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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich diese Masterarbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt und die aus fremden Quellen direkt oder indirekt übernommenen Gedanken als solche kenntlich gemacht habe.

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Wien, 2017

Index of Abbreviations

4-APEBA	4-(2-((4-bromophenethyl)dimethylammonio)ethoxy)benzenaminiumdibromide
AA	Amino Acid
abWAT	Abdominal white adipose tissue
ACN	Acetonitrile
AD	Autoimmune disease
BAT	Brown Adipose Tissue
CBT	Core body temperature
CLA	Conjugated linoleic acid
CR	Caloric restriction
DPP	Dipeptidyl peptidase
dWAT	Dorsal white adipose tissue
EDC	(N- (3-Dimethylaminopropyl) -N'- ethylcarbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
ESI	Electrospray ionization
eWAT	Epididymal white adipose tissue
FA	Fatty acid
FFA	Free Fatty Acid
IGF	Insulin-like growth factor
LA	Lauric Acid
LCFA	Long Chain Fatty Acid
MCFA	Middle Chain Fatty Acid
LCT	Long Chain Triglyceride
MeOH	Methanol
MG	Monoglyceride
MCT	Middle Chain Triglyceride
MIC	Minimum Inhibitory Concentration
MO	Microorganism

ND	Neurodegenerative disease
NHS	N-Hydroxysuccinimide
NK	Natural killer
PPAR	Peroxisome Proliferator Activated Receptor
ROS	Reactive oxygen species
SCFA	Short Chain Fatty Acid
STD_O	Caprylate analytical standard
STD_R	Isotopically labelled caprylate
TG	Triglyceride
UCP	Uncoupling Protein
WAT	White Adipose Tissue

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1 Abstract

Caprylate is a medium chained fatty acid (MCFA), mostly known for activating the orexigenic hormone ghrelin. Therefore, caprylate is especially important while fasting. In order to elucidate the effect of caloric restriction (CR) on caprylate plasma level, mice were subjected to a CR diet for two weeks. By using wood, corncob and cellulose beddings, the impact of the bedding type on CR-outcomes as well as on the caprylate plasma level were evaluated.

As there was no well-established method for the detection of caprylate in the plasma of mice, a new method was developed. The sample preparation included the derivatization of caprylate with the derivatization reagent 4-(2-((4-bromophenethyl) dimethylammonio) ethoxy) benzenaminiumdibromide (4-APEBA) which was analysed by LC-MS.

This thesis supports the observation that CR mice tend to eat their bedding, preferably the wooden one, the second favourite being the corncob and the least favoured being the cellulose bedding (1.39g ± 0.05g vs 1.01g ± 0.06g vs 0.61g ± 0.06g, respectively; $p<0.001$). It was observed that the bedding type did neither significantly influence the daily body weight loss, nor the total body weight loss, nor the time it took the mice to start the meal, nor were the glucose levels or the stomach weight expressed in percent of the body weight affected. However, the cecum weight was influenced by the highest fiber and probably by the cellulose intake.

In the feces, elevated caprylate concentrations in the CR groups cellulose and wood were observed. This may be due to the overall

higher cellulose intake, which can be metabolized by the microbiota into caprylate. The caprylate plasma levels were lower in the CR groups (6.62µg/ml ± 0.29µg/ml) than in the groups fed *ad libitum* (8.38µg/ml ± 0.24µg/ml). There was no difference in the plasma caprylate levels within the CR or the control group. Interestingly, the caprylate plasma level of the CR group with the wooden bedding, and thus with the highest fiber intake, did not differ from the one of the control groups.

Altogether, these results show that CR does have an effect on the plasma caprylate concentration and that the bedding type may have an impact on the feces as well as on the plasma caprylate concentrations.

2 Zusammenfassung

Caprylsäure zählt zu den mittelkettigen Triglyceriden und ist vorwiegend als wichtigster Aktivator von Ghrelin bekannt. Die vorliegende Masterarbeit untersucht die Auswirkung von Kalorienrestriktion auf den Caprylsäurespiegel von Mäusen. Einerseits wurden die Auswirkungen des Einstreutyps auf die Ergebnisse der kalorischen Restriktion untersucht, wofür Einstreu aus Holz, Maiskolben und Zellulose verwendet. Andererseits wurde der Einfluss des Caprylsäuregehalts im Blutplasma oder im Stuhl überprüft.

Da es für die Plasma-Caprylsäure-Analyse noch keine etablierte Methode gibt und eine solche trotzt intensiver Recherchen nicht auffindbar war, wurde eine neue Methode mit LC-MS/MS entwickelt. Diese schließt die Derivatisierung der Caprylsäure mit dem Reagens 4-(2-((4-bromophenethyl) dimethylammonio) ethoxy) benzenaminiumdibromide (4-APEBA) ein.

Die vorliegende Arbeit bestätigt die Beobachtung, dass Mäuse unter Kalorienrestriktion die Einstreu fressen. Es ließ sich beobachten, dass jene Streu, die am meisten konsumiert wurde, Holz war, gefolgt von Maiskolben. Jene aus Zellulose war am unbeliebtesten (1,39g ± 0,05g vs 1,01g ± 0,06g vs 0,61g ± 0,06g; $p<0,001$). Der Genuss der Einstreu hat weder einen signifikanten Einfluss auf den täglichen Körpergewichtsverlust, den gesamten Körpergewichtsverlust, die Dauer, bis die Mäuse zu fressen beginnen, noch auf die Blutzuckerwerte oder das Magengewicht (gemessen in Prozent des Körpergewichtes). Jedoch wurde der Caecumgewicht durch die Ballaststoffzufuhr und wahrscheinlich der Zelluloseaufnahme beeinflusst.

Jene Versuchsgruppen, die unter Kalorienrestriktion eine erhöhte Stuhl-Caprylsäure-Konzentrationen aufwiesen, waren diejenigen, die Zellulose und Holz gefressen hatten. Als Ursache für eine solche Konzentration kann die erhöhte Zellulose-Aufnahme gesehen werden, welche die Anzahl der Caprylsäure-produzierenden Bakterien fördern könnte. Die Plasma-Caprylsäure-Konzentration war in der Kontrollgruppe (8,38µg/ml ± 0,24µg/ml) höher als in den Gruppen unter Kalorienrestriktion (6,62µg/ml ± 0,29µg/ml). Innerhalb der Kontrollgruppen und jene die der Kalorienrestriktion ausgesetzt wurden, waren keine signifikante Unterschiede bezüglich des Plasma-Caprylsäure-Spiegels zu beobachten. Interessanterweise war auch zwischen der kalorienreduzierten Gruppe mit der Holz-Einstreu und den Kontrollgruppen kein signifikanter Unterschied zu erkennen.

Schlussendlich deuten die Ergebnisse des für diese Arbeit durchgeführten Experiments auf eine Beeinflussung des Plasmagehalts und der Caprylsäure-Konzentration im Stuhl durch Kalorienrestriktion und Einstreutyp hin.

3 Introduction

CR is a reduction of the *ad libitum* calorie intake. It is given as a percentage of calories, so that the *ad libitum* consumption represents 100%. Due to its life and health span extending characteristics, caloric restriction has often been studied across all species from yeast to primates. Most of these studies were conducted on mice. For CR experiments in different laboratories mice were housed under similar light/dark cycle, temperature and in sterile cages, but different types of cage bedding materials were used, such as spruce, poplar, hardwood, red cedar, corncob and cellulose.

It has been observed that CR mice tend to eat the bedding but the effects of the bedding type on the metabolome, transcriptome or microbiome of the mice have not been assessed.

The objective of the present experiment is to determine the influence of the bedding on body weight and body weight composition, the organs´ weight, glucose tolerance, hunger, calorie content of the feces and the assessment of the amount of the bedding eaten. For this purpose, three popular types of bedding were used: wood (poplar), corncob and cellulose pellets.

Furthermore, a specific MCFA, the caprylate circulating concentration and its appearance in the feces were of interest. Caprylate is well known for its antibacterial properties and its key role in the acylation of ghrelin but there is no data concerning caprylate or any other MCFA plasma level. The main aim of this thesis is to analyse how CR and the bedding type influences caprylate levels in the plasma and feces of mice.

As the only published method to quantify caprylate in plasma proved to be not sensitive in our lab, a new method, with the lowest limit of detection possible, was developed. The caprylate levels in the feces were assessed with nuclear magnetic resonance (NMR) spectroscopy.

This master thesis will present a new laboratory method for plasma caprylate analysis and focus on the relation between caprylate concentrations in plasma and feces and CR and bedding type.

3.1 Caloric restriction

CR is a reduction of the daily calorie intake. Most of the studies investigating the effects of a CR diet restrict the calorie intake between 20-40%. But, there are also more rigorous ones where the restriction is higher than 50%. [Blondhein et al., 1981]

As a reaction to CR, animals lose weight, mostly from the white adipose tissue (WAT), whereas the lean body mass is less effected or unchanged. [Bertrand et al., 1980] Therefore, CR is a commonly used strategy to treat obesity. [Dahlman et al., 2005] With the weight loss, the body composition changes and the resting metabolic rate (RMR) drops. [Hambly and Speakman, 2005] The physical activity patterns remain unchanged or reduced and the most active phase is prior to the meal arriving. [Mistlberger, 1994] Insulin and glucose levels decline as well as the plasma adipokines [Speakman and Mitchell, 2011] and the metabolism shifts from carbohydrate to fat metabolism. [Chen et al., 2008]

3.1.1 Positive Effects of CR

For at least 500 years, CR diets have been used for life extension and for life quality improvement. [Speakman and Mitchell 2011] One of the first experimental works on CR, published by McCay and Crowell [1935], showed a prolongation of the lives of CR-restricted rats and thus, confirmed the observations of several hundred years. Later, numerous studies observed similar processes on rodents such the one of McCay and Crowell, [Heilbronn and Ravussin, 2003] showing that a lifelong CR could extend the lifespan for nearly 50% on rodents. [Abalan et al., 2010] In order to obtain the highest possible life extending effect a CR of 55%-60% seems to be necessary, a higher restriction lowers the lifespan. [Speakman and Mitchell 2011] One explanation for the prolongation of life may be the decreased core body temperature (CBT) which occurs during CR. [Rikke and Johnson 2004] Lowering the CBT by 0.3-0.5 °C in mice by affecting thermoregulatory mechanisms showed an increase in the median life expectancy in absence of CR group up to a 20%. [Conti et al., 2006] Another example to explain the longevity may be the optimized cell recycling process called autophagocytosis (autophagy). Rubinsztein et al. (2011) showed a neutralization of CR´s protective effects on aging by the inhibition of autophagy.

CR may not just extend the lifespan, but it may also lower the risk for age related diseases such as type II diabetes mellitus, cancer, neurodegenerative disorders, cardiovascular diseases and autoimmune diseases. [Speakman and Mitchell, 2011]

3.1.1.1 Diabetes

In 2014, 422 million people were diagnosed with diabetes which is a major cause of heart attacks, strokes, kidney failure, blindness and lower limb amputations. [Roglic, 2016] In type II diabetes, CR lowers the hepatic triacylglycerol content and normalizes the fasting plasma glucose levels. [Lim et al., 2011] The normalization of the glucose levels take place within the first week of the CR therapy. In this short amount of time there is no change in the muscular insulin resistance, which is considered the earliest detectable abnormality leading to type II diabetes. The reason for the improved fasting glucose levels may be due to its rapid drop within the liver fat content as well as the improved hepatic insulin sensitivity. [Taylor, 2013]

In some cases of diabetes and/or obesity, CR is not an option and has to be carried out for a lifetime due to a bariatric surgery, for example after sleeve-gastrectomy or gastric bypass. Here, one can distinguish between restrictive and malabsorptive methods: restrictive methods limit the food intake, whereas malabsorptive ones limit the bioavailability of food. Both of them lead to a lower supply of macronutrients to the body. The same happens when undergoing a CR diet. Nevertheless, a meta-analysis on 6 131 people suffering from type II diabetes showed that on the long run, a bariatric operation is 10–16 times more efficient than a traditional diet. Although a conventional therapy may achieve excellent results with very low calorie diets, the main problem, is the low compliance due to strict dietary restrictions. [Ribaric et al., 2014]

3.1.1.2 Cancer and autoimmune diseases

The number of cancer cases around the world is expected to increase from 14.1 million (2012) to 24 million by 2035. [WCRFI, 2017] CR may lower the risk and development of certain cancer types. There are several theories for the anti-cancer properties of CR including the change in the body composition, elevation of adiponectin level and reduction of glucose and insulin-like growth factor 1 (IGF-1) levels. [Speakman and Mitchell, 2011] Although, when CR was applied on mice from the weaning, CR had a negative effect on the number of natural killer (NK) cells, which are believed to defend against tumor growth. Applying CR from an older age on, the cytolytic response to poly I:C, a known stimulator of NK cells, was improved. [Weindruch et al., 1983]

In western societies, the frequency of autoimmune diseases (AD) is steadily rising. This is especially the case with celiac disease, type I diabetes and myasthenia gravis. [Lerner et al., 2015] Furthermore, CR mice have greater lymphocyte production and activity in later life and show a delay and reduction in the number of AD. [Speakman and Mitchell, 2011] It is suggested that plasma ghrelin and leptin levels mediate the immune system. In fact, ghrelin regulates T-cell-activation and inflammation. Thus, a ghrelin infusion is capable of partially reverse age related thymic involution. [Dixit et al., 2007] CR also decreases oxidative stress caused by reactive oxygen species (ROS), which protects T-cells and contributes to defending against the development of AD and cancer. In contrary, ROS is essential for fighting off pathogens and thus, a lower number of ROS may lead to infections. In general the anticancer and anti-AD properties of CR exceed the probable negative effects on the immune system. [Speakman and Mitchell, 2011]

3.1.1.3 Neurodegenerative diseases

For neurodegenerative diseases (ND), such as Alzheimer´s, Parkinson´s and Hungtington´s diseases, the greatest risk factor is age thus, the anti-aging properties of CR might extenuate the burden of such diseases. [Ntsapi and Loos, 2016] It is anticipated that CR might improve neurogenesis, synaptic plasticity and cellular self-repair [Prolla and Mattson, 2001]. One mechanism to explain such positive effects could be an induced autophagy. This is a mechanism for breaking down cellular components and recycling them into bioenergetic and biosynthetic materials in order to maintain homeostasis. [Nobelprize, 2017] The suppression of autophagy causes ND in mice [Hara et al., 2006; Komatsu et al., 2006; Alirezaei et al., 2008]. An optimal autophagy might avert the plaque formation associated with neurodegeneration and thus, protects against ND. [Martinez-Vicente and Cuervo, 2007; Ntsapi and Loos, 2016]. In order to fight Alzheimer´s disease, there have already been attempts of developing drugs that upregulate neuronal autophagy. [Nixon 2007; Yu et al., 2005; Geisler et al., 2016] A drug-free possibility to increase autophagy may be CR or even a regimen consisting of extended intervals between meals (intermittent fasting). [Jeong et al., 2016]

3.1.1.4 Cardiovascular diseases

Every year, more people die of cardiovascular diseases, including coronary heart disease and stroke, than from any other cause. In 2015, this meant 31% of all global deaths. [WHO, 2017] CR significantly lowers triglycerides, fasting glucose and fasting insulin as well as systolic and diastolic blood pressure and acts cardioprotective. [Walford et al., 1992; Fontana et al., 2004] CR also contributes to weight loss which can improve heart function and positively influences risk factors of all metabolic coronary heart diseases. [Fontana et al., 2007] The mediation of the enhanced cardiovascular functions may be carried out by cellular and biochemical adaptations such as oxidative stress, mitochondrial function, inflammation, apoptosis and autophagy. [Speakman and Mitchell, 2011]

3.1.2 Negative Effects of CR

A chronic low calorie diet might not deliver the calories and nutrients necessary to maintain homeostasis. Also, the population size and the fertility of any species is closely linked to the energy available in the form of food and thus, a CR diet may impair the reproductive functions. [Martin et al., 2008]

Furthermore, very low calorie diets may cause undesirable symptoms such as insomnia, food craving, fatigue, dry mouth, dizziness and constipation. [Alabdali et al., 2013] Thus, that kind of CR diets should only be carried out for a limited period of time and with a lot of care.

3.2 Caprylate

3.2.1 Introduction

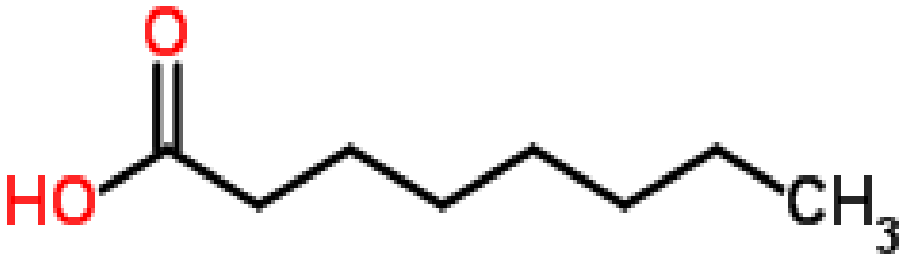

Figure 1: Caprylate. [Chemspider, 2017]

Caprylate (Figure 1) is the common name for the eight-carbon saturated middle chained fatty acid (FA), the octanoic or caprylic acid (C8:0). Caprylate oil is a colorless to light yellow liquid with a rancid, slightly sour taste and an unpleasant irritating odor. Further information is shown in Table 1.

Fatty acids such as caproic acid (C6:0), caprylic acid and capric acid (C10:0) have a very strong odor similar to the smell of a goat. Therefore, their trivial names derive from the Latin word "capra" meaning "goat". All together they are called "capra fatty acids". [Shipley, 2001]

Table 1: Caprylic acid properties. [Chemspider, 2017]

Chemical formula	$C_8H_{16}O_2$
Molar mass	144.21
Density	0.91 g/cm^3
Melting point	16 °C
Boiling point	237 °C
Solubility in water	680 mg/L

3.2.2 Where can caprylate be found?

The highest concentrations of naturally occurring C8:0 can be found in coconut oil (6.8%), palm kernel oil (3.3%) and in butter (1,2%), but it also occurs in cheese (parmesan: 0.3%), goat milk (0.1%) or cow's milk (0,04%). [USDA, 2017]

C8:0 is one of the four MCFAs naturally occurring in human nutrition. The other three MCFAs are the remaining two capra fatty acids and the lauric acid (C12:0 or LA). Middle chain triglyceride (MCT) oil is a processed food that can be extracted from coconut or palm kernel oil, containing only MCFAs. The major part of the oil (65-75%), is the C8:0, the rest of the compounds are 1-2% C6:0, 25-35% C10:0 and 1-2% C12:0. [Bach and Babayan, 1982] Therefore, MCT oil is a suitable food for experiments researching the impacts of MCFAs or C8:0.

Most of the studies do not focus on only one fatty acid but rather on a fatty acid group like MCFAs. Therefore, it is not always possible to value one outcome to just one FA, but, as MCTs are made up mostly from C8:0, those studies are also relevant to assess the effects of C8:0.

3.2.3 MCTs' occurrence in human nutrition, digestion, absorption, storage and metabolism

In human nutrition, the only natural source of C8:0 is milk, but, as food industry improves, humans gain access to an ever-increasing number of products containing C8:0.

The only product containing a very high concentration of C8:0 is the MCT oil with 65%-75% of C8:0 content. [Bach and Babayan 1982]

There was an intention of introducing MCT oil, but, as it has a low smoking point, this idea was soon abandoned. A possible alternative to this may be laurate-rich MCTs as they have a higher smoking point. But currently food industry considers this type of oil too expensive (US $32/liter). [McCarty and DiNicolantonio, 2016] Although the availability of C8:0-containing foods grew in the western diet, its intake is still less than 2%. [Lemarié et al., 2016]

Due to the preduodenal lipase (gastric lipase in humans) activity, which contributes 15–20% to the hydrolysation of triglycerides, MCFAs can be released early in the process of digestion [Gargouri et al., 1986] and thus, MCFAs can be absorbed in the stomach. Next, the preduodenal lipase triggers the activity of pancreatic lipase and the escaped MCTs from the stomach are digested and absorbed in the gut (Figure 2). [Papamandjaris et al., 1998]

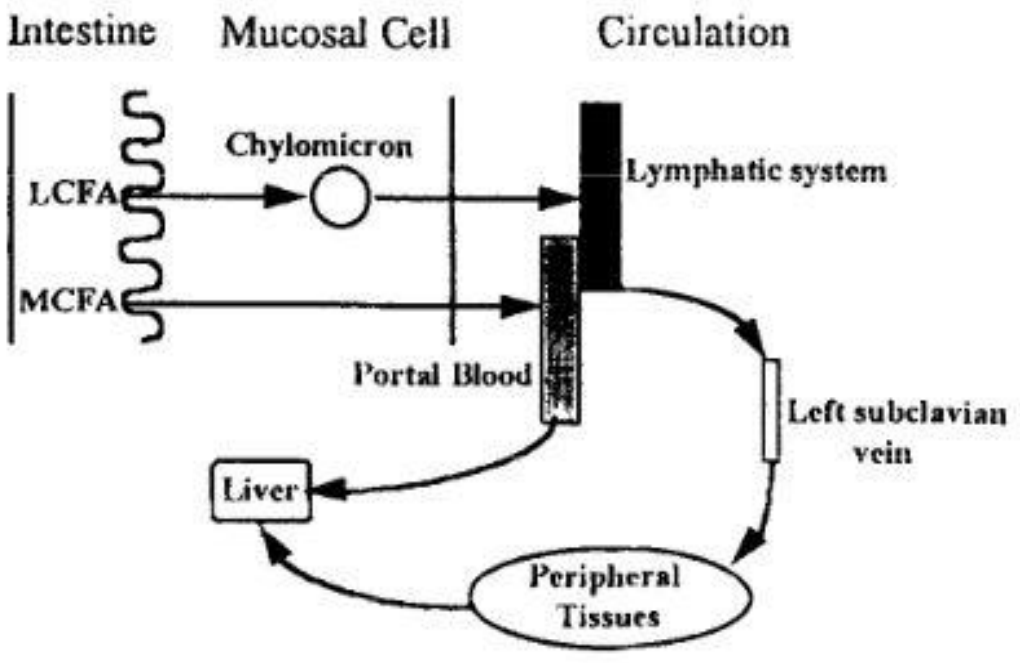

Figure 2: Intestinal fatty acid absorption. [Papamandjaris et al., 1998]

Due to their solubility, MCFAs are transported directly to the portal circulation as free fatty acids (FFAs) (Figure 2). Within the stream of blood they cling to albumin and thus rapidly access the liver. [You et al., 2008] As illustrated in studies on rats, LA is an exception, because it is more likely to be transported to the intestinal lymph system than to the portal vein. Thus, LA has a delayed access to the liver compared to capra fatty acids. [Sigalet et al., 1997; Sigalet and Martin 1999]

The body stores less MCFA than long chain fatty acid (LCFA). Probable reasons for this phenomenon lie in the limited storage capacity and fast metabolism. The carnitine independent transport across the mitochondria membrane allows MCFAs to quickly and efficiently produce ketone bodies. [Hoppel, 1982; Bach et al., 1996] Thus, MCTs are unlikely to be directly adipogenic. This theory is supported by a study on Sprague-Dawley rats that was able to show that MCTs aid loss of weight with the help of downregulating adipogenic genes, such as peroxisome proliferator activated receptor (PPAR) gamma, and improve insulin sensitivity as well as glucose tolerance. [Han et al., 2003a]

A study on rats showed that even after 6 weeks of a C8:0-free diet, C8:0 can be detected in the stomach, but not in other tissues. This could indicate a specific storage for C8:0 in stomach cells or be a sign of endogenous synthesis. [Lemarié et al., 2015] As shown on young infants and on rats, some storage in the adipose tissue only occurs if C8:0 is supplemented in a high dose. [Sarda et al., 1987; Han et al., 2003b]

FA metabolism follows several catabolic pathways such as beta-, peroxisomal-, and omega oxidation. Omega oxidation only occurs when the beta oxidation is exceeded. [Christensen et al., 1991]

Comparing the hepatic metabolism of C8:0 to the one of C14:0 in rats shows that the C8:0 degradation to CO_2 is more than ten times faster than that of C14:0 and that only 5% of C8:0 is used for lipid synthesis. [Scheig and Klatskin, 1968] The re-esterification of triglycerides (TG) from MCFAs is rare as MCFAs undergo rapid beta-oxidation in the liver or are absorbed by various tissues. [Papamandjaris et al., 1998]

3.2.4 Caprylate concentration in plasma and faeces

Lemarié et al. (2015) tried to measure the plasma caprylate concentration in Sprague-Dawley male rats with a gas chromatography coupled to mass spectrometry. These rats were fed for 6 weeks with chow containing up to 21% caprylate of total FA content. After an 18h fast no caprylate was detected. The results were explained by the rapid metabolization of the MCFAs. There is no data on plasma analysis in a non-fasted state. No detection limit for the analysis was given and no other research data concerning the analysis of plasma caprylate levels were to be found.

There is no data on caprylate occurrence in faeces of rodents or other animals, including humans.

3.2.5 Health advantages

The unique characteristics, including fast catabolism, low adipose tissue storage and the down regulation of PPAR gamma, made caprylate a potentially beneficial fatty acid.

Through the Western style diet, the increased incidences of obesity are frequently causing many diseases such as diabetes type II and hypertonia. The meta-analysis provided by Mumme and Stonehouse

[2015] suggests that the metabolism of MCTs´ supports weight loss and is thus useful in the treatment of obesity. Accordingly, the replacement of long chain triglycerides (LCTs) by MCTs, especially with C8:0 and C10:0, may induce modest but positive changes in body weight and composition without any adverse effects on the blood lipid profile. Long term studies (>8 weeks) on rats concluded that the weight reducing effect is short-lasting, a result that may be due to the phenotypic adaptation to a new metabolic condition. [Ferreira et al., 2014]

In a ketogenic diet, MCTs generate more ketones than long chained fatty acids. Therefore, a MCT-based ketogenic diet can include more carbohydrates which has the effect that the diet becomes more palatable and less strict. [Sills et al., 1986] This is especially important in pediatrics during the ketogenesis-treatment of epilepsy, when children have problems in holding on to the ketogenic diet. [Misiewicz and So, 2012]

MCFAs, unlike LCFAs, are not increasing the serum TG concentration and the TG accumulation in the skeletal muscle and the liver. This would promote insulin resistance and metabolic syndrome. Thus, they may be considered healthier for obese and for people suffering from total lipodystrophy, type II diabetes, metabolic syndrome or insulin resistance. [Wilson et al., 1983; Nagao and Yanagita, 2010; Wein et al., 2009]

3.2.5.1 Antibacterial, -fungal and -viral properties

FFAs are well known for their antifungal, -bacterial and viral nature. [Kabara et al., 1972] The most potent antibacterial and -viral MCFA is probably C12:0 [Batovska et al., 2009] or C10:0 if only the capra fatty acids are compared. [Lemarié et al., 2016] Testing on *B.*

megaterium and *Ps. phaseolicola* the minimum inhibitory concentration (MIC) of caprylate was, with 288.42µg/ml, at least twice as high as the concentration of C10:0. [Galbraith et al., 1971] In 1972, caprylate was tested on 8 gram-negative and 12 gram-positive microorganisms with a higher concentration (1124.84µg/ml) and no inhibitory effect was found. [Kabara et al., 1972] Nevertheless, the monoglyceride ester of caprylate is active against food-borne pathogens such as *Escherichia coli O157:H7*, *Listeria monocytogenes*, *Streptococcus spp.*, *Yersinia spp.*, *Penicillium spp.* and *Aspergillus spp.*, and *herpes simplex virus*. [Hyldgaard et al., 2012] According to a study comparing the MIC of MCFAs as FFAs or as their monoglycerides (MGs) found that the effectiveness of monocaprylin or caprylate on gram-positive cultures is lower than that from monocaprin or monolaurin and from their FFA-form. The MIC for caprylic acid and monocaprylin is almost always higher than 500µg/ml whereas the MIC of the two other FAs are mostly under 500µg/ml. [Batovska et al., 2009]

The bacterial cell membranes are the most influenced by the antimicrobial effects of FFAs. This can be seen in their disruption of the electron transport chain and/or the interference with oxidative phosphorylation. Furthermore, FFAs can induce cell lysis, autolysis and it is able to disrupt nutrient uptake. By interrupting the membrane integrity, FFAs can even cause leakage of cell metabolites (Figure 3). [Desbois and Smith, 2010] Monoglycerides act similar to FFAs by forming pores on the cell membrane or even provoke cell lysis. [Hyldgaard et al., 2012]

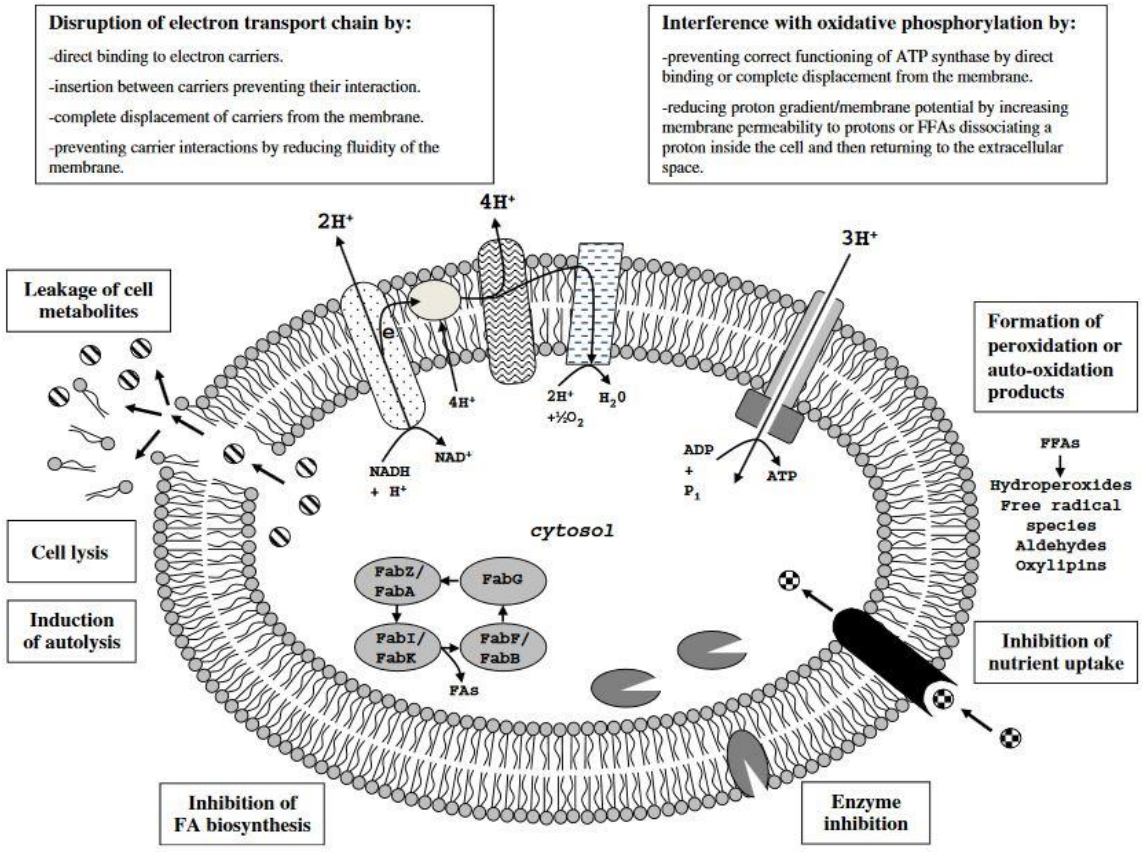

Figure 3: Possible mechanisms of the antibacterial effects of FFA. [Desbois and Smith, 2010]

According to its concentration, caprylate has either an anti-algal effect (2,800µg/ml) [McGrattan et al., 1976] or with a higher concentration of 10,000µg/ml an antiviral effect [Thormar et al., 1987] When supplemented for ruminants, caprylate reduce the protozoal count. [Dohme et al., 2001]

Furthermore, C8:0 may reduce the symptoms of frequent urination in case of coexisting infections of *Candida albicans*, *Helicobater pylori* and *Cytomegalovirus*. And it may function as an anti-cancer, anti-aging, anti-Alzheimer's disease as well as an anti-Autism agent. [Omura et al., 2011]

3.3 Intestinal microbiome

In the intestine, the colonization of epithelial surfaces by microorganisms (MOs) represents a large variety of organisms like bacteria and fungi. De facto, there is approximately the same number of MOs in the human gastrointestinal tract as nucleated cells forming the body (3.8·1013). [Sender et al., 2016]

As a response to endogenous and exogenous factors such as infections, xenobiotics, drugs, environmental microbial exposure, and the mode of a neonate´s delivery, the intestinal MOs change. [Backhed et al., 2015; Cho et al., 2012; Fujimura et al., 2014] Starting in the early life with weaning, nutrition has a major influence on this kind of adaptation. Later in life, the characteristics and the quantity of ingested food is crucial. [David et al., 2014]

3.3.1 Metabolic products

MOs coevolve with the host providing many benefits: they play an important role in the education of the immune system, [Fulde and Hornef, 2014] protect the intestinal lumen from pathogen overgrowth, [Kamada et al., 2013] control intestinal endocrine activity, [Neuman et al., 2015] and signal neurologically [Yano et al., 2015]. MOs are also able to produce ammonia, hydrogen sulfide, amines, phenols and organic acids, [Blachier et al.,] vitamins (vitamin K and several components of vitamin B) [Yatsunenko et al., 2012] and energy sources such as conjugated linoleic acid (CLA) [Devillard et al., 2009] and short chain fatty acids (SCFAs). [Macfarlane and Macfarlane, 2003]

SCFAs can be synthesized from digestible carbohydrates escaped proximal digestion, indigestible carbohydrates (e.g. resistant starch),

proteins and peptides by gut MOs like Bacteroides, Roseburia, Bifidobacterium, Fecalibacterium and Enterobacteria. [Blachier et al., 2007] Acetate, propionate and butyrate constitute more than 95 % of the gut SCFA content, while formate, valerate and caproate make up the remaining part. [Cummings et al., 1987] The proximal large intestine and the cecum contain the highest number of fermenting bacteria and available substrates, hence producing the major amount of SCFAs. [Wong et al., 2006] SCFAs produced by the gut microbiota can be absorbed in the colon which is efficient (>95%) and can be managed in two ways: the protonated molecules are absorbed either by nonionic diffusion in the presence of CO_2 or by ionic diffusion of the Na or K salt of the SCFAs. [Ruppin et al., 1980]

4 Materials and Methods

4.1 Bedding and chow

- **Arbocel Performance Small**, pellets, cellulose; J. Rettenmaier & Söhne GmbH + Co KG (Vienna, Austria)
- **Lignocel select**, poplar, cubic; J. Rettenmaier & Söhne GmbH + Co KG (Vienna, Austria)
- **RehoFix MK 3500**, maize grains; J. Rettenmaier & Söhne GmbH + Co KG (Vienna, Austria)
- **V1535 R/M-H Extrudate**, autoclavable; ssniff Spezialdiäten GmbH (Soest Germany)

4.2 Materials

4.2.1 Equipment

- **Lauda Alpha A6** thermostatic bath; temperature range between 25 and 100 °C; LAUDA DR. R. Wobser GmbH & CO. KG (Lauda-Königshofen, Germany)
- **Arium®pro** ultrapure water systems; Sartorius Lab Instruments GmbH & Co KG (Goettingen, Germany)
- **Centrifuge 5418R**; 100 rpm – 14 000 rpm; 0 °C to +40 °C; Eppendorf AG (Hamburg, Germany)
- **LCMS-8040 Liquid Chromatograph Mass Spectrometer**; Shimadzu Corporation (Kyōto, Japan)
    - High Pressure Switching Valve FCV-20AH2
    - UV/VIS Detector SPD-20A
    - Column Oven CTO-20AC

    - Autosampler SIL-20AC HT
    - Liquid Chromatograph LC-20 AD
    - Degassing Unit DGU-20A5
    - Kinetex® 5 µm EVO C18 100 Å LC Column 150 x 3.0 mm
    - LabSolutions (Software)
- **Vortex mixer**, lab dancer; VWR Chemical (Fontenay-sous-Bois, France)
- **Sartorius Entris®** 224i-1S analytical balance; Sartorius Lab Instruments GmbH & Co KG (Goettingen, Germany)
- **Sample Concentrator EVA-EC1-S** for 24 Samples; VLM GmbH (Bielefeld, Deutschland)

4.2.2 Accessories

- **Microcentrifuge tubes**; 1.5 mL; Eppendorf Austria GmbH (Vienna, Austria)
- **Tips** TipOne®; 10µl, 200µl, 1000µl; STARLAB International GmbH (Hamburg, Germany)
- **Centrifuge tubes**; 15 mL, 50mL; Corning Inc. (Corning, New York, USA)
- **HPLC vial**; 2mL; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Shell style insert** for HPLC vial; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Pipette**, Research plus; 2.5µl, 10µl, 20µl, 100µl, 200µl, 1000µl; Eppendorf Austria GmbH (Vienna, Austria)

4.3 Reagents

4.3.1 Chemicals

- **4-APEBA** (4-(2-((4-bromophenethyl) dimethylammonio) ethoxy) benzenaminium bromide, $C_{18}H_{25}Br_3N_2O$), CAS: 1226984-28-6; AxonMedChem (Groningen, Netherlands)
- **Acetonitrile** (CH3NH), HiPerSolv CHROMANORM® Super gradient for HPLC; CAS: 75-05-8; VWR Chemical (Fontenay-sous-Bois, France)
- **Aprotinin** ($C_{284}H_{432}N_{84}O_{79}S_7$) from bovine lung; CAS: 9087-70-1; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **ddH2O**; produced in the lab with Arium®pro
- **DPP IV inhibitor** (dipeptidylpeptidase IV, $C_{17}H_{31}N_3O_{4)}$; Merck Millipore (Vienna, Austria)
- **EDC** (N- (3-Dimethylaminopropyl) -N'- ethylcarbodiimide hydrochloride, $C_8H_{17}N_3 \cdot HCl$); CAS: 25952-53-8; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **EDTA** ≥98.0%, (Ethylenediaminetetraacetic acid, $(HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)_2$); CAS: 60-00-4; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Formic acid** 98% - 100% for LC-MS LiChropur®; CAS: 64-18-6; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Hydrochloric acid** 37% (HCl), AR grade; CAS: 7647-01-0; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Methanol** (CH3OH), HiPerSolv CHROMANORM® gradient for HPLC; CAS: 67-56-1; VWR Chemical (Fontenay-sous-Bois, France)
- **NHS** (N-Hydroxysuccinimide, $C_4H_5NO_3$), CAS: 6066-82-6; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)

- **Octanoic acid** ≥98 % ($CH_3(CH_2)_6COOH$); CAS: 124-07-2; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Octanoic acid (1,2,3,4-13C4, 99%)** , ($CH_3(CH_2)_3(*CH_2)_3*COOH$; CAS: 124-07-2; Cambridge Isotope Laboratories Inc. (Tewksbury, USA)
- **Sodium hydroxide** ≥99 % (NaOH); CAS:1310-73-2; Carl Roth (Karlsruhe, Germany)
- **Water**, HiPerSolv CHROMANORM® for HPLC; CAS: 7732-18-5; VWR Chemical (Fontenay-sous-Bois, France)

4.3.2 Preparation of solvents

- **HCl** – 3 M in ddH_2O
- **NaOH** – 3 M in ddH_2O
- **NHS** – 190 mM in ddH_2O
- **EDC** – 290 mM in ddH_2O
- **4-APEBA** – 3g/ml in ddH_2O
- **Octanoic acid** – 0.91 mg in 200 µl ddH_2O
- **Octanoic acid (1,2,3,4-13C4, 99%)** – 0.91 mg in 200 µl ddH_2O

4.3.3 Eluent

Mobile phase A: for one liter eluent 950 ml H_2O for HPLC, 50 ml MeOH for HPLC and 1 ml formic acid were added.

Mobile phase B: for one liter eluent 50 ml H_2O for HPLC, 950 ml MeOH for HPLC and 1 ml formic acid were added.

Mobile phase C: One liter ACN (abbreviation) was mixed with 1 ml formic acid.

4.4 Animals and experimental protocol

Male C57BL/6NRj mice were housed in cages in an animal room, lived under standard SPF conditions using Tecniplast IVC system (cage type 2L, blue line). The room temperature and its humidity were electronically controlled and constantly supervised. The lighting was set to a 12-hour light-dark cycle. The mice were purchased from Janvier Inc. Labs (Le Genest, France) and lived in groups of maximum four per cage, with free access to food and water. Aggressive mice were separated to prevent fights. The animals were divided into one control and one CR group; Each of these groups was separated into three subgroups by the bedding type: wooden, corncob, cellulose (Table 2).

Table 2: Experimental groups. The asterisk means the loss of a mouse during the CR in the wooden group.

	Bedding type	**Group name**	**Number of mice**
Control	Wood	Control W	10
	Corncob	Control CC	10
	Cellulose	Control C	10
CR	Wood	W	9*
	Corncob	CC	10
	Cellulose	C	10

Prior and during the experiment, the control group was fed with commercial chow *ad libitum*. The CR mice were fed with commercial chow *ad libitum* for 6 weeks. The mice were kept in cages with mixed beddings for acclimatization. At the beginning of the experiment, the mice were separated and put into single cages with one of the chosen bedding. After the separation, the mice were subject to a two-week

CR with 75% (2.8–2.9g) of their daily food intake. The measured portion of food was delivered daily between 3 and 6 pm after measuring the mice's body weight.

To estimate the hunger, each food pellet was placed in the cage and the mice were allowed to start the meal. The time it took the mice to start eating was measured with a stopwatch. From a small cut in the tail, whole-blood samples were collected and the fasting glucose concentrations were measured.

On day 11, the bedding of the CR mice cage was changed. On day 12, 13 and 14 the bedding was collected and dried. The amount of eaten bedding was measured and feces were collected on day 12 and 13.

Fecal samples were sorted out of the bedding used for one mouse and one day. The samples were measured with a laboratory balance and sent for an analysis by calorimeter (IKA-Kalorimeter C2000; IKA®-Werke GmbH & Co. KG; Staufen, Germany).

4.4.1 Tissue collection

On day 15, the CR mice were anaesthetized (isoflurane in oxygen) and euthanized with blood drawn by cardiac puncture and eventually dissected. The blood was mixed with 10µl ethylenediaminetetraacetic acid (EDTA) to ensure blood in fluid form. Than 20µl aprotinin and 10µl dipeptidyl peptidase (DPP) IV was added and centrifuged for 10min at 3,600rpm 4°C, the plasma was stored at –80°C.

The following parameters were measured: cecum, liver, stomach and white adipose tissue (WAT) weight. WAT includes dorsal WAT (dWAT), epididymal WAT (eWAT), abdominal WAT (abWAT) and the sum of WAT, the subcutaneous white adipose tissue (sWAT) weight.

Stomach, liver, WAT biopsies, cecum content, intestinal scrapings, and hypothalamus were collected and were snap frozen and stored at –80°C.

4.5 NMR

Bedding was changed daily, 3 days long. Between the change, the mice were kept for 10–20 minutes in their cage without bedding and fresh fecal samples were collected, snap frozen and stored at –80°C. The samples were sent for NMR analysis with NMR spectrometer machine (Bruker; DRX-600-Avance; Wissembourg, France).

4.6 Plasma caprylate detection

A caprylate analysis was made with a combined system of a high-pressure liquid chromatograph (HPLC) and a mass spectrometer (MS). As there was no well-established method for a caprylate analysis on this kind of analytical instrument a new method has been developed: Based on our experience in the MCFA detection in plasma, we came to the conclusion, that the most sensitive method would include the derivatization of caprylate with the help of 4-APEBA.

For this purpose, octanoic acid standard ($STD_{O,}$ analyte) and isotopic labelled 13C-octanoic acid ($STD_{R,}$ internal standard) were prepared in a concentration of 9.1mg/ml in ddH_2O. The preparation was the following:

Based on the work of Kretschmer et al. (2011), 100 µl ddH_2O was mixed with 2,5 µl STDo. After this step, a dilution with 200 µl 3mg/ml 4-APEBA, 50 µl 290 mM EDC and 50 µl 190mM NHS followed. The solution was vortexed thoroughly and left for 60 minutes in a water

bath of 60°C for derivatization. Afterwards, this solution was transferred into a HPLC vial and injected (Figure 4).

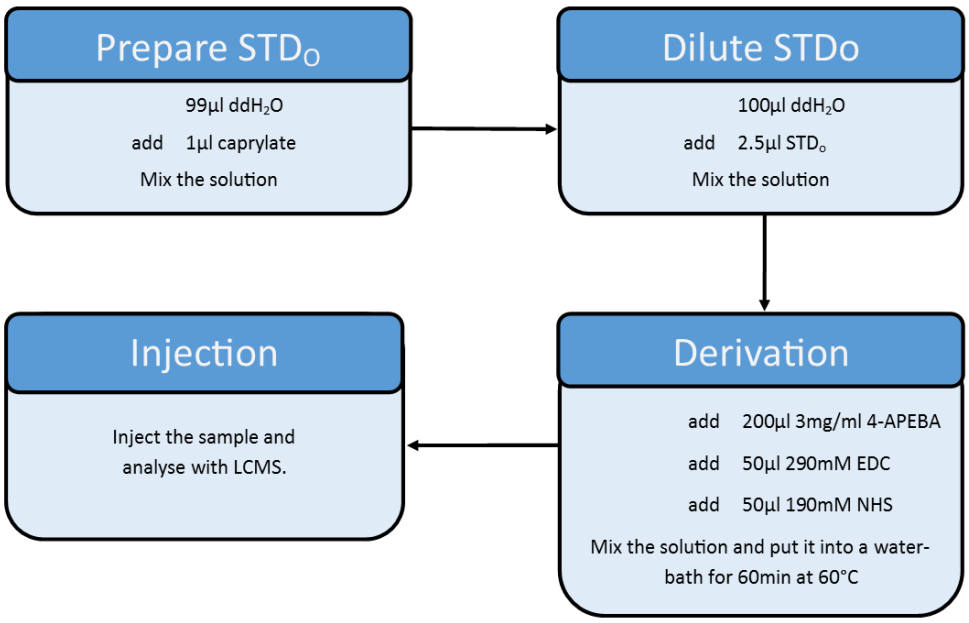

Figure 4: Pipetting scheme for establishing the method for derivatization.

4.6.1 Sample preparation

For the analysis of the mice samples, 100µl plasma was mixed with the isotopically labelled analytical standard (STD_R) and 6.5µl 3M HCl was added to lower the pH to 1. This solution was mixed and put into a water bath of 60°C for a duration of 60min. The effect of this procedure is the following: The low pH and the higher temperature frees the bound FAs and in this way, both, the bound and non-bound FAs can be analysed.

The procedure is problematic because there is, on the one hand, a need of a low pH for the hydrolyzation, on the other hand it would disrupt the derivatization. In order to avoid this problem, the HCl had to be neutralized with 6.5µl 3M NaOH. After this, it was possible to extract the FAs by mixing the solution with ice-cold 100µl ACN for one minute and centrifuge it for 20min at 4°C with 13,600rpm. The supernatant (tube 1) was mixed with 100µl 3mg/ml 4-APEBA, 25µl 290mM EDC and 25µl 190 mM NHS. The leftover was extracted once more (tube 2), dried under N_2 and re-suspended with 100µl 4-APEBA, 25µl EDC and 25µl NHS. After adding ACN the samples were kept at +4°C to prevent any counteraction with caprylate. After the addition of 4-APEBA, no cooling was needed any more.

Tube 1 and 2 were united and left in the 60°C hot water bath for 60min for derivatization. After this, the samples were directly injected and analysed. This sample preparation is illustrated in Figure 5.

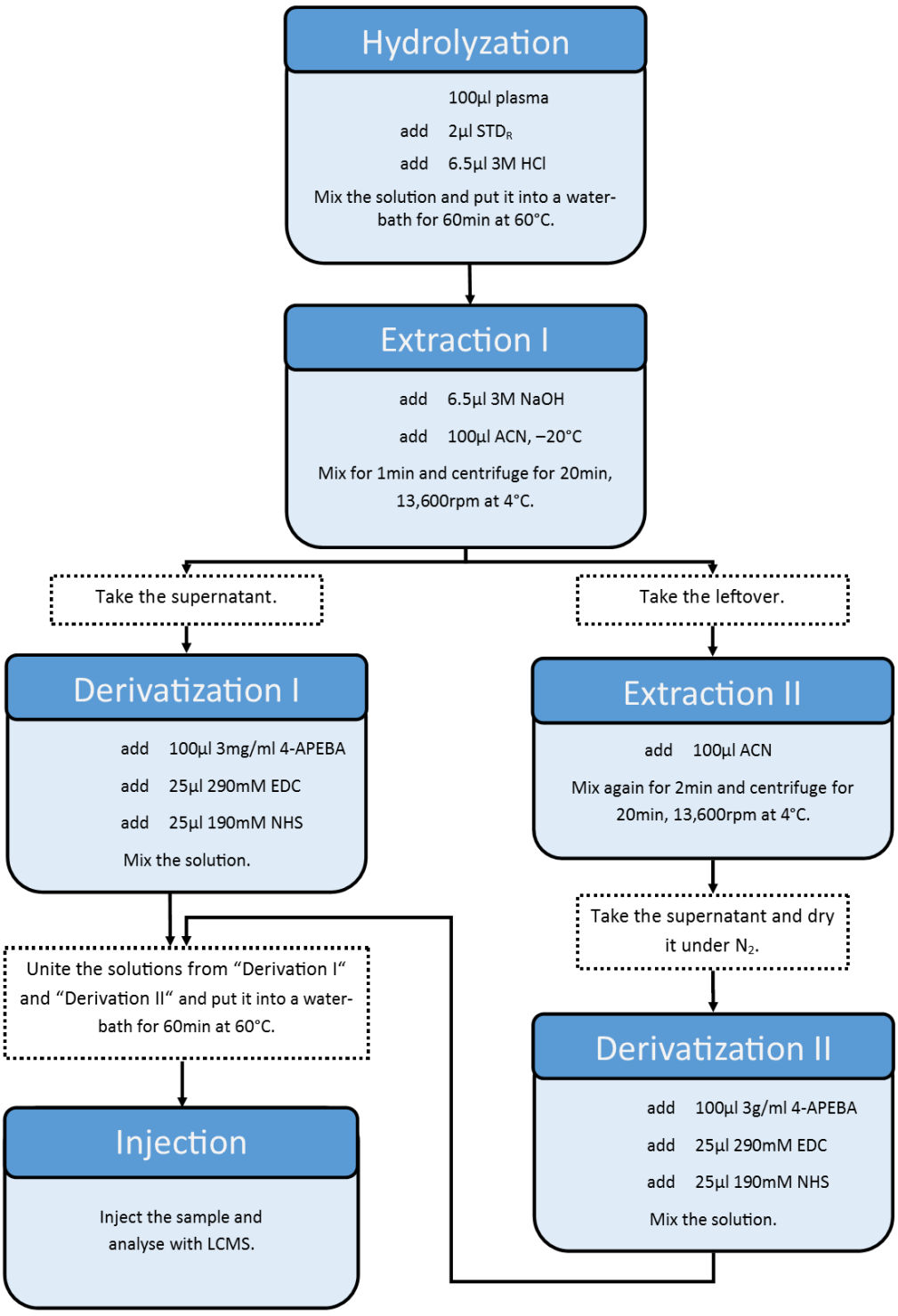

Figure 5: Scheme for hydrolyzation, extraction and derivatization.

4.6.2 Measurement with HPLC-MS/MS

After the hydrolyzation, the extraction and the derivatization an analysis using a HPLC-MS/MS-system was made. The functioning is as follows: By pressing the eluent with the sample (mobile phase) through a column (stationary phase), the HPLC separates the chemical substances. The column is filled with a solid adsorbent material that interacts with the molecules in the solvent and is thus causing different flow rates.
The settings of the combined system are shown in Table 3.

Table 3: HPLC-MS/MS conditions.

Stationary phase	Kinetex® 5µm EVO C18; 150 x 3.0mm
Mobile phase A	95% H_2O, 5% MeOH, 0.1% formic acid
Mobile phase B	5% H_2O, 95% MeOH, 0.1% formic acid
Flow	0.5 mL/min
DL temperature	180°C
Heat block temp.	130°C
Oven temp.	25°C
Drying gas flow	12L/min
Nebulizing gas flow	3L/min
CE	-10V
Run time	30min
Dwell	100msec
ESI mode	Positive

4.6.3 Validation

4.6.3.1 Selectivity

For a successful analysis with LC-MS/MS it is essential to distinguish between the analyte and other substances within the biological matrix. As there are diverse metabolites, globulins, coagulation factors and so forth in a plasma sample, a certain selection has to be made.

To optimize this procedure a stable retention time must be established which variability should not exceed 5%.

To illustrate the selectivity of this method with the MS/MS, Kretschmer et al. (2011) provided a variety of product ions for caprylate (Figure 6), from which m/z 307.24 should be the one specific for caprylate.

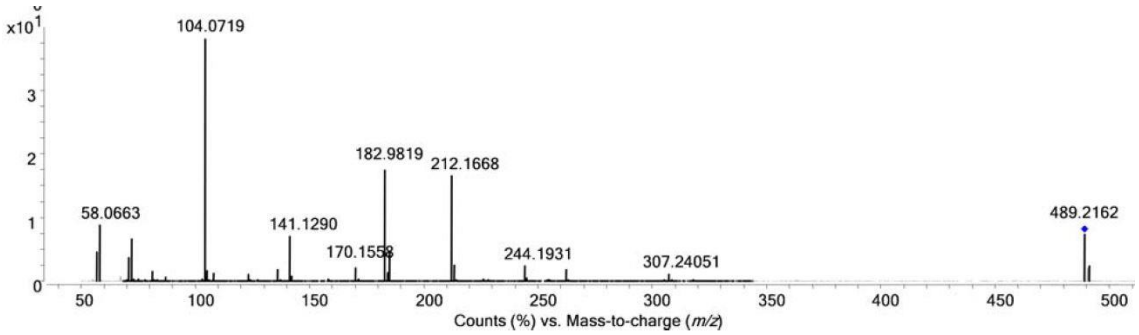

Figure 6: A variety of product ions for caprylate. The given specific precursor by Kretschmer et al. [2011] is m/z 307.24.

4.6.3.2 Retention Time

The time required for the analyte to travel from the injector to the detector is called retention time. Here, the interaction with the chromatographic column is crucial. The column used by Kretschmer et al. [2011] is a Waters XTerra MS reversed-phase column (C18, 100x2.1 mm, 3µm) but for this present experiment, a slightly different column (Kinetex® 5 µm EVO C18 100 Å LC; 150 x 3.0 mm) was used. Therefore, its retention time had to be evaluated: To do so, an analytical standard with a concentration of 500µl/ml was prepared and analysed with the conditions shown in Table 4.

Table 4: Gradient elution program.

Mobile Phase (A:B)	Time (min)
100:0	0
100:0	5
95:5	5.5
10:90	20
10:90	25
100:0	27
100:0	35

4.6.3.3 Linearity

The regression line, which should be established for every analyte – in this case for caprylate - shows the correlation between the response of the LC-MS/MS and the applied concentration of the analyte. In order to identify all disruptive factors, native caprylate and isotopic labelled caprylate were analysed separately as well as together.

For the interpretation of the response-concentration, a serial dilution with at least 5 different concentrations – all of them being close to the expected range of the plasma levels - is requested (Table 5). Furthermore, a specific precursor ion and at least one product ion have to be identified.

Table 5: Serial dilution for the analyte.

STD 1	45.5 µg/sample
STD 2	35.5 µg/sample
STD 3	27.5 µg/sample
STD 4	21.5 µg/sample
STD 5	15.5 µg/sample

4.6.3.4 Limit of quantification/detection

The term limit of quantification (LOQ) designates the lowest possible concentration that can be analysed with precision and accurateness. For the identification of the LOQ, the signal-to-noise (S/N) ratio was used, which, according to the recommendation of the European Union, should be at least 10.

The limit of detection (LOD) on the other hand, is the lowest possible concentration from which the deduction of the presence of the

analyte is possible. Here, the S/N ratio should be at least 3. [Torfs et al., 2012]

To identify the S/N ratio, the LabSolutions software and the ASTM method was used.

4.6.4 Precursor ion

The scan of the precursor ions was realized in the positive electrospray ionization (ESI) mode. After travelling through a capillary, the liquid sample is ionized with the help of nitrogen. Then, the multiply charged ions flow into the analytical part of the mass spectrometer (Figure 7).

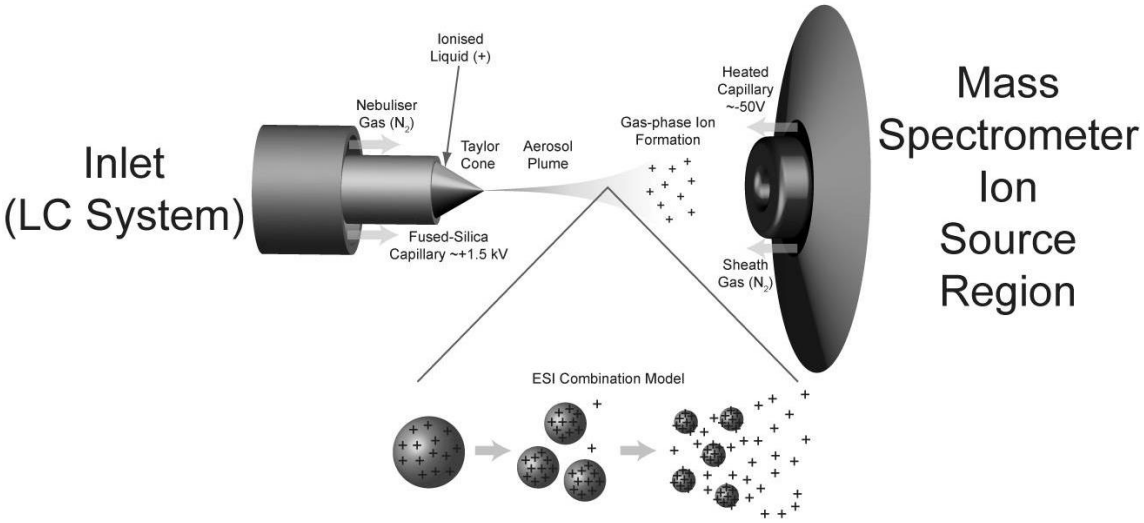

Figure 7: Electrospray ionisation and ion source overview. [NanotechnologySolutions, 2017]

For the present analysis of caprylate in plasma, a triple-quadrupole mass spectrometer with a collision cell stringed together with two analysers was used. Before reaching the first quadrupole, the ionized sample flows through the desolvation line (DL) and multipole 1 and 2. Normally, the analysis is done by the first and third quadrupoles: The first quadrupole analyses the precursor-ion, the second one

fragments the precursor-ion and the third measures the fragments. In scan-mode, only the third quadrupole is activated to analyse the precursor, but not the product ions.

4.6.4.1 Product Ion

In the first quadrupole measuring the mass-to-charge (m/z) ratio of the precursor ion and selecting the ones of interest, product ions derive from the ionized sample. The advantage of this first step is that nonanalyte ions are discarded. Furthermore, the collision cell (hexapole) applies voltages to the analyte which causes more fragmentation. In the third quadrupole, a spectrum of the resulting product ions is generated which can be analysed (Figure 8).

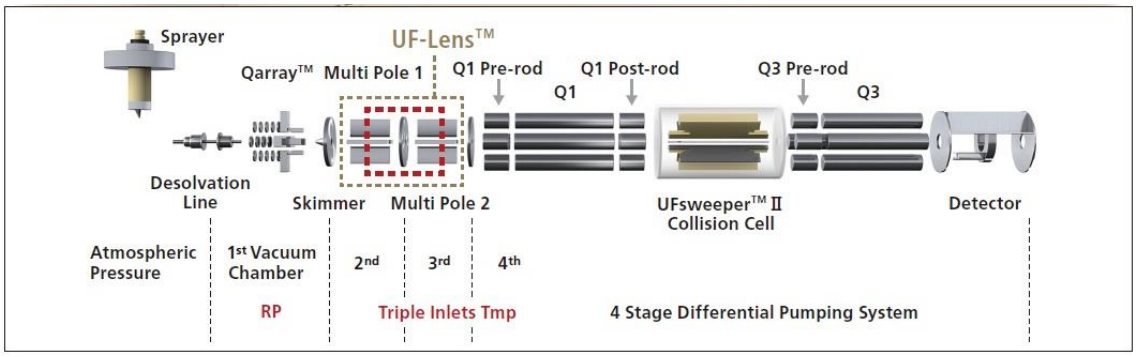

Figure 8: Triple quadrupole mass spectrometer. The Q1 area analyze the precursor ion and the Q3 analyze the product ion. [JAYA, 2017]

4.6.5 Quantifier and Qualifier

Quantifiers are used for the quantification of the analyte. When analysing a standard solution, the highest signal intensity should be considered the quantifier. In the present study, the use of 4-APEBA in the standard solution produces a variety of caprylate-independent product ions and makes it thus harder to find a fragment that is specific for caprylate only.

This problem may be illustrated as follows: The major fragments of the adduct of 4-APEBA and caprylate are the products "A" and "B" in the Figure 9. Product "A" is also a fragment of nonanal and thus, not specific enough when analysed in a complex matrix such as mouse plasma. Product "B" is an abundant fragment ion that, due to the 4-bromophenethyl cation, indicates the successful derivatization with 4-APEBA (Figure 9). Both products demonstrate a successful derivatization but none of these fragments can be used as a qualifier or a quantifier for caprylate. [Kretschmer et al., 2011]

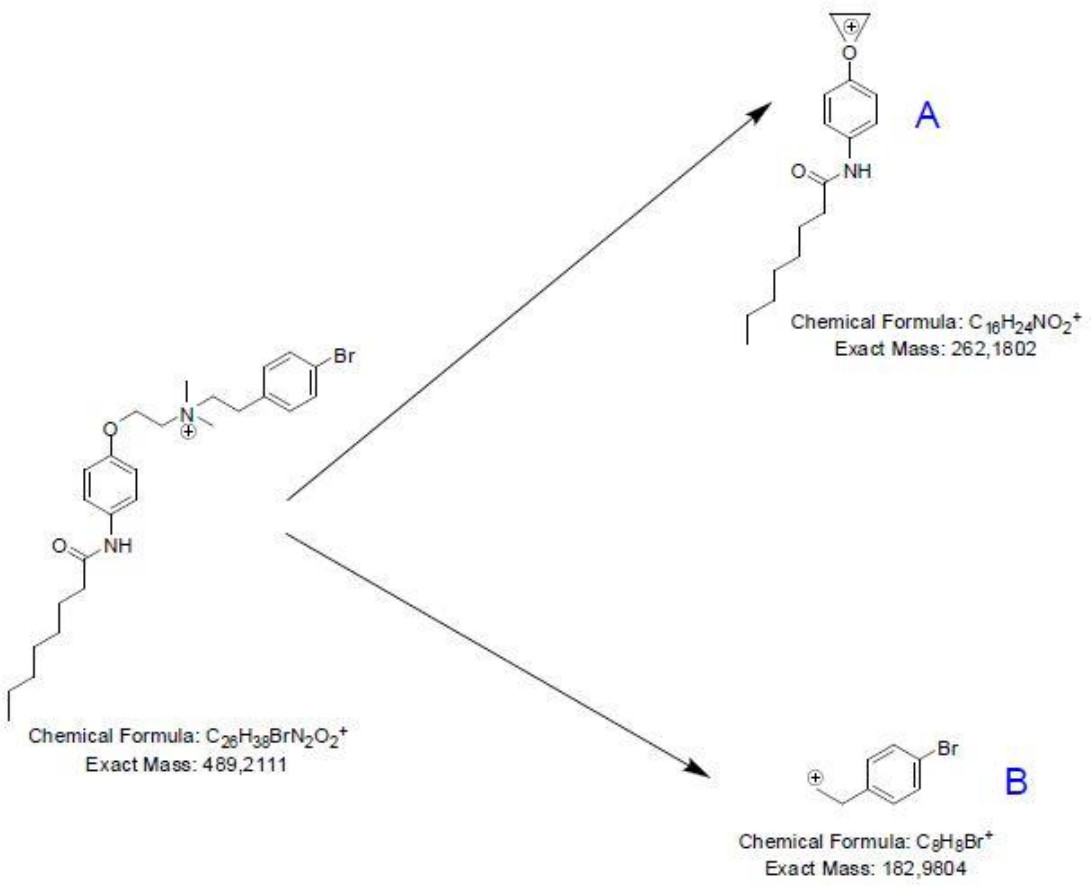

Figure 9: Major fragmentations of the adduct of 4-APEBA and caprylate. [Kretschmer et al., 2011]

4.6.6 Statistical analysis

Statistical analysis was performed with Microsoft Excel. The single factor ANOVA and T-Tests were used to show the significant difference between the groups. Significance was defined by a p-value <0.0083.

5 Results

5.1 Bedding

This experiment was conducted on male C57BL/6NRj mice. Wood, cellulose and corncob beddings were used. For acclimatization, the mice were first housed in cages with mixed beddings. Then, at the beginning of the two weeks of experiment, the mice were separated and put into single cages with one of the chosen beddings. The control group was fed ad libitum before and during the experiment, whereas the CR group was first fed ad libitum and then these mice were subjected to an energy-restricted diet for two weeks. The weight of the eaten bedding was measured. The results showed a significant difference between the three groups. The wooden bedding was the favorite, the cellulose one was the least consumed. Mice ate 1.39g ± 0.05g from the wooden bedding, 0.61g ± 0.06g from the cellulose bedding and 1.01g ± 0.06g from the corncob bedding (Figure 10).

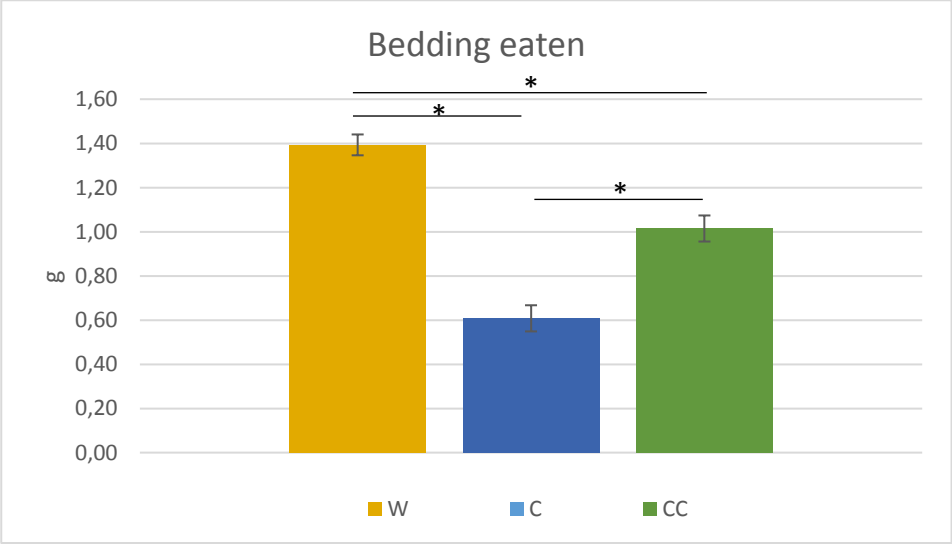

Figure 10: Bedding eaten. Asterisk means a significant difference of $p<0.0083$ between the groups. Mice in the groups: W (9), C (10) and CC (10).

The exact fiber content of these specific beddings for this present study were not given by the producer. Therefore, the following data was used for the calculation of the fiber eaten by mice.

Wood contains 15%-25% lignin, 23%-32% Hemicellulose, 38%-50% and mineral components, here the median of the given percentages was used. [Forest Bioenergy, 2017] Corncob consist of 35.2% lignin, 13% hemicellulose, 41.5% cellulose and contains 1.33% minerals [Publishing, 2015], whereas the raw material for the cellulose bedding is pure cellulose (Table 6).

Table 6: Fiber content.

Fiber type	**Bedding type**		
	W	C	CC
Lignin	20%	0%	35.2%
Hemicellulose	27.5%	0%	13%
Cellulose	44%	100%	41.5%

Figure 11 shows that in the groups W and C the cellulose intake is similar. In these groups the caprylate levels are higher compared to those of the control group. This could be explained by the higher intake of cellulose.

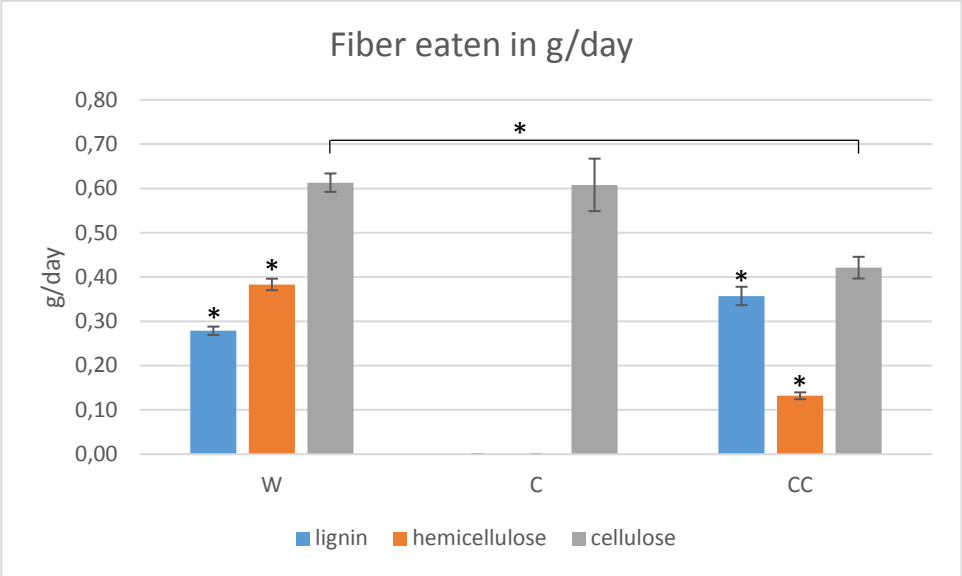

Figure 11: Fiber eatin in g/day. Mice in the groups: W (9), C (10) and CC (10). Asterisk depicts the significant difference $p<0.0083$.

In group W, the total fiber-intake was the highest, whereas no significant difference between the groups CC and C were observable (Figure 12).

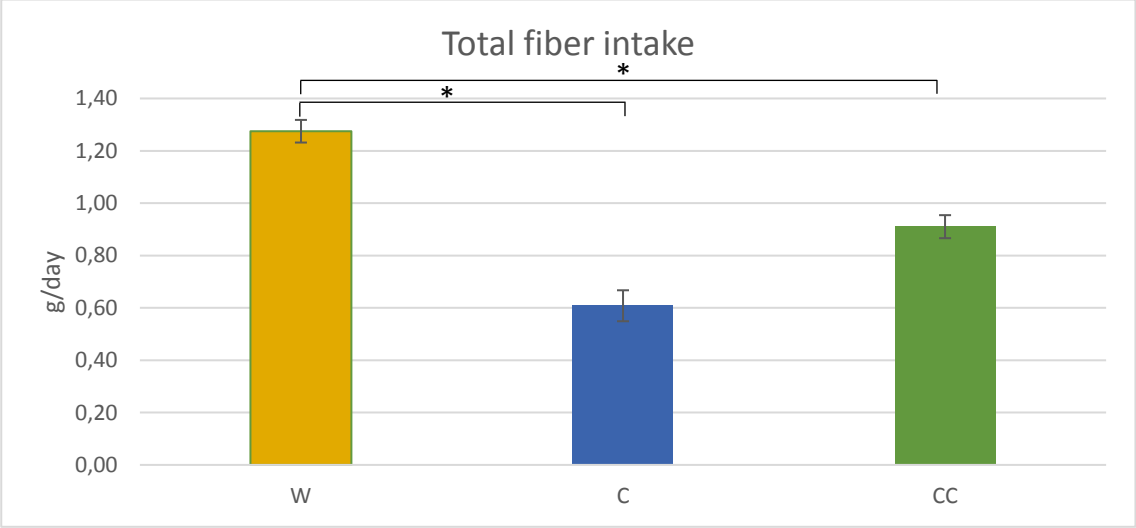

Figure 12: Total fiber intake. Mice in the groups: W (9), C (10) and CC (10). Asterisk depicts the significant difference $p<0.0083$.

5.2 Starting meal

During the period of CR, the appetite was assessed by measuring the time it took for the mice to start the meal. In the first two days, this procedure usually took more than 10sec but from the third day on less than 10sec were needed to start eating. The only significant differences were to be observed on days 4 and 5, when CC took more time than W before starting to eat (Figure 13).

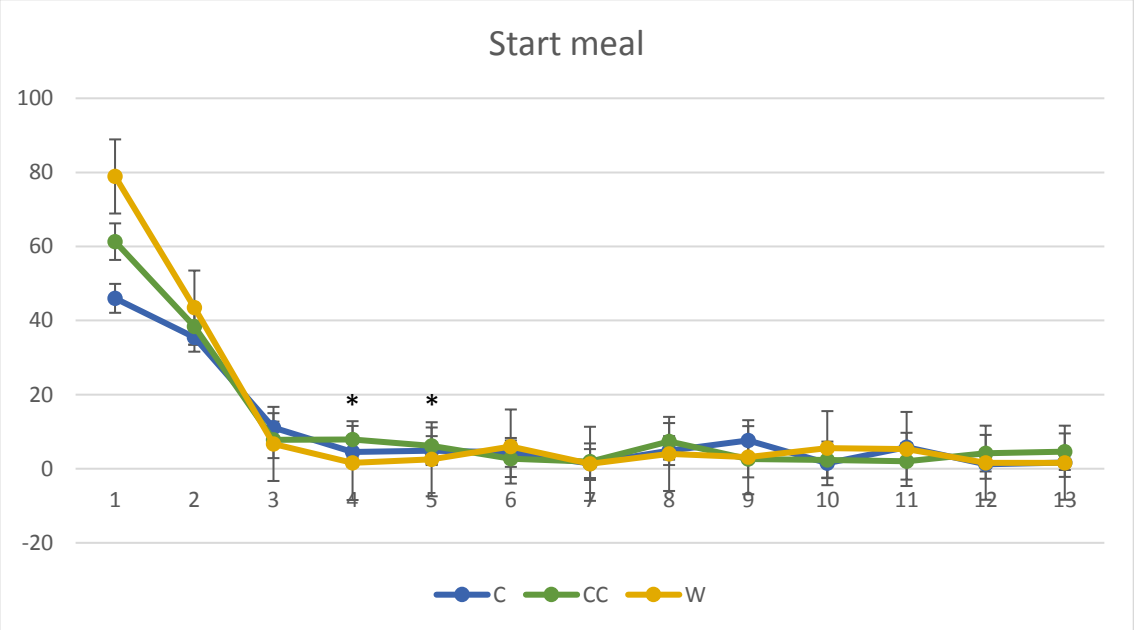

Figure 13: Start meal. Asterisk means means a significant difference of p<0.0083 *on day 4 between CC and W and on day 5 between CC and W.* Mice in the groups: W (9), C (10) and CC (10).

5.3 Glucose

Fasting glucose was measured which shows how hard the fasting for mice is. Generally, the glucose levels in the CR groups are significantly lower than in the control group. No difference by the bedding type can be obtained (Figure 14).

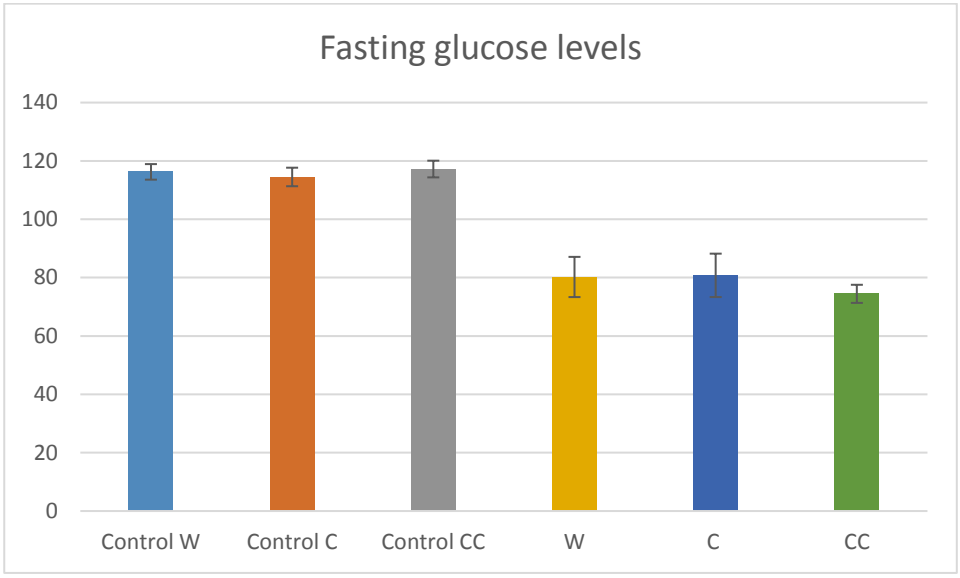

Figure 14: Fasting glucose levels. There is a significant difference between the CR and the control group, but no significant difference within the CR and control group.

5.4 Body weight

The body weight was measured throughout the two weeks of CR, the BW changes and loss were calculated. As the BW loss in the CR groups was not significantly different, no significant effect of the bedding on the body weight change was observed (Figure 15).

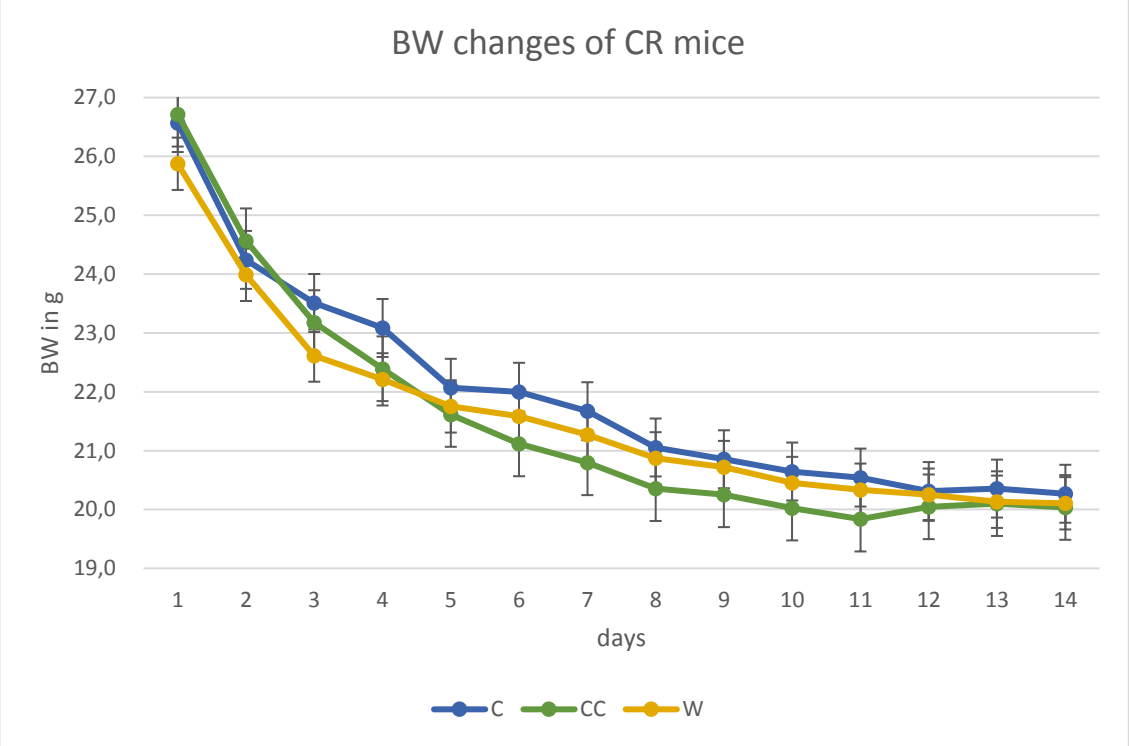

Figure 15: BW changes of CR mice. Mice in the groups: W (9), C (10) and CC (10).

After two weeks of CR, mice had lost in average 24% of their body weight. ANOVA showed no significant difference between the CR groups. The T-Test showed a difference between CC (25%) and W (22%) with a p-value of 0.02 which is close to the significance border (Figure 16).

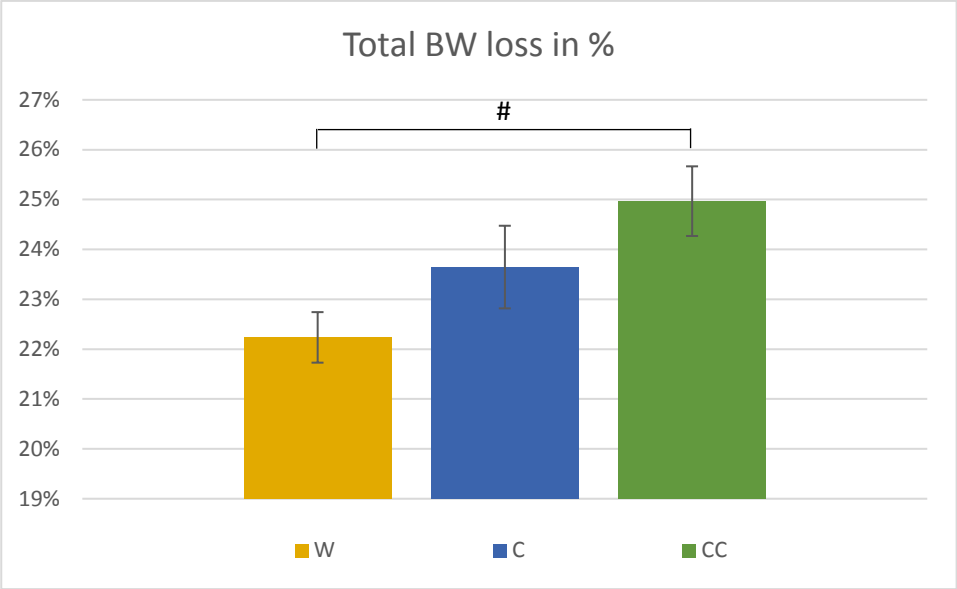

Figure 16: Total BW loss in %. # depicts the almost significant difference of p=0.02 between CC vs W. Mice in the groups: W (9), C (10) and CC (10).

5.5 Weight of the organs

The cecum stores food material, which is hard to digest, such as cellulose, which in turn, can be broken down by bacteria. Thus, the cecum weight calculated in percent of the body weight correlates with the amount of indigestible food. Figure 17 shows that CR mice have a proportionally heavier cecum than the control groups.

In addition, there is a significant difference in the size of the cecum between the wooden CR and the corncob CR groups. Group W had the biggest and group CC showed the smallest cecum.

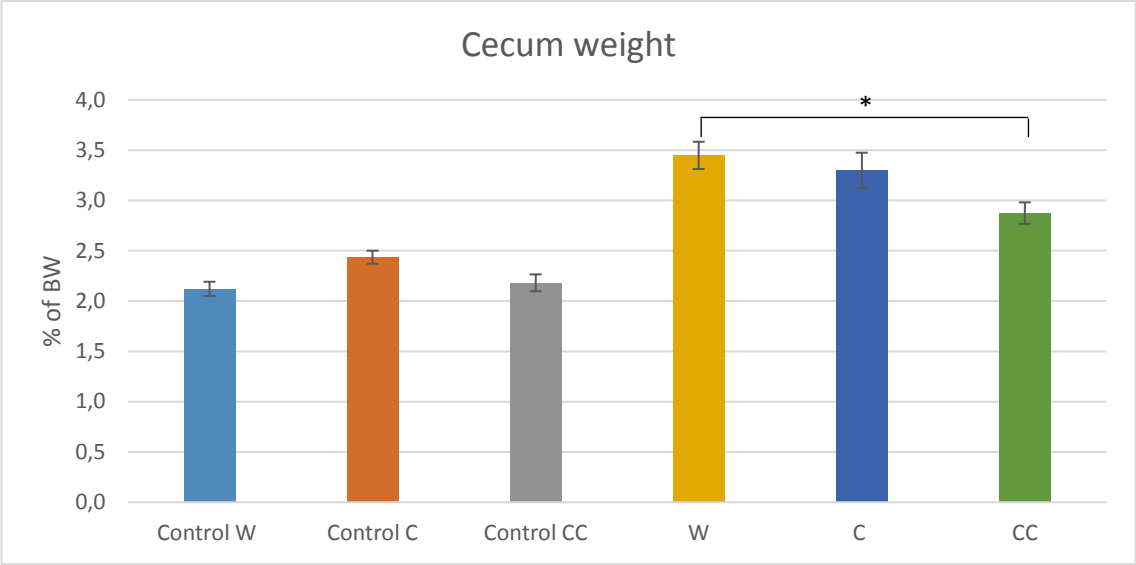

Figure 17: Cecum weight. Asterisk means significant difference and there is also a significant difference between the control and the CR group. Mice in the groups: W (9), C (10), CC (10), control W (4), control C (10), control CC (9). Asterisk depicts the significant difference $p<0.0083$.

The stomach weight calculated in percent of the body weight shows no difference between the groups. The stomach size always stayed proportional to the body weight (Figure 18).

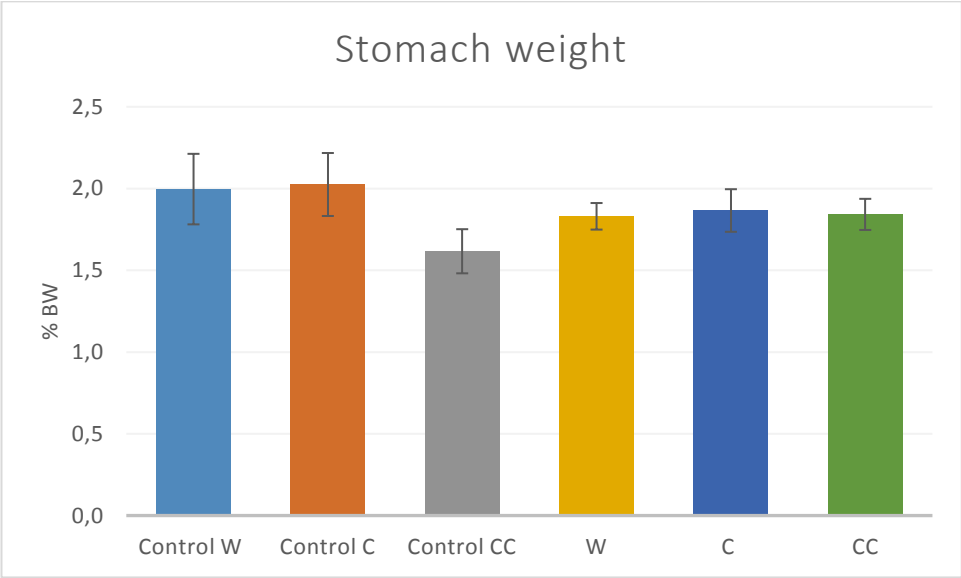

Figure 18: Stomach weight. Mice in the groups: W (9), C (10), CC (10), control W (9), control C (10), control CC (10).

Between the CR groups, no significant difference in the sum of subcutaneous WAT (sum sWAT) expressed in percent of BW was being detected. But in the control group, an almost significant (p=0.04) difference between control C and control W as well as between control CC and control W could be shown. Comparing the control and the CR groups it was possible to observe that the control C and control CC are significantly higher than any of the CR groups. Nevertheless, there is no significant difference between control W and the CR groups. There is no significant difference between the CR groups (Figure 19).

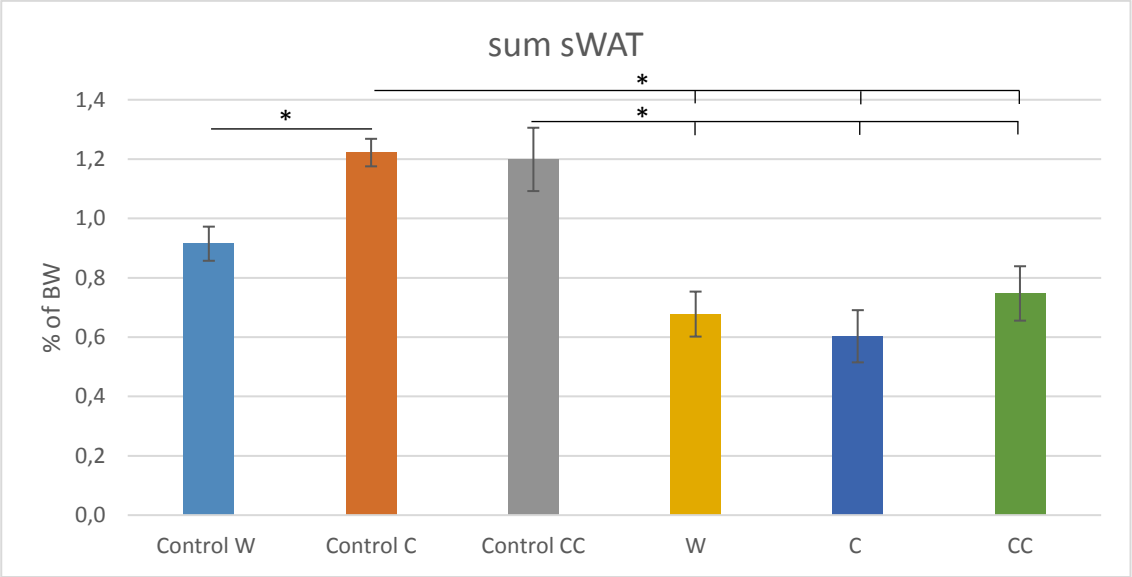

Figure 19: Sum sWAT. Mice in the groups: W (9), C (10), CC (10), control W (10), control C (10), control CC (9). Asterisk depicts the significant difference p<0.0083.

5.6 Calorimetry

Faeces was collected, dried, weighted and the calorie content pro gram was measured. There is no significant difference between the groups, but control C is almost significantly higher than W (p=0.018) and C (p=0.018). The difference between control W and W is also close to the significance border (p=0.045) shown in Figure 20.

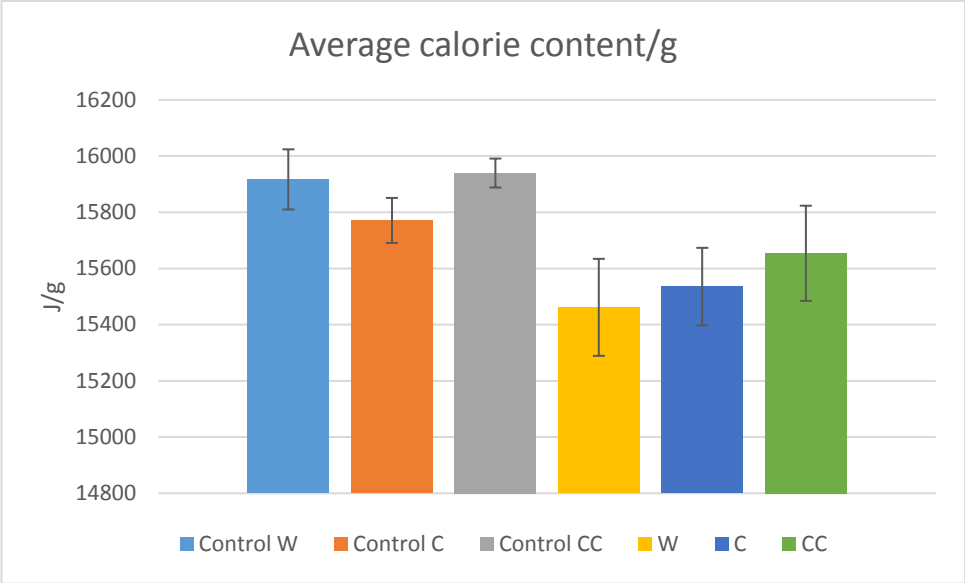

Figure 20:Average calorie content/g. Mice in the groups: W (9), C (10), CC (10), control W (10), control C (10), control CC (10). *There is a significant difference between the CR and the control group, but no significant difference within the CR and control group.*

The calorie content per gramm was multiplied with the weight of the feces produced, which showed significant differences between the control and the CR group, but not within the CR or the control groups (Figure 21).

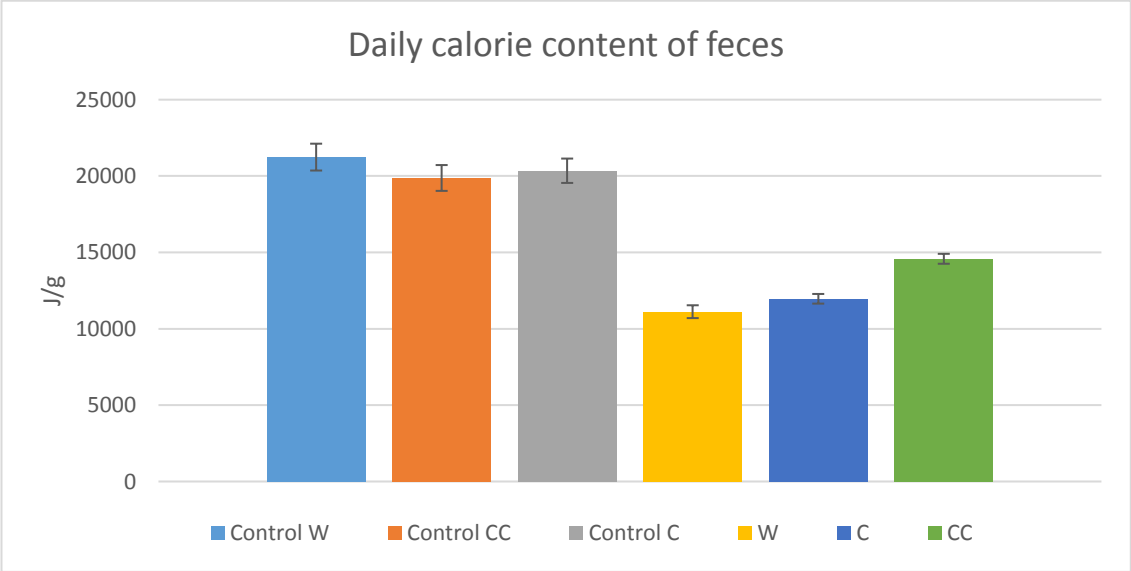

Figure 21: Daily calorie content of feces. Mice in the groups: W (9), C (10), CC (10), control W (10), control C (10), control CC (10). *There is a significant difference between the CR and the control group, but no significant difference within the CR and control group.*

5.7 HPLC-MS/MS detection of caprylate in plasma

5.7.1 Optimization of caprylate detection

The molecular weight of C8:0 is 144.21g/mol, from 13C-C8:0 it is 148.21g/mol and that from 4-APEBA is 365.00g/mol. Both, C8:0 and 13-C8:0 had to be derivatized by 4-APEBA. Therefore, a scan in the range of 300 to 600 was made, assuming, that the precursor ion had to be in this range. For the scan a standard with a concentration of 9.1mg/ml was used. The molecule of interest was the 4-APEBA derivatized caprylate with the mass of 489.2111 (Figure 22). [Kretschmer et al., 2011]

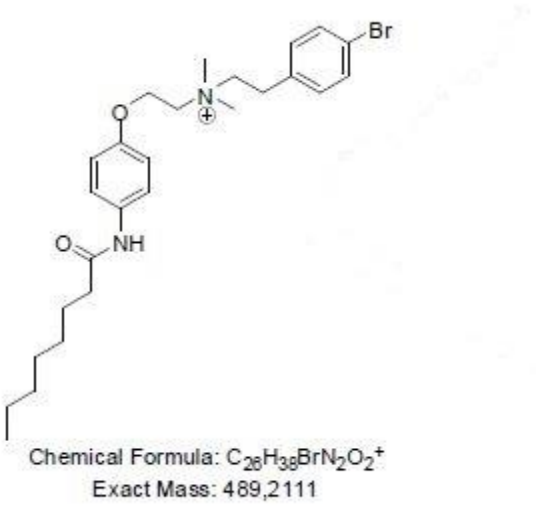

Figure 22: Caprylate bound to 4-APEBA. [Kretschmer et al., 2011]

In this study, the scan results showed an m/z ratio of 489.0 for 4-APEBA derivatized C8:0 in ESI positive mode. Compared to native caprylate, the C13-C8:0 consists of 4 additional hydrogen atoms, thus the mass should be 4g/mol higher. This was confirmed by the scan, showing a precursor ion for C13-C8:0 with an m/z of 493.0.

5.7.2 Selectivity

The determination of the stability of the retention time and the chromatographic separation was carried out with standards in different concentrations (500, 250, 100 µg/ml). The retention time of caprylate was 19.4 (Figure 23), the relative standard error is 0.14%.

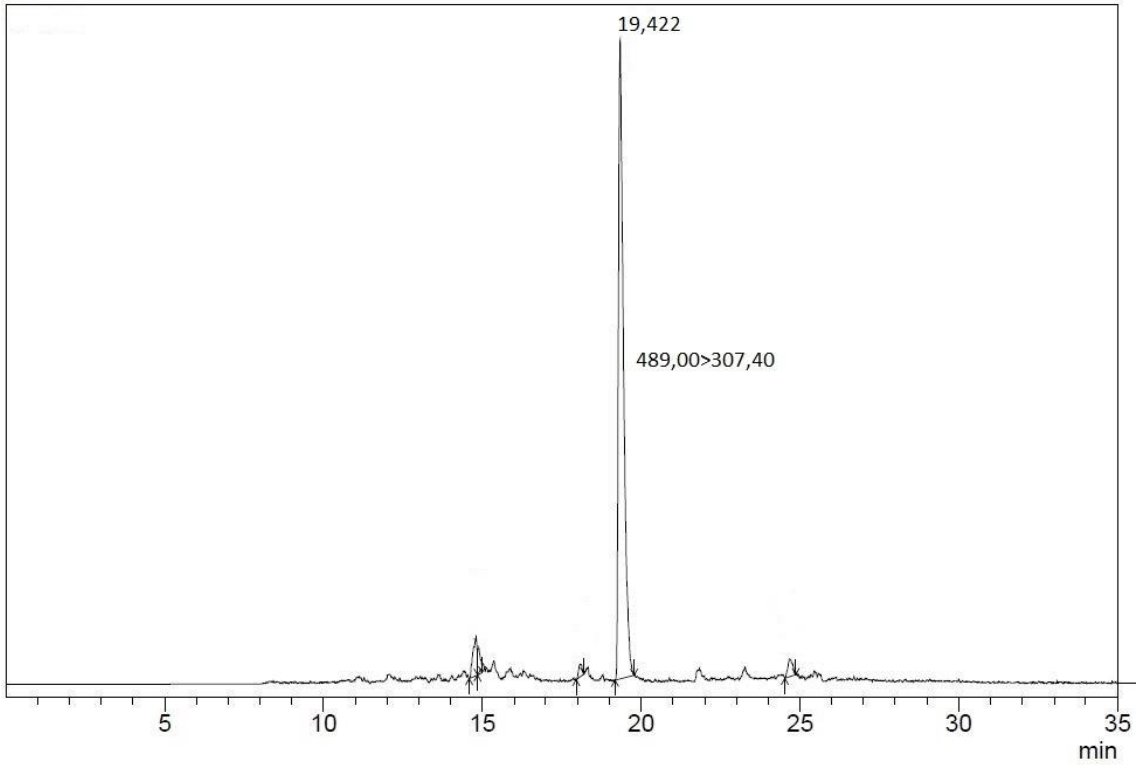

Figure 23: Caprylate has a retention time at 19.4, a precursor ion of 489.0 and an m/z of 307,40.

Isotopic labeled caprylate is in its nature similar to the native caprylate. In this experiment, the most important difference between the two FAs is their weight, which does not have a notable influence on the chromatographic separation or on the retention time. The retention time of 19.4 (Figure 24) is very similar to the retention time of native caprylate, the relative standard error is 0.14%.

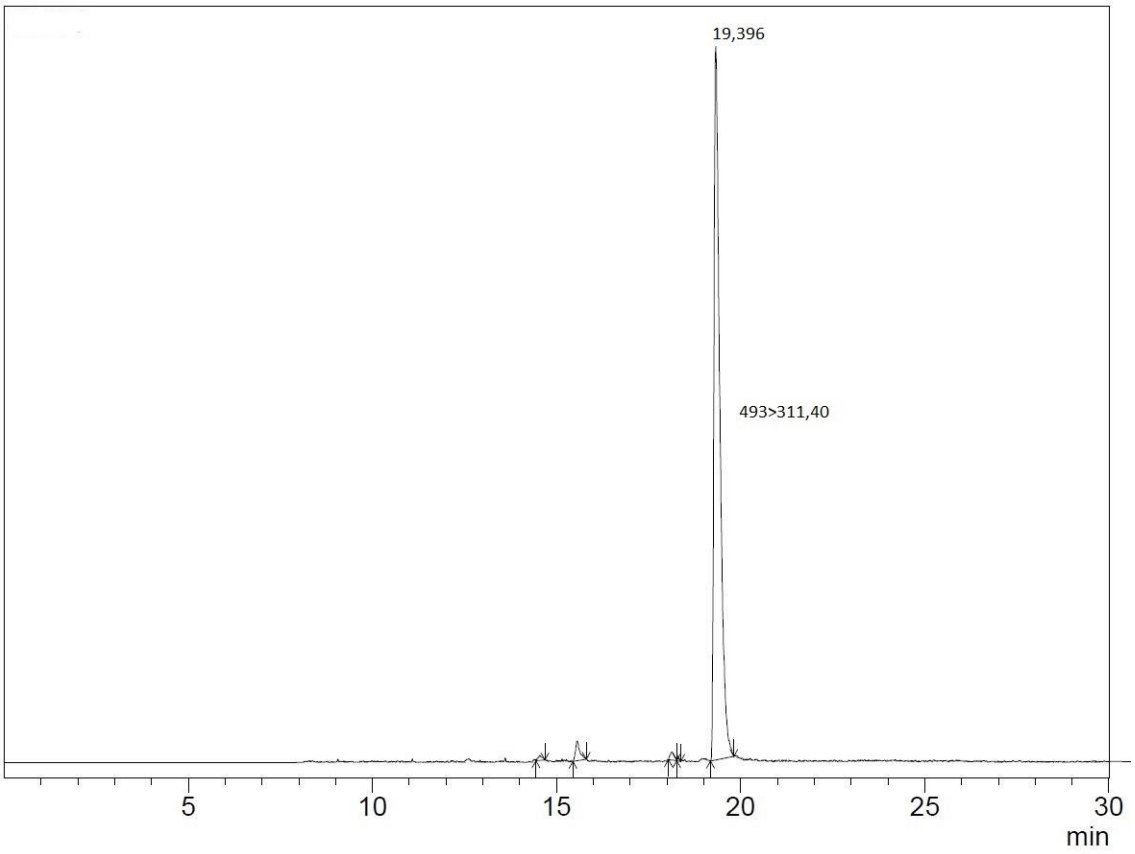

Figure 24: Isotopically labeled caprylate has a retention time at 19.4, a precursor ion of 493.0 and an m/z of 311,4.

Kretschmer et al showed a specific product ion for caprylate with m/z 307.24 shown in Figure 6. In this experiment the same caprylate-specific product ion with m/z 307.4 was found.

5.7.3 Limit of quantification/detection

For this method, the calculated LOQ is 4.23µg/ml and the LOD is 1.27µg/ml.

We were able to show that all of the analysed samples contained caprylate. Altogether, 3.85% of the CR sample concentrations were below the LOQ and thus, a minor optimization of the method is needed. Within the control group, all the samples showed a caprylate

concentration higher than the LOQ, the lowest caprylate concentration was 5.49µg/ml.

5.7.4 Plasma caprylate concentration

For the method development human plasma was used with added caprylate. After finishing the method and before analysing the samples of interest, this human plasma, without added caprylate, was analysed (n=1) and no caprylate was found (Figure 25).

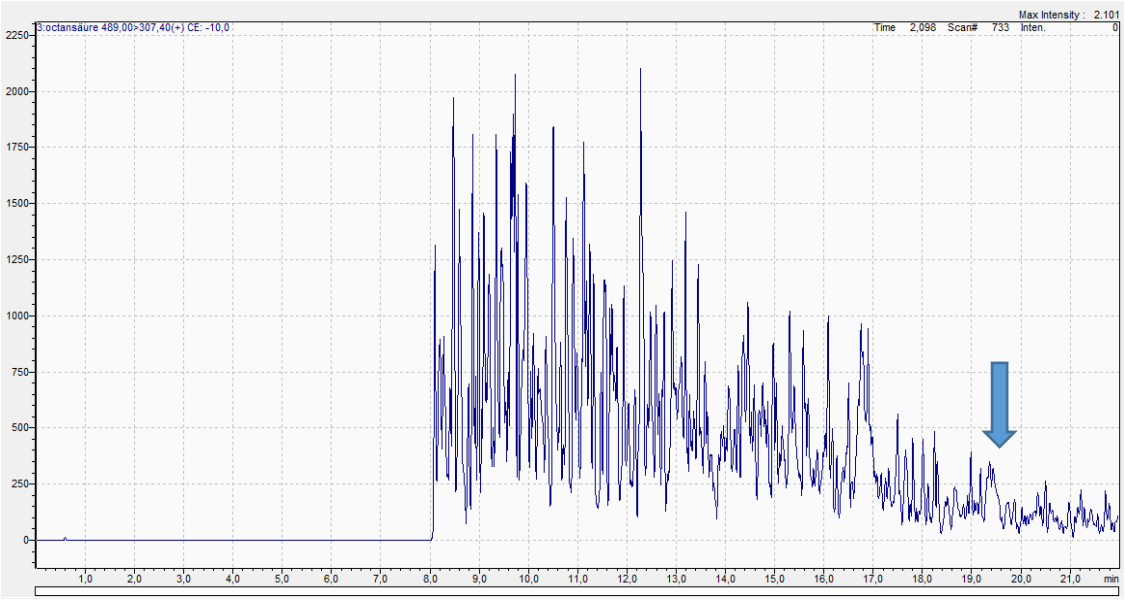

Figure 25: Caprylate detection in human plasma. The arrow points at 19.5min which is where caprylate would be expected.

In samples of the mice, caprylate was always present within every group, no matter if it were the control or the CR groups. Figure 26 shows that control W and control CC are significantly higher than CC and C. Control C is almost significantly higher than CC (p=0.035) and C (p=0.044).

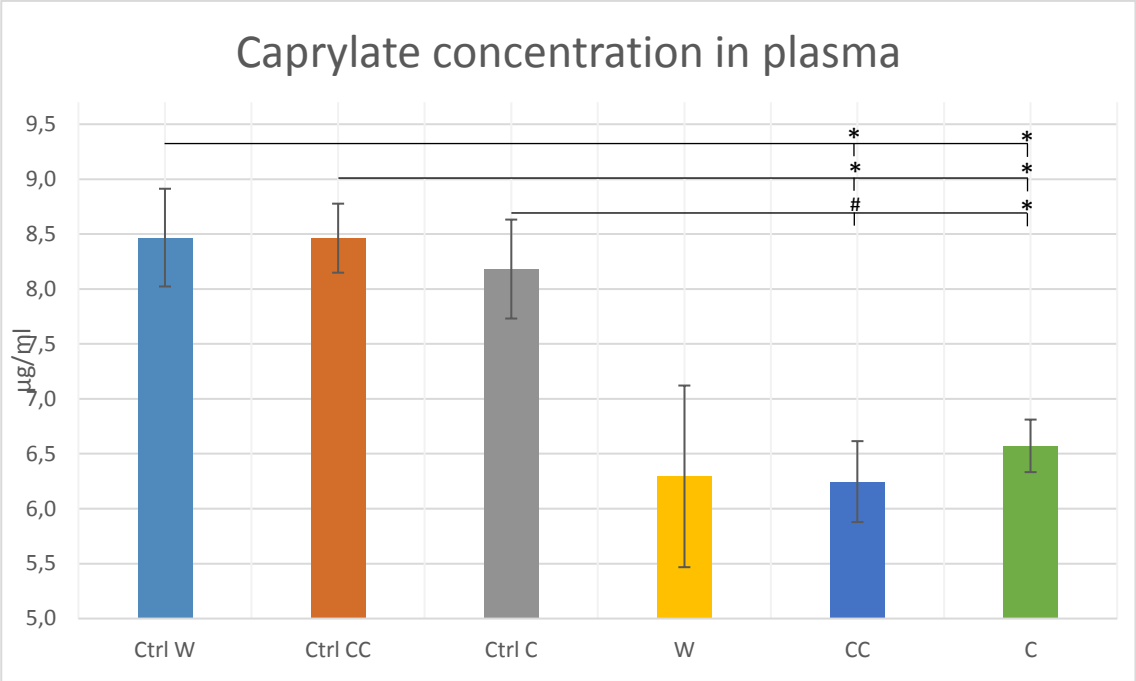

Figure 26: Caprylate concentration in plasma. Mice in the groups: W (7), C (10), CC (9), control W (7), control C (6), control CC (7). Asterisk depicts the significant difference $p<0.0083$. # depicts the almost significant difference of $p=0.0085$. The statistical significance was verified using the Student´s t-test. The data is shown as mean values ± SEM error bar.

5.7.5 Further optimization

For future purposes the amount of 4-APEBA (200µl/sample) and of mouse plasma (100µl) could be lowered and the volume of the other solutions may be adjusted. In the control groups, derivatizing 100µl of blood plasma with 150µl 3mg/ml 4-APEBA showed the same results as when 200µg/ml 4-APEBA was used. The concentration or the amount of EDC and NHS were not changed.

As CR mice tend to have less plasma than the mice in the control group, mostly 80µl of CR mice plasma was used. The analysis of this volume delivered results above the LOQ and thus rendering it a more ideal plasma volume.

For the extraction of 80µl plasma, the equivalent amount of ACN is needed. Usually, such an extraction is carried out two to three times.

In the present experiment, the multiple extraction did not lead to any advantage. This may be due to the suitable analytical standard.

Eventually, the volume adjustment to 80µl plasma should be conducted for 3M HCL, 3M NaOH, 3mg/ml 4-APEBA, 290mM EDC and 190mM NHS by lowering the volume by 20 %. Due to the limited sample availability, these changes have not been tested. A comparison of the method used and the method foreseen for future use is shown in Table 7.

Table 7: Caprylate detection method comparison. Not tested volumes are marked with an asterisk. Volumes in brackets shows volumes adjusted to the lowered volume of 4-APEBA.

	Original method for 100µl plasma	**New method for 100µl plasma**	**New method for 80µl plasma**
Plasma	100µl	100µl	80µl
3M HCL	6.5µl	6.5µl	5.2µl*
3M NaOH	6.5µl	6.5µl	5.2µl*
ACN	100µl	100µl	80µl
4-APEBA	200µl	150µl	120µl*
EDC	50µl	50µl (37.5µl*)	40µl* (30µl*)
NHS	50µl	50µl (37.5µl*)	40µl* (30µl*)
End volume	513µl	463µl (438µl)	370.4µl (340µl)

Lowering the dilution results in a higher end concentration of 4-APEBA derivatized caprylate. For example: A 100µl standard solution with a concentration of 9.1mg/ml is prepared and diluted as shown in Table 7. The final concentration for the final volume of 513µl is 1.77mg/ml, whereas for the final volume of 438µl the concentration is 2.08mg/ml. Thus, the higher final concentration of caprylate could

lower the LOQ and LOD by approximately 17%. This possible new method is shown in Figure 27.

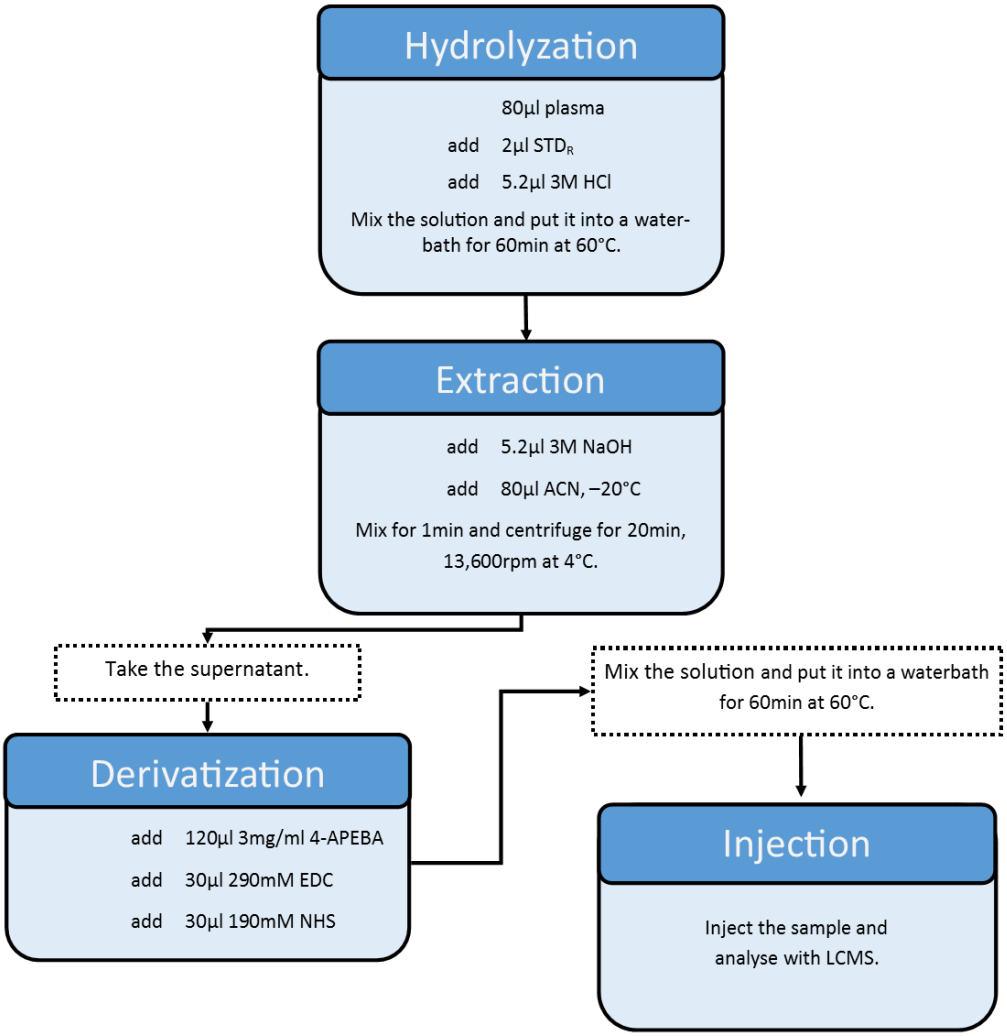

Figure 27: Method forseen for future purposes.

5.8 NMR

It has been evaluated that bedding has an impact on the appearance of caprylate in mouse feces during CR. Figure 28 shows an NMR-based metabolic profiling in fecal water from CR mice (red) and the same sample with addition of a standard solution of caprylate (blue). When adding the standard solution, the signals overlapping with the signals of interest are increased indicating the presence of caprylate.

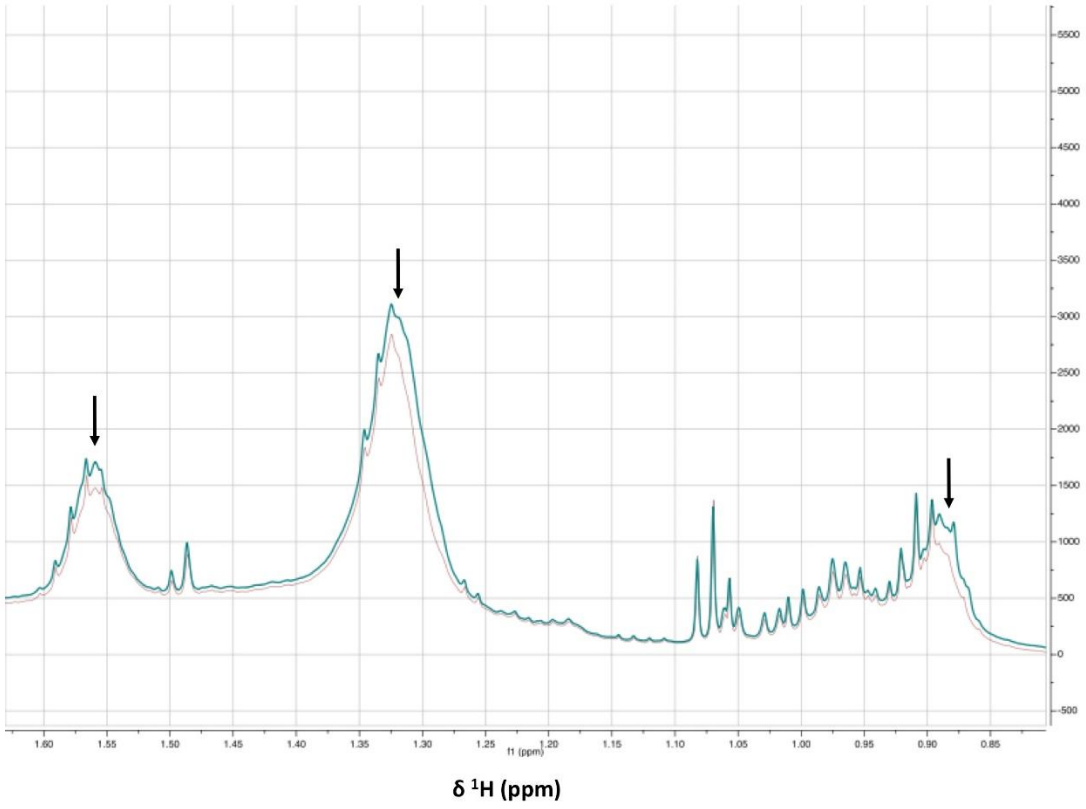

Figure 28: Caprylate in mouse faeces. Red: fecal water from CR mouse. Blue: The same sample wit addition of a standard solution of caprylate. Arows shows the signals of interest.

The Principal Component Analysis (Figure 29) shows that CR strongly influences the fecal metabolic profiles independently of the bedding. There is a strong clustering of the CR groups cellulose and wood. The corncob CR group samples seem to be distinct from the control and also from the other CR groups.

No apparent difference can be observed between the control group samples.

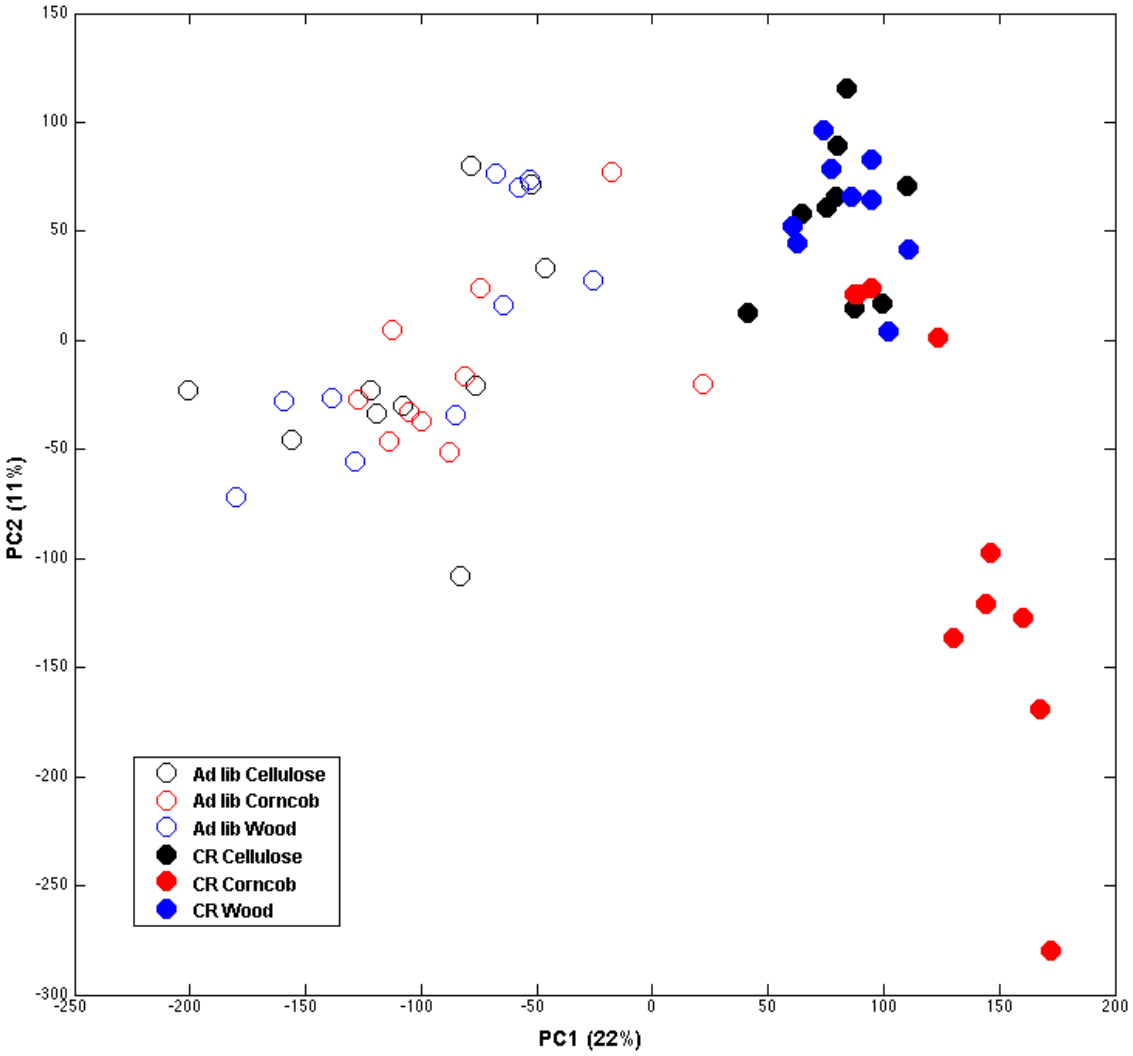

Figure 29: Principal Component Analysis.

The NMR shows caprylate in the fecal water in both CR and control groups. Cellulose and wood, but not corncob, increases the signal for caprylate at 1.3 ppm compared to the control groups (Figure 30).

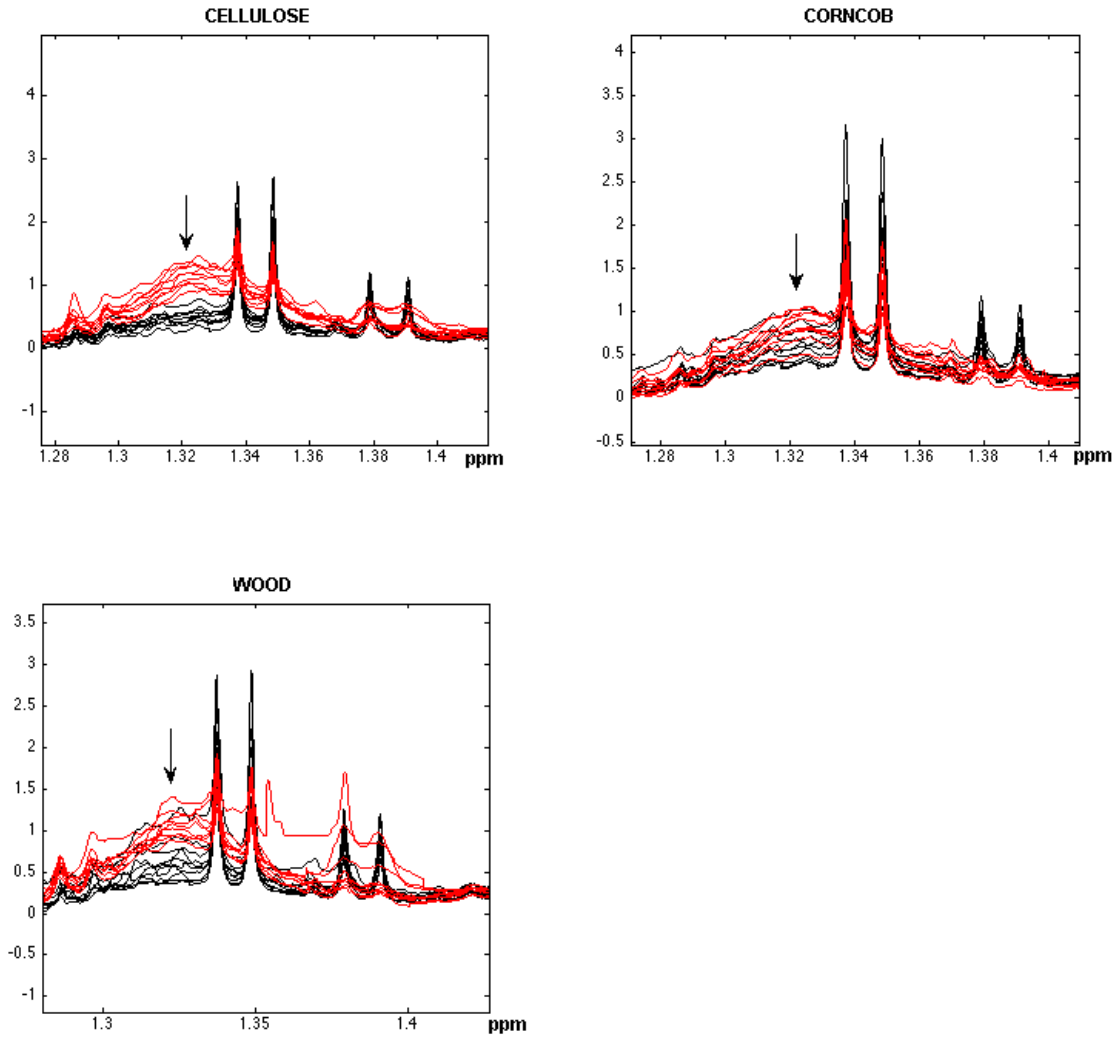

Figure 30: 1H-NMR spectroscopy. Black indicates the control groups and red the CR groups. Arrow indicates the resonance of interest.

6 Discussion

The observation that CR mice tend to eat the bedding is supported by our data, showing that mice eat up to 1.39g ± 0.05g of bedding daily. The amount of bedding eaten correlates strongly with the type of the bedding. From the wood, cellulose and corncob beddings used in this experiment the most ingested one was the wooden bedding and the least ingested the cellulose bedding. The higher fiber intake may enhance the number and activity of cellulolytic bacteria in the digestive tract of mice [Boulahrouf et al., 1990] which could lead to a higher supply of nutrients, such as SCFAs. Thus, the higher bedding ingestion could alleviate the effects of CR. However, the daily body weight loss, total body weight loss, the glucose levels and the stomach weight expressed in percent of the body weight were not significantly influenced by the bedding type. On two days of CR, the time it took the mice to start the meal was significantly higher in group CC than in group W. There was no significant difference between any other group on any other day. This indicates that the two weeks of fasting were equally difficult to both groups.

On the other hand, the cecum weight given in percent of the body weight was significantly higher in the CR group W than in the CR group CC which might be because of the overall higher fiber intake in the CR group W. Group C had the lowest bedding intake but the cecum size in percent of the body weight was not significantly lower than that of the group W. This could be because of the high cellulose intake which does not significantly differ from the cellulose intake of group W. (or: Cellulose might have a key role in this relation, as the cellulose intake matches with that of group W and CC.)

In the sum sWAT, there was no significant difference between the CR groups but surprisingly, the sum sWAT of control group W was not significantly higher than that of any of the CR groups and it was significantly lower than the one of the control group C. Extrapolating from the data on CR mice, a probable explanation could be, that mice fed *ad libitum* also consume the wooden bedding which was the favorite in the CR groups. This could lead to a lower chow consumption resulting in a lower sum sWAT. However, there is no other data supporting this theory, as this was the only significant difference found between the control groups.

There was no difference between the feces calorie content of CR mice, but control mice produced more feces than CR mice and therefore the total calories in the feces of the control mice was higher than that from CR mice.

In studies on ghrelin-acylation, in which rodents were fed with caprylate or other MCFAs, the caprylate intake is known and the effect on acylation can be assessed. In this present study, the impact of CR on the possible intestinal caprylate production, which can be absorbed from the gut and used for ghrelin activation, was assessed. Dietary fibers have been proved to modify the microbial composition, which could lead to the phenomenon of higher caprylate production in the gut. Only in the feces of the CR groups W and C a higher caprylate level – compared to the one of the control group – was observable. Thus, cellulose may serve as a probable enhancer of the intestinal caprylate production.

Despite the fact, that the concentration of fecal caprylate is higher in the CR groups W and C, the plasma concentrations are significantly lower in the CR group C than in all the control groups. Because of the high variability, the concentration of plasma caprylate in the CR

group W neither differs from the control nor from the other CR groups, whereas the CR group CC significantly differs from the control groups W and CC and almost significantly differs from control C. One possible reason for the tendency of lower plasma caprylate concentrations in the CR groups could be that the caprylate absorbed from the gut is rapidly used by the mitochondria for energy production, whereas in the control group there is less need for energy from FAs. Bearing this in mind, it may be possible that after a longer CR, when the glucose levels stay low for a longer time, the concentration of caprylate drops further.

In a study of Lemarié et al. (2016), the plasma caprylate levels were assessed on Sprague-Dawley male rats. The rats were either on 70% CR, high fat or on moderate fat diets containing different concentrations of caprylate or no caprylate at all. After 18 hours fasting, blood was drawn and plasma was analysed but no detectable amount of caprylate was found in the moderate fat group. As there was no caprylate found in the liver either, it was stated that after such a long fasting period, caprylate is taken up and catabolized by the liver. There is no data shown on the caprylate plasma levels of the CR rats.

Usually, CR mice eat their daily meal rapidly within a couple of hours. [Speakman and Mitchell, 2011] In one recent study CR mice ate their meal within two hours, afterwards mice fasted until the next food delivery. [Acosta-Rodríguez et al., 2017]

In this present study, caprylate-free food was delivered from 3 p.m. to 4 p.m. Therefore, it can be assumed that the food was surely eaten at midnight and that the mice were fasting until the dissection, which happened on the next day between 9 a.m. to 11 p.m. After 9 hours to 11 hours of fasting, and although the chow contained no caprylate it was still detectable in the plasma both in CR mice and in

mice fed *ad libitum*. This discrepancy between the study of Lemarié et al. and the present study may be due to the different animal models, the longer fasted state of the rats or the higher LOD of the method of Lemarié et al.

7 Conclusion

In this thesis, the impact of caloric restriction on the caprylate concentration in the feces and plasma is documented, and a new method for the detection of caprylate is provided. Furthermore, it has been assessed whether wood, corncob or cellulose bedding affects the CR outcomes and whether the bedding type influences the caprylate concentration in feces and plasma.

Eventually, when subjected to a CR diet, the bedding type influences the amount of bedding eaten. Of the three types of bedding offered, the wooden one was the favourite and cellulose bedding the least consumed one. The bedding type, and therefore the fiber intake, may also influence the cecum size expressed in percent of body weight, where the fibers are broken down by bacteria.

After two weeks of CR, there was no observable effect on the daily body weight loss, the total body weight loss, the time it took the mice to start the meal, the glucose levels and the stomach weight expressed in percent of body weight.

The only significant difference in the control group was observed in the sum sWAT, showing that the sum sWAT of the control group W was significantly lower than the sum sWAT of the control group C and did not differ from the CR groups. In the control group W, like in the CR group W, could be a tendency for eating the bedding, resulting in smaller fat depots.

The impact of CR became visible in a higher concentration of caprylate in the feces of the CR groups W and C compared to the control groups. Despite the higher concentration of feces caprylate in two of the CR groups, the plasma concentrations in the CR groups

tended to be lower than in the control groups. This was the case also in the CR group CC, in which the concentration of feces caprylate was similar to the one of the control group CC. The CR group C was the only one in which the plasma caprylate concentration was significantly lower than in all the control groups. In contrary, there was no significant difference between the CR group W and the control groups although the feces caprylate concentration was similar to the CR group C.

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