher Sirt1 gene expression and that of the Acsl3, Acot4 and Acox2 (see Figure 8 A). Moreover, cecum microbiota analysis showed a completely different bacterial composition between the two control groups. Interestingly, the CR diet was the only restrictive diet having the ability to increase *Lactobacillus* content, whereas the amount of all of the other investigated bacteria was reduced in this group in comparison to Ad lib (see Figure 10 A).
Furthermore, the CR diet led to an elevation in the expression of genes connected to taurine and BAs metabolism, which was more pronounced in the liver than in the jejunum mucosa (see

Figure 9 A and Figure 11 A). Accordingly, this resulted in an increase in the content of taurine and BAs after CR in the ileum and liver (see Figure 12 A, 12 D; Figure 14 A-E and Figure 15 A-I), although the increased taurine levels in the liver and the ones of the BAs in the ileum did not reach statistical significance. However, observing more pronounced changes in the taurine content in the ileum after CR is in line with previous reports [3].
Although the impact was not that strong, decrease in insulin signaling (pAKT), as well as higher markers of autophagy (ATG7) and mitochondrial functions (TOMM20) were measured in the jejunum mucosa of CR mice (see Figure 18 A), which are processes typically connected to the influence of CR in the body and are one of the beneficial effects of restriction on the GIT [2]. Hereby, the presented results extend the knowledge of the response of the GIT to CR in the context of oxidative stress, inflammation and restriction-related markers, as well as microbiota composition and taurine and BAs' metabolism. However, the exact influence of CR and its outcomes need to be further investigated.

4.2 Impact of macronutrient restriction on the GIT

To the best of the current knowledge, this is the first investigation to explore the restriction of all the three macronutrients and its effect on intestinal inflammation, microbiota, as well as metabolism of taurine and BAs. The few existing studies have addressed restrictions separately in terms of ageing but none of them compared the limitations to other macronutrients or a typical energy restriction [19–22].

4.2.1 Antioxidative processes

The low-macronutrient interventions did not seem to have a great statistically significant impact on antioxidative processes compared to a standard *ad libitum* feeding. However, an Ad lib LC diet showed a similar or even a partly stronger effect than CR in terms of its activity on GSH regulation, since the Nrf2 expression was even lower than in the CR group and the Gsta3 and Mgst1 genes were upregulated in the jejunum mucosa of the Ad lib LC with a statistically significant difference observed for the Gsta3 gene (see Figure 6 A). This coincided with the lower levels of GSH in the ileum mucosa and in the liver of the Ad lib LC group (see Figure 12 C and 12 F). Interestingly, an increase of carbohydrates during CR showed a reverse effect in the expression of Gsta3 and Mgst1, although not statistically significant (see Figure 6 B). However, this dietary intervention also statistically significantly reduced GSH levels in the ileum mucosa (see Figure 13 C). These results suggest that carbohydrates play a major role in the effect of diet on GSH activity, but further research is needed to define the exact mechanism.

4.2.2 Inflammation

Contradictory to the observations of Ling et al. where a low-protein diet increased inflammation [23], the measurements of inflammatory markers in the jejunum here indicate that an Ad lib LP could be an alternative diet to CR to reduce inflammation in the mucosa, since it resulted in decreased the expression of Stat1, Reg3g, MyD88, Tlr3 and Rsad2 with the last four genes adding up to a statistically significant difference in comparison to the Ad lib group (see Figure 7 A). Interestingly, the applied CR HP diet induced lower expression of inflammatory genes than CR in the jejunum mucosa, although not statistically significant (see Figure 7 B). This is in line with the results of Lan et al. [35], where the use of a high-protein diet decreased cytokine's gene expression in the ileum. However, it needs to be considered, that the diet here was also a CR one and suggest that the role of protein on inflammation in the gut needs further investigation. On the other hand, an Ad lib LC diet had a similar effect as a standard *ad libitum* diet on the measured inflammatory parameters (see Figure 7 A), whereas an increase of carbohydrates during CR was not able to erase the anti-inflammatory effect on the gut (see Figure 7 B). These observations were further confirmed by the observed IκB content in the jejunum mucosa. Namely, the amount of this protein was increased in the Ad lib LP and CR HP and decreased in the Ad lib LC group (see Figure 18 A and Figure 19 A). Contradictory to the observed expressions of inflammatory genes in the jejunum mucosa, IκB content was also decreased after a CR HC diet, although none of the observations were statistically significant. The IκB kinase is responsible for inhibiting the inflammatory NF-κB pathway. As already described, this mechanism is known to be stimulated by ROS and is associated with ageing [32]. To sum up, these observations imply that concerning inflammation in the jejunum mucosa, there might be an interplay between carbohydrates and protein consumption. Interestingly, an increase of the previously mentioned substances during CR was not able to induce a strong reverse reaction, which underlines the role of CR on inflammation.

4.2.3 Metabolism

Interestingly, this investigation demonstrated that an Ad lib LC could have a similar effect on longevity as CR. Namely, the relative mRNA expression of Sirt1, which is considered one of the most important markers of CR and longevity [60], was almost the same between the two groups and statistically significantly higher compared to Ad lib ($p \leq 0.01$). Moreover, the Ad lib LC diet had an even stronger upregulating effect than CR on fatty acid metabolism-related genes (see Figure 8 A). Age-related and metabolic diseases are linked to problems in lipid homeostasis. Food reduction has been shown to positively affect this through shift in energy

metabolism with increased fatty acid oxidation [36]. Accordingly, an Ad lib LC diet could be considered beneficial in its influence on these pathways.
Interestingly, the increase of carbohydrates during CR did not induce any negative changes in the Sirt1 expression, but reduced the expression of Acsl3, Acot4, Scd1 and Acox2. The increase of the fatty acid metabolism-related genes during CR HF suggests that fat is essential for the positive influence of CR on fatty acid oxidation (see Figure 8 B).

4.2.4 Metabolism of taurine and BAs

A standard CR diet elevated the expression of genes of taurine and BAs metabolism in the jejunum. However, neither the low-macronutrient *ad libitum*, nor the high-macronutrient CR diets showed a consistent impact on the measured parameters (see Figure 9 A and 9 B). To further investigate this, related genes in the liver of the mice were observed. The results demonstrated a partly similar or an even stronger upregulating effect than CR of an Ad lib LC diet on the measured parameters in the liver. In contrast to CR, however, the relative mRNA expression of Fxr in the jejunum and, correspondingly, Shp in the liver of Ad lib LC were upregulated. That might be responsible for the lower Cyp7a1 gene expression observed in the Ad lib LC group and implies that this diet can only partly positively influence the associated genes (see Figure 9 A and Figure 11 A). This data leads to the suggestion that lowering of carbohydrates in an *ad libitum* diet could be able to partly induce a CR-similar phenotype, but needs further investigations since its effects were not reproduced on all of the measured genes connected to taurine and BAs metabolism.
Interestingly, the increase of the selected macronutrients during CR lowered the measured genes in the liver, although the only statistical significance to CR was observed for Cdo and Ntcp (see Figure 11 B). The only exception remains the Cyp7a1 gene. A CR HP diet statistically significantly increased its expression in comparison to CR. However, the exact mechanisms behind these observations remain unknown.
The taurine measurements after macronutrient restriction *ad libitum* suggested that none of the diets would be capable of inducing the observed CR phenotype of increased taurine levels neither in the ileum, nor in the liver. Interestingly, an Ad lib LC, which is similar to CR on some terms, even resulted in statistically significantly lower levels of taurine and its 249 conjugate in the liver (see Figure 12 D and 12 E). However, it needs to be noted that previous results have shown an increase in taurine levels during CR in the gut, but not in the liver [3]. As already described above, an Ad lib LC diet also statistically significantly decreased GSH content in the ileum in comparison to Ad lib (see Figure 12 C). This might be pointing at the previously described mechanism of conjugation of taurine to GSH to enhance its uptake in the intestine during CR, although the current results only indicated that in the Ad lib LC and not in the CR

group [3]. However, as before, these novel observations still need further research, since the exact mechanism is still largely unclear. A statistically significant decrease in taurine content and its presented conjugate in the liver was also observed after a CR HP diet (see Figure 13 D and 13 E). It needs to be noted that both Ad lib LC and CR HP diets had a high percentage of dietary protein (see Table 1 and Table 2), which might influence the need for internal synthesis of taurine and confirms a role of dietary protein in the regulation of taurine levels.

The impact of protein intake was further acknowledged in the BAs measurements. Although not statistically significant, the Ad lib LP continuously showed the lowest levels of BAs in the ileum (see Figure 14 A-I). However, this effect was not that distinct in the liver (see Figure 15 A-I), but would suggest that lowering of protein *ad libitum* and with this of dietary taurine would be detrimental to a CR phenotype of increased BAs content. Interestingly, the Ad lib LF and Ad lib LC led to higher levels than Ad lib of TUDCA, TDCA and TLCA in the ileum (see Figure 14 F-I), which partly coincides with the expression pattern of BAs metabolism-associated genes in the liver of the low-macronutrient groups (see Figure 11 A). The Ad lib LC diet, same as CR, also statistically significantly elevated the levels of CDCA, DCA and UDCA in the liver in comparison to Ad lib. However, the influence of the low-macronutrient diets on BAs content in the liver was not that consistent as in the ileum, suggesting an organ-specific role of the restrictions on the metabolism of BAs (see Figure 15 A-I). The contribution of dietary protein to BAs metabolism was further confirmed in the increased amounts of taurine-conjugated BAs in the ileum and liver in the CR HP group. However, the elevation was not statistically significant (see Figure 16 F-I and Figure 17 F-I). Moreover, in the CR HF and CR HC groups statistically significantly lower levels of BAs were observed in comparison to CR, not only in the ileum, but also in the liver (see Figure 16 A-I and Figure 17 A-I). Both of these diets had a low protein content, in order to compensate for the increase in the other macronutrient during CR (see Table 2). These results suggest that the increase of carbohydrates or fat is a possible way to erase the effects of CR on BAs' metabolism, whereas an increase of protein would have a similar or even a partly stronger effect than CR, especially concerning the taurine-conjugated BAs. To sum up, macronutrient restriction seems to influence taurine and BAs metabolism, but the exact mechanisms need to be further investigated.

4.2.5 Microbiota

On the one hand, the effect of BAs as antimicrobials is largely known as it is a way to protect the intestine from infections. On the other hand, bacteria such as *Lactobacillus*, *Clostridium*, *Bifidobacterium* and *Bacteroides* are also responsible for modifications of BAs, which makes this a symbiotic relationship [50]. It has already previously been shown that there is a tight

connection between microbiota and the CR-triggered alterations in taurine and BAs levels [18]. The here presented results indicate again an interplay between the three. The increase of taurine and unconjugated BAs in the ileum and liver of the CR group was reflected by the lowest content of *Clostridium leptum*, *Akkermansia mucinphila, Bifidobacterium* and *Bacteroides fragilis* in the cecum of the same group (see Figure 10 A). This could be pointing out the antimicrobial effect of BAs or it could also be indicating a shift in the abundance of these bacteria elsewhere. Namely, these results contribute to the previously described model where during CR the microbiota increases deconjugation of BAs from taurine and correspondingly raises the levels of free taurine and taurine-deconjugated species in the gut [18].

In contrast, the lowering of protein in an *ad libitum* diet seemed to be the one intervention that would increase the abundance of *Akkermansia mucinphila, Bifidobacterium* and *Bacteroides fragilis* to an even higher extent than Ad lib, although a statistically significant difference was observed only for the content of the first bacterium (see Figure 10 A). This higher amount of the named bacteria in the cecum of Ad lib LP was mirrored by the lowest BAs levels in the ileum of the same group (see Figure 14 A-I). However, the influence of this diet on free taurine levels was not that distinct (see Figure 12 A). These observations again not only confirm a role of microbiota in the metabolism of BAs, but also suggest that the lowering of protein *ad libitum* would be detrimental in inducing a CR-similar phenotype, which might be dependent on microbiota. Interestingly, the increase of protein during CR was the one intervention that induced similar low levels of the same three bacteria as a standard CR diet (see Figure 10 B). Although not statistically significant in comparison to CR, in the CR HP group higher levels of taurine-conjugated BAs in the ileum and liver were observed, whereas the unconjugated compounds were not that strongly influenced (see Figure 16 A-I and Figure 17 A-I). On the other hand, the CR HF and CR HC showed a continuous significant lower BAs content in comparison to CR not only in the ileum, but also in the liver (see Figure 16 A-I and Figure 17 A-I), which resulted in higher levels of *Akkermansia mucinphila, Bifidobacterium* and *Bacteroides fragilis* in the cecum of the mice (see Figure 10 B). These observations raise further questions on the exact impact of microbiota on the metabolism of BAs during CR and what the role of the macronutrients is on this mechanism.

Generally, none of the low-macronutrient diets were in the position to reproduce the effects of CR on microbiota. Interestingly, the Ad lib LC, which showed similar measurements to CR in terms of longevity and metabolism, levels of taurine-conjugated BAs in the ileum, as well as those of CDCA, DCA and UDCA in the liver, showed an opposite impact on *Clostridium leptum*, *Akkermansia mucinphila* and *Bacteroides fragilis* content. It also to no extent positively regulated the amount of *Lactobacillus*. This bacterium is known to alleviate inflammation in the gut and although here not measured, to support mucin production [46]. Its decrease has been

also associated with ageing [27]. As previously mentioned, it has been shown to be stimulated by CR, which was again confirmed by the current results (see Figure 10 A) [46]. However, none of the low-macronutrient *ad libitum* diets or even the high-macronutrient caloric restricted ones had the same upregulating effect on this bacterium's content as CR. The CR HC, same as Ad lib, even led to a statistically significantly lower measurements compared to CR (see Figure 10 B). Interestingly, all of the *ad libitum* diets had elevated *Bacteroides fragilis* levels. Although *Firmicutes* content here was not measured, an increase of *Firmicutes* to *Bacteroidetes* ratio has been connected to obesity [16, 41]. Statistically significantly higher measurements than CR of *Bacteroidetes* were also observed in the CR HF and CR HC groups (see Figure 10 B). However, it is not possible propose a statement based on these results without knowing the available *Firmicutes* content.

In conclusion, these observations validate the important role of microbiota in the metabolism of BAs and suggest a correlation in the observed microbiota composition in the cecum and BAs levels in the ileum and liver. In general, the bacteria content varied between the groups and implied that the composition of the macronutrients in a diet is a deciding factor in shaping the gut microbiome even during CR. It intensified the need to further research the role of *Akkermansia, Bifidobacterium* and *Bacteroides* in BAs metabolism. In reverse, the antimicrobial and anti-inflammatory properties of taurine and BAs on the intestine might also be partly responsible for the bacteria composition in the cecum and the anti-inflammatory effect of CR in the intestine. The current results implied that protein might be the deciding factor in the diet in terms of microbiome and BAs levels. Furthermore, the increase of carbohydrates or fat might have an adverse effect on this particular metabolism during CR. It is also important to mention that the influence of the diet on the body and BAs content might be independent on bacteria to some extent or that other bacteria such as *Firmicutes* play a role in the mentioned processes. The current observations strengthened previous proposed theories, but also raised further questions and encourage the need for more investigations on the influence of macronutrient restriction on the gut microbiota in terms of BAs metabolism.

4.2.6 Restriction-related processes

Reduction in insulin concentration and with this improved insulin sensitivity is one of the main ways that CR affects the organism. A decrease in insulin signaling during CR has been shown to extend maximal life-span [61]. As confirmed by the current measurements of pAKT in the gut, the insulin signaling pathway was less active during a CR diet. Interestingly, the Ad lib LC group also showed a potential positive influence on this marker, although not statistically significant (see Figure 18 A). An increase in autophagy has been connected to an improvement in the functionality of the GIT [2]. The Ad lib LC and Ad lib LF stimulated a raise in ATG7 protein

content in the jejunum in a similar way as CR, although none of the diets made a statistically significant difference to Ad lib. Whereas based on the measurements of TOMM20, the mitochondrial function could not be increased by any of the low-macronutrient diets (see Figure 18 A).

The high-macronutrient CR diets induced an even further downregulation than CR of the protein amount of pAKT. The CR HP showed the strongest decrease of this protein, which indicates that this macronutrient will not only not erase the effect of CR on insulin signaling, but it would rather stimulate it. This diet was also the only one that showed similar measurements as CR on the markers of autophagy and transport along the mitochondrial membrane. The CR HF and CR HC led to a downregulation of the last two parameters. However, the observed impact was relatively mild (see Figure 19 A).

4.2.7 Histological alterations of the gut

The *ad libitum* diets did not induce any drastic changes in the morphology of the ileum and its villi length (see Figure 20 A). This was further confirmed by no significant alterations in the length of the small intestine (see Figure 5 A). However, it needs to be noted that the Ad lib LC diet did reduce the villi's length. This observation was not statistically significant, but it might be in correlation with the measured expression of inflammatory genes in the jejunum (see Figure 7 A).

The thickness of the *muscularis externa* of the colon was lowered after an Ad lib LP diet, but not statistically significant (see Figure 23 A). This has previously been observed during CR with the assumption that a thinning of the colon's wall during energy restriction could be due to a reduced intake of nutrients, especially of amino acids, with this resulting in shrinkage of muscle mass [37]. However the impact here was relatively mild and did not lead to any changes in the colon's length (see Figure 5 C). It needs to be pointed out that the investigations on the morphology of the gut were only done in the *ad libitum* groups and that a further observation in the high-macronutrient CR diets might be needed.

4.3 Limitations and outlook

It is important to emphasize that the used diets are custom-made for the designed investigation and have been applied in an experimental setup. The nutritional composition, the degree of restriction and the duration of the intervention in real-life can differ strongly. In this experiment, the applied restriction of 20 % is relatively mild. The CR chow contains more than the minimal micronutrient requirements for mice and was applied for a short period of time so it should not result in malnutrition or starvation. For this reason, the calorie restricted groups did not receive any supplementation, which is a common procedure during CR. However, their diet

composition was still different than that of the *ad libitum groups*. It also needs to be considered that the low-macronutrient groups were fed *ad libitum,* whereas the CR groups received chow only once a day.

Out of the *ad libitum* low-macronutrient diets, the Ad lib LC showed a few similarities to CR. It is a dietary approach without reduction in energy intake and induction of hunger, which is typical for a CR diet. However, one needs to note that a strong reduction in carbohydrates can also be hard to maintain for some individuals. Interestingly, a CR HP diet boosted some of the CR-typical phenotypes and raised further questions on the role of protein on the health of the GIT. However, this diet still consists of a reduced caloric intake and led to weight loss. It can also possibly be associated with hunger. It cannot be entirely excluded that the positive effects are primarily due to CR. While this research puts focus on macronutrient restriction during an *ad libitum* feeding, it would be curious to investigate the influence that an increase in either protein, fat or carbohydrates *ad libitum* (without excess energy intake) might have. This approach would give an overview if a particular macronutrient could benefit gut health without the inconveniences of reduced energy intake. In addition, a period of 4 weeks is relatively short and cannot be used to make a conclusion for a life-long treatment. What is more, the investigations were done on healthy animals. It would be interesting to compare the measured markers in an already present inflammation of the GIT. Furthermore, due to the lack of data on hunger and appetite markers, the results cannot confirm if a certain intervention is more feasible than CR. Based on these consideration, future studies with a modified setup and measurements of additional parameters are required. Moreover, an investigation in a female model is needed. For these reasons, a generalizability of the current results is hard to achieve and needs further research.

5 Conclusion

The here presented results deepen the current knowledge on the pathways involved with CR and introduce novel information about the effect of macronutrient restriction on the GIT. The first objective of the current project was to find a CR-mimic intervention during an *ad libitum* diet. The observations suggest that an Ad lib LC might be similar to CR on several terms. Firstly, the results indicate that carbohydrates in the diet could be a major factor in GSH regulation. Their lower intake during an *ad libitum* feeding can contribute to increased conjugation of GSH, whereas their increase during CR could have an adverse effect. This was indicated by the upregulated GSH transferases in the Ad lib LC group and an observed downregulation during a CR HC diet in the jejunum mucosa. Secondly, a reduction of carbohydrates in an *ad libitum* diet seemed to influence longevity and fatty acid metabolism in a similar way as CR. Thirdly, the measurements of taurine and BAs metabolism-related genes in the jejunum and liver also showed a partial upregulation in the Ad lib LC group. Yet, Ad lib LC diet was not able to increase taurine levels neither in the ileum, nor in the liver. Although it was not statistically significant, the lowering of carbohydrates *ad libitum* elevated the levels of taurine-conjugated BAs in the ileum more than a CR diet and significantly increased those of CDCA, DCA and UDCA in the liver in comparison to Ad lib. However, the Ad lib LC did not show the same anti-inflammatory effect in the jejunum as CR. On the other hand, the Ad lib LP diet downregulated some of the measured inflammatory markers. However, the lowering of protein was recognized as decreased BAs levels in the ileum, although not statistically significant. It also needs to be noted that none of the *ad libitum* diets was able to achieve a similar microbiota composition in the cecum, as the one of the CR group and that these observations might partly be connected to the different BAs levels.

The second objective of the current project was to recognize if the increase of a certain macronutrient would diminish the positive influence of CR on the body. In general, some slight changes in the high-macronutrient diets were observed, but with no great statistical significances. None of the high-macronutrients diets was in a position to erase the antioxidative and anti-inflammatory effect of CR, as well as its positive influence on autophagy and mitochondrial function. However, it need to be noted that the CR HF and CR HC diets did reduce ATG7 and TOMM20 in the jejunum, but without statistical significance. On the other hand, a CR HP group seemed to keep up best with some of the typical CR phenotypes, as it had partly a similar microbiota composition as CR and was able maintain the stimulation of markers of autophagy and mitochondrial function. The increase of protein in a CR diet was also able to cause higher levels of taurine-conjugated BAs compared to CR in both ileum and liver. In contrast, the increase of fat or carbohydrates caused a statistically significant downregulation in the levels of BAs in the ileum and liver during CR.

To sum up, an Ad lib LC diet showed similarities to CR in the jejunum in terms of GSH regulation, longevity, fatty acid metabolism, as well as taurine and BAs metabolism, but it was not able to reduce inflammation markers in the gut. However, this diet could offer an advantage in achieving a broad spectrum of the positive effects of CR, but without the inconveniences of reduced energy intake and hunger and might be a new therapy measure for a lot of diseases, including those of the GIT. The increase in none of the macronutrients seemed to be in the position to completely erase a CR phenotype. However, the influence of CR on taurine and BAs metabolism might partly be dependent on composition of the diet. This was suggested by higher levels of taurine-conjugated BAs in the CR HP group and lower levels of BAs in the CR HF and CR HC groups, both in the ileum and liver.

Currently, the knowledge on the response of the GIT to CR is still largely unclear. This study extends the information about the influence of CR on the intestine and the liver. It also delivers new data about the effects of macronutrient restriction. However, the exact physiological mechanisms behind macronutrient restriction and CR and their outcomes remain to a large extend undetermined and trigger the need for further research.

References

1. Duszka, K.: Versatile Triad Alliance: Bile Acid, Taurine and Microbiota. Cells **11**(15), 2337 (2022). doi: 10.3390/cells11152337
2. Gregor, A., Huber, L., Auernigg-Haselmaier, S., Sternberg, F., Billerhart, M., Dunkel, A., Somoza, V., Ogris, M., Kofler, B., Longo, V.D., König, J., Duszka, K.: A Comparison of the Impact of Restrictive Diets on the Gastrointestinal Tract of Mice. Nutrients **14**(15), 3120 (2022). doi: 10.3390/nu14153120
3. Gregor, A., Pignitter, M., Fahrngruber, C., Bayer, S., Somoza, V., König, J., Duszka, K.: Caloric restriction increases levels of taurine in the intestine and stimulates taurine uptake by conjugation to glutathione. The Journal of nutritional biochemistry **96**, 108781 (2021). doi: 10.1016/j.jnutbio.2021.108781
4. Speakman, J.R., Mitchell, S.E.: Caloric restriction. Molecular aspects of medicine **32**(3), 159–221 (2011). doi: 10.1016/j.mam.2011.07.001
5. Maestri, E., Duszka, K., Kuznetsov, V.A.: Immunity Depletion, Telomere Imbalance, and Cancer-Associated Metabolism Pathway Aberrations in Intestinal Mucosa upon Short-Term Caloric Restriction. Cancers **13**(13), 3180 (2021). doi: 10.3390/cancers13133180
6. Duszka, K., Gregor, A., Guillou, H., König, J., Wahli, W.: Peroxisome Proliferator-Activated Receptors and Caloric Restriction-Common Pathways Affecting Metabolism, Health, and Longevity. Cells **9**(7), 1708 (2020). doi: 10.3390/cells9071708
7. Osborne, T.B., Mendel, L.B., Ferry, E.L.: The effect of retardation of growth upon the breeding period and duration of life of rats. Science (New York, N.Y.) **45**(1160), 294–295 (1917). doi: 10.1126/science.45.1160.294
8. McCay, C.M., Crowell, M.F., Maynard, L.A.: The Effect of Retarded Growth Upon the Length of Life Span and Upon the Ultimate Body Size: One Figure. J Nutr **10**(1), 63–79 (1935). doi: 10.1093/jn/10.1.63
9. Fontana, L., Partridge, L.: Promoting health and longevity through diet: from model organisms to humans. Cell **161**(1), 106–118 (2015). doi: 10.1016/j.cell.2015.02.020
10. Fontana, L., Partridge, L., Longo, V.D.: Extending healthy life span-from yeast to humans. Science (New York, N.Y.) **328**(5976), 321–326 (2010). doi: 10.1126/science.1172539
11. Masoro, E.J.: Caloric restriction and aging: an update. Experimental gerontology **35**(3), 299–305 (2000). doi: 10.1016/s0531-5565(00)00084-x
12. Most, J., Tosti, V., Redman, L.M., Fontana, L.: Calorie restriction in humans: An update. Ageing research reviews **39**, 36–45 (2017). doi: 10.1016/j.arr.2016.08.005
13. Colman, R.J., Anderson, R.M., Johnson, S.C., Kastman, E.K., Kosmatka Kristopher J., Beasley Mark T., Allison David B., Cruzen, C., Simmons Heather A., Kemnitz Joseph

W., Weindruch, R.: Caloric Restriction Delays Disease Onset and Mortality in Rhesus Monkeys. Science (New York, N.Y.) **325**(5937), 201–203 (2009). doi: 10.1126/science.1176225

14. Fontana, L., Klein, S.: Aging, adiposity, and calorie restriction. JAMA **297**(9), 986–994 (2007). doi: 10.1001/jama.297.9.986
15. Masoro, E.J.: Overview of caloric restriction and ageing. Mechanisms of ageing and development **126**(9), 913–922 (2005). doi: 10.1016/j.mad.2005.03.012
16. Duszka, K., Ellero-Simatos, S., Ow, G.S., Defernez, M., Paramalingam, E., Tett, A., Ying, S., König, J., Narbad, A., Kuznetsov, V.A., Guillou, H., Wahli, W.: Complementary intestinal mucosa and microbiota responses to caloric restriction. Scientific reports **8**(1), 11338 (2018). doi: 10.1038/s41598-018-29815-7
17. Gabandé-Rodríguez, E., Gómez de Las Heras, M.M., Mittelbrunn, M.: Control of Inflammation by Calorie Restriction Mimetics: On the Crossroad of Autophagy and Mitochondria. Cells **9**(1), 82 (2019). doi: 10.3390/cells9010082
18. Gregor, A., Pignitter, M., Trajanoski, S., Auernigg-Haselmaier, S., Somoza, V., König, J., Duszka, K.: Microbial contribution to the caloric restriction-triggered regulation of the intestinal levels of glutathione transferases, taurine, and bile acid. Gut microbes **13**(1), 1992236 (2021). doi: 10.1080/19490976.2021.1992236
19. Schulz, T.J., Zarse, K., Voigt, A., Urban, N., Birringer, M., Ristow, M.: Glucose restriction extends Caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress. Cell metabolism **6**(4), 280–293 (2007). doi: 10.1016/j.cmet.2007.08.011
20. Sanz, A., Caro, P., Barja, G.: Protein Restriction Without Strong Caloric Restriction Decreases Mitochondrial Oxygen Radical Production and Oxidative DNA damage in Rat Liver. Journal of bioenergetics and biomembranes **36**(6), 545–552 (2004). doi: 10.1007/s10863-004-9001-7
21. Lee, B.C., Kaya, A., Ma, S., Kim, G., Gerashchenko, M.V., Yim, S.H., Hu, Z., Harshman, L.G., Gladyshev, V.N.: Methionine restriction extends lifespan of Drosophila melanogaster under conditions of low amino-acid status. Nature communications **5**, 3592 (2014). doi: 10.1038/ncomms4592
22. Marte, M.L. de, Enesco, H.E.: Influence of low tryptophan diet on survival and organ growth in mice. Mechanisms of ageing and development **36**(2), 161–171 (1986). doi: 10.1016/0047-6374(86)90017-5
23. Ling, P.-R., Smith, R.J., Kie, S., Boyce, P., Bistrian, B.R.: Effects of protein malnutrition on IL-6-mediated signaling in the liver and the systemic acute-phase response in rats. American journal of physiology. Regulatory, integrative and comparative physiology **287**(4), R801-8 (2004). doi: 10.1152/ajpregu.00715.2003

24. Ling, P.-R., Bistrian, B.R.: Comparison of the effects of food versus protein restriction on selected nutritional and inflammatory markers in rats. Metabolism: clinical and experimental **58**(6), 835–842 (2009). doi: 10.1016/j.metabol.2009.03.002
25. Pal Yu, B.: Aging and oxidative stress: modulation by dietary restriction. Free Radical Biology & Medicine **21**(5), 651–668 (1996). doi: 10.1046/j.0013-0427.2003.00027.x
26. Navarro, A., Boveris, A.: The mitochondrial energy transduction system and the aging process. American journal of physiology. Cell physiology **292**(2), C670-86 (2007). doi: 10.1152/ajpcell.00213.2006
27. Ferrucci, L., Fabbri, E.: Inflammageing: chronic inflammation in ageing, cardiovascular disease, and frailty. Nature reviews. Cardiology **15**(9), 505–522 (2018). doi: 10.1038/s41569-018-0064-2
28. Jackson, D.N., Theiss, A.L.: Gut bacteria signaling to mitochondria in intestinal inflammation and cancer. Gut microbes **11**(3), 285–304 (2020). doi: 10.1080/19490976.2019.1592421
29. Bagherniya, M., Butler, A.E., Barreto, G.E., Sahebkar, A.: The effect of fasting or calorie restriction on autophagy induction: A review of the literature. Ageing research reviews **47**, 183–197 (2018). doi: 10.1016/j.arr.2018.08.004
30. Randall-Demllo, S., Chieppa, M., Eri, R.: Intestinal epithelium and autophagy: partners in gut homeostasis. Front. Immunol. **4**, 301 (2013). doi: 10.3389/fimmu.2013.00301
31. Collier, J.J., Suomi, F., Oláhová, M., McWilliams, T.G., Taylor, R.W.: Emerging roles of ATG7 in human health and disease. EMBO molecular medicine **13**(12), e14824 (2021). doi: 10.15252/emmm.202114824
32. Chung, H.Y., Kim, H.J., Kim, J.W., Yu, B.P.: The inflammation hypothesis of aging. Annals of the New York Academy of Sciences **928**(1), 327–335 (2001). doi: 10.1111/j.1749-6632.2001.tb05662.x
33. Molodecky, N.A., Soon, I.S., Rabi, D.M., Ghali, W.A., Ferris, M., Chernoff, G., Benchimol, E.I., Panaccione, R., Ghosh, S., Barkema, H.W., Kaplan, G.G.: Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology **142**(1), 46-54 (2012). doi: 10.1053/j.gastro.2011.10.001
34. Vidal-Lletjós, S., Beaumont, M., Tomé, D., Benamouzig, R., Blachier, F., Lan, A.: Dietary Protein and Amino Acid Supplementation in Inflammatory Bowel Disease Course: What Impact on the Colonic Mucosa? Nutrients **9**(3), 310 (2017). doi: 10.3390/nu9030310
35. Lan, A., Andriamihaja, M., Blouin, J.-M., Liu, X., Descatoire, V., Desclée de Maredsous, C., Davila, A.-M., Walker, F., Tomé, D., Blachier, F.: High-protein diet differently modifies intestinal goblet cell characteristics and mucosal cytokine expression in ileum

and colon. The Journal of nutritional biochemistry **26**(1), 91–98 (2015). doi: 10.1016/j.jnutbio.2014.09.007

36. Mezhnina, V., Pearce, R., Poe, A., Velingkaar, N., Astafev, A., Ebeigbe, O.P., Makwana, K., Sandlers, Y., Kondratov, R.V.: CR reprograms acetyl-CoA metabolism and induces long-chain acyl-CoA dehydrogenase and CrAT expression. Aging cell **19**(11), e13266 (2020). doi: 10.1111/acel.13266
37. Schoffen, J.P.F., Vicentini, F.A., Marcelino, C.G., Araújo, E.J.A., Pedrosa, M.M.D., Natali, M.R.M.: Food restriction beginning at lactation interferes with the cellular dynamics of the mucosa and colonic myenteric innervation in adult rats. Anais da Academia Brasileira de Ciencias **86**(4), 1833–1848 (2014). doi: 10.1590/0001-3765201420140163
38. Chwen, L.T., Foo, H.L., Thanh, N.T., Choe, D.W.: Growth performance, plasma Fatty acids, villous height and crypt depth of preweaning piglets fed with medium chain triacylglycerol. Asian-Australasian journal of animal sciences **26**(5), 700–704 (2013). doi: 10.5713/ajas.2012.12561
39. Hermes, C., Fernandes de Azverdo, J., José de Almeida Araújo, Eduardo, Mello Gonçales Sant'Ana, D. de: Intestinal Ascending Colon Morphometrics in Rats Submitted to Severe Protein Malnutrition. Internation Journal of Morphology **26**(1), 5–11 (2008). doi: 10.4067/S0717-95022008000100001
40. Valdes, A.M., Walter, J., Segal, E., Spector, T.D.: Role of the gut microbiota in nutrition and health. BMJ (Clinical research ed.) **361**, k2179 (2018). doi: 10.1136/bmj.k2179
41. Franklin, C.L., Ericsson, A.C.: Microbiota and reproducibility of rodent models. Lab animal **46**(4), 114–122 (2017). doi: 10.1038/laban.1222
42. Duszka, K., Wahli, W.: Enteric Microbiota-Gut-Brain Axis from the Perspective of Nuclear Receptors. International journal of molecular sciences **19**(8), 2210 (2018). doi: 10.3390/ijms19082210
43. Fujimura, K.E., Slusher, N.A., Cabana, M.D., Lynch, S.V.: Role of the gut microbiota in defining human health. Expert review of anti-infective therapy **8**(4), 435–454 (2010). doi: 10.1586/eri.10.14
44. Rothschild, D., Weissbrod, O., Barkan, E., Kurilshikov, A., Korem, T., Zeevi, D., Costea, P.I., Godneva, A., Kalka, I.N., Bar, N., Shilo, S., Lador, D., Vila, A.V., Zmora, N., Pevsner-Fischer, M., Israeli, D., Kosower, N., Malka, G., Wolf, B.C., Avnit-Sagi, T., Lotan-Pompan, M., Weinberger, A., Halpern, Z., Carmi, S., Fu, J., Wijmenga, C., Zhernakova, A., Elinav, E., Segal, E.: Environment dominates over host genetics in shaping human gut microbiota. Nature **555**(7695), 210–215 (2018). doi: 10.1038/nature25973

45. Zhang, C., Zhang, M., Wang, S., Han, R., Cao, Y., Hua, W., Mao, Y., Zhang, X., Pang, X., Wei, C., Zhao, G., Chen, Y., Zhao, L.: Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. The ISME journal **4**(2), 232–241 (2010). doi: 10.1038/ismej.2009.112
46. Gregor, A., Fragner, L., Trajanoski, S., Li, W., Sun, X., Weckwerth, W., König, J., Duszka, K.: Cage bedding modifies metabolic and gut microbiota profiles in mouse studies applying dietary restriction. Scientific reports **10**(1), 20835 (2020). doi: 10.1038/s41598-020-77831-3
47. Bäckhed, F., Jill, K.M., Clay, F.S., Gordon, I.J.: Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proceedings of the National Academy of Sciences **104**(3), 979–984 (2007). doi: 10.1073/pnas.0605374104
48. Schuller-Levis, G.B., Park, E.: Taurine: new implications for an old amino acid. FEMS microbiology letters **226**(2), 195–202 (2003). doi: 10.1016/S0378-1097(03)00611-6
49. Yu, H., Guo, Z., Shen, S., Shan, W.: Effects of taurine on gut microbiota and metabolism in mice. Amino acids **48**(7), 1601–1617 (2016). doi: 10.1007/s00726-016-2219-y
50. Guzior, D.V., Quinn, R.A.: Review: microbial transformations of human bile acids. Microbiome **9**(1), 140 (2021). doi: 10.1186/s40168-021-01101-1
51. Aguiar Vallim, T.Q. de, Tarling, E.J., Edwards, P.A.: Pleiotropic roles of bile acids in metabolism. Cell metabolism **17**(5), 657–669 (2013). doi: 10.1016/j.cmet.2013.03.013
52. Parséus, A., Sommer, N., Sommer, F., Caesar, R., Molinaro, A., Ståhlman, M., Greiner, T.U., Perkins, R., Bäckhed, F.: Microbiota-induced obesity requires farnesoid X receptor. Gut **66**(3), 429–437 (2017). doi: 10.1136/gutjnl-2015-310283
53. Pols, T.W.H., Noriega, L.G., Nomura, M., Auwerx, J., Schoonjans, K.: The bile acid membrane receptor TGR5: a valuable metabolic target. Digestive diseases (Basel, Switzerland) **29**(1), 37–44 (2011). doi: 10.1159/000324126
54. Guo, C., Chen, W.-D., Wang, Y.-D.: TGR5, Not Only a Metabolic Regulator. Frontiers in physiology **7**, 646 (2016). doi: 10.3389/fphys.2016.00646
55. Joyce, S.A., Gahan, C.G.M.: Disease-Associated Changes in Bile Acid Profiles and Links to Altered Gut Microbiota. Digestive diseases (Basel, Switzerland) **35**(3), 169–177 (2017). doi: 10.1159/000450907
56. Ward, J.B.J., Lajczak, N.K., Kelly, O.B., O'Dwyer, A.M., Giddam, A.K., Ní Gabhann, J., Franco, P., Tambuwala, M.M., Jefferies, C.A., Keely, S., Roda, A., Keely, S.J.: Ursodeoxycholic acid and lithocholic acid exert anti-inflammatory actions in the colon. American journal of physiology. Gastrointestinal and liver physiology **312**(6), G550-G558 (2017). doi: 10.1152/ajpgi.00256.2016
57. Pan, X.-L., Zhao, L., Li, L., Li, A.-H., Ye, J., Yang, L., Xu, K.-S., Hou, X.-H.: Efficacy and safety of tauroursodeoxycholic acid in the treatment of liver cirrhosis: a double-blind

randomized controlled trial. Journal of Huazhong University of Science and Technology. Medical sciences = Hua zhong ke ji da xue xue bao. Yi xue Ying De wen ban = Huazhong keji daxue xuebao. Yixue Yingdewen ban **33**(2), 189–194 (2013). doi: 10.1007/s11596-013-1095-x

58. Wang, W., Zhao, J., Gui, W., Sun, D., Dai, H., Xiao, L., Chu, H., Du, F., Zhu, Q., Schnabl, B., Huang, K., Yang, L., Hou, X.: Tauroursodeoxycholic acid inhibits intestinal inflammation and barrier disruption in mice with non-alcoholic fatty liver disease. British journal of pharmacology **175**(3), 469–484 (2018). doi: 10.1111/bph.14095
59. Hardison, W.G.: Hepatic Taurine Concentration and Dietary Taurine as Regulators of Bile Acid Conjugation with Taurine. Gastroenterology **75**(1), 71–75 (1978). doi: 10.1016/0016-5085(78)93767-8
60. Chang, H.-C., Guarente, L.: SIRT1 and other sirtuins in metabolism. Trends in endocrinology and metabolism: TEM **25**(3), 138–145 (2014). doi: 10.1016/j.tem.2013.12.001
61. Fontana, L., Klein, S., Holloszy, J.O.: Effects of long-term calorie restriction and endurance exercise on glucose tolerance, insulin action, and adipokine production. Age (Dordrecht, Netherlands) **32**(1), 97–108 (2010). doi: 10.1007/s11357-009-9118-z