Master's thesis

NTNU
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Department of Circulation and Medical Imaging

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Investigation of the role of carbohydrate intake on gut microbiota and short-chain fatty acids

Master's thesis in Clinical Health Science - Obesity and Health
Supervisor: Catia Martins
Co-supervisor: Jessica Ann Røkenes
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Kari Nerhoel

Abstract

Background: Ketogenic diets have become increasingly popular, likely because they are able to induce weight loss (WL), while preventing the increase in appetite otherwise seen with weight reduction. However, these diets contradict evidence-based nutrition guidelines, as a low-carbohydrate (CHO) intake is needed to induce ketosis. Low-CHO diets are likely to have a negative impact on gut microbiota, but results are scarce and contradictory. Therefore, the aim of this thesis was to assess how low-energy diets (LED) with different amounts of CHO impacts on gut microbiota and short-chain fatty acids (SCFA).

Methods: 101 healthy adults (51 females), with obesity (mean ± SEM; BMI 34.6 ± 0.4 kg/m²) were randomized to 1 out of 3 isocaloric LED prescriptions (1000 kcal [4184 kJ]/day) with 70, 100 or 130 g CHO/day (Low, Medium and High CHO groups, respectively), for 8 weeks, followed by 4 weeks of refeeding (weight stabilization phase). Body weight, plasma concentration of beta hydroxybutyrate (βHB)), gut microbiota and fecal SCFA concentrations were measured at baseline (BL), end of the diet intervention (W9) and end of 4 weeks refeeding (W13).

Results: Overall, participants lost 14 % ($P<0.001$) of their initial BW, with no differences between groups. All groups were in ketosis (0.7 ± 0.1 mM, $P<0.001$) at W9, but not at W13, but there were significantly higher βHB-values in the low CHO group compared with the medium ($P<0.01$) and high ($P<0.001$) CHO groups. An overall increase in the relative abundance of Alistipes ($P<0.001$), and Ruminococcaceae ($P<0.01$), and a decrease in Eubacterium rectale group ($P<0.001$) was seen between BL to W9, and at W13 the difference from BL was still significant for the Eubacterium rectale group ($P<0.01$). Also, there was a decrease in the proportion of acetic, propionic, butyric, valeric ($P<0.001$ for all) and isobutyric acid ($P<0.01$) at W9, and they remained low at W13, even though the acetic, propionic and butyric acids increased significantly from W9 to W13 ($P<0.01$, $P<0.01$ and $P<0.001$, respectively). At W9, the low CHO group had lower proportions of Blautia, Eubacterium rectale group and isobutyric acid than the high CHO group ($P<0.05$ for all), and for Blautia this difference was also seen between the low and medium CHO groups, with lower proportion in the low CHO group ($P<0.01$). Moreover, the decrease in Eubacterium rectale group was weakly and positively correlated with CHO intake at W9 ($P<0.05$).

Conclusion: A low CHO intake seems to have a negative effect on gut microbiota and fecal SCFA production. More studies are required to establish this relationship.

Sammendrag

Bakgrunn: Ketogene dietter har stadig økt i popularitet de siste årene, antakelig fordi de har vist at de kan føre til vekttap, samtidig som de forebygger en økning i appetitt som vanligvis forekommer ved vektreduksjon. Disse diettene har derimot en tendens til å gå imot evidensbaserte ernæringsretningslinjer, siden et lavt inntak av karbohydrater (KHO) er nødvendig for å indusere ketose. Et kosthold med lavt innhold av KHO er antatt å ha en negativ effekt på tarmmikrobiotaen, men resultatene fra forskning er begrensede og motsigende. Derfor vil målet med denne oppgaven være å vurdere hvordan lav-energi dietter (LED) med forskjellige mengder KHO påvirker tarmmikrobiota og produksjon av kortkjedete fettsyrer (SCFA).

Metode: 101 friske voksne (51 kvinner), med fedme (GJ. Snitt ± SEM; BMI 34.6 ± 0.4 kg/m²) ble randomisert til 1 av 3 isokaloriske LEDs (1000 kcal [4184 kJ]/dag) med 70, 100 eller 130 g KHO/dag (i hhv. lav, medium, høy KHO gruppe), i 8 uker, etterfulgt av 4 uker med en vektstabiliseringsfase. Vekt, plasmakonsentrasjoner av beta-hydroxybutyrat (βHB), tarmmikrobiota og SCFA ble målt ved oppstart (BL), ved slutten av 8 uker kostholds-intervensjon (U9) og ved slutten av vektstabiliseringsfasen (U13).

Resultater: I alt så gikk deltakerne ned 14% av opprinnelig vekt ($P<0.001$), og det var ingen forskjell mellom gruppene. Alle gruppene var under ketose (0.7 ± 0.1 mM, $P<0.001$) ved U9, men ikke ved U13, men den lave KHO gruppen hadde signifikant høyere βHB-verdier enn medium ($P<0.01$) og høy ($P<0.001$) KHO-gruppene. Det var en økning i den relative forekomsten av Alistipes ($P<0.001$), og Ruminococcaceae ($P<0.01$), og en redusert forekomst av Eubacterium rectale gruppen ($P<0.001$) mellom BL og U9, men ved U13 var forskjellen fra BL fortsatt signifikant for Eubacterium rectale gruppen ($P<0.01$). Det var også en redusert forekomst av acetat, propionat, butyrat, valerat ($P<0.001$ for alle), og isobutyrat ($P<0.01$) ved U9, og forekomsten av disse forble lave ved U13, selv om acetat, propionat og butyrat økte signifikant fra U9 til U13 (hhv. $P<0.01$, $P<0.01$ og $P<0.001$). Ved U9 hadde lav KHO gruppe lavere forekomst av Blautia, Eubacterium rectale gruppen og isobutyrat enn høy KHO gruppe ($P<0.05$ for alle), og for Blautia var også denne forskjellen observert mellom lav og medium KHO gruppe, med lavere andel hos lav KHO gruppe ($P<0.01$). I tillegg var reduksjonen i andelen av Eubacterium rectale gruppen svakt positivt korrelert med KHO inntak ved U9 ($P<0.05$).

Konklusjon: Et lavt KHO inntak ser ut til å ha en negativ effekt på tarmmikrobiotaen og på produksjon av SCFA. Flere studier på feltet er nødvendig for å etablere en slik sammenheng.

Abbreviations

AcAc - acetoacetate

BHB/β-HB - β-hydroxybutyric acid

BL - baseline

BMI - body mass index

BW - body weight

CHO - carbohydrate

F/B – firmicutes/bacteroides

KDs – ketogenic diets

KLCDs - ketogenic low carbohydrate diets

LED - low energy diets

MACs – microbiota accessible carbohydrates

NDC – nondigestible carbohydrate

ObeCe - regional Center of Obesity Research and Innovation

REK - regional ethics committee

SCFA - Short chain fatty acids

SEM – standard error of the mean

VLEDs - very-low energy diets

W9 – week 9

W13 – week 13

WL - weight loss

WHO - World Health Organization

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1.0 Background

1.1 Introduction

Obesity is highly prevalent in most parts of the world and its prevalence has nearly tripled since 1975 (1). This is alarming considering the association between obesity and comorbidities such as type 2 diabetes and cardiovascular diseases (1). Finding methods that will effectively induce and maintain weight loss (WL) has been an issue for decades. These methods include all types of interventions such as energy-restricted diets, meal replacements and exercise programs, education programs related to lifestyle and diet, and different behavioral techniques (2). Even when WL is successfully achieved, about a third of the weight is expected to be regained with diet and exercise related WL alone after one year, and most of the weight is regained within 3 to 5 years (3).

An increase in subjective feelings of hunger is cited as a major side effect of WL attempts, followed by an increase in the secretion of the hunger hormone ghrelin, and a reduction in the postprandial secretion of several satiety peptides, such as glucagon-like peptide-1 (GLP-1), peptide YY (PYY) and Cholecystokinin (CKK) (4-6). Although this increase in appetite due to diet-induced WL is likely a normalization towards a lower body weight (7), this side effect is reported to be an important contributing factor to the high attrition rates seen with energy restricted diets (8). Ketogenic diets (KDs) have, however, become more popular in recent years because of its supposed ability to induce WL without this increase in hunger feelings, or ghrelin secretion (4, 9). However, considering that KDs require a severe restriction of entire food groups that are thought to be beneficial for health, such as fruits and vegetables, whole-grains and other fiber rich foods, these diets are considered to be contradictory to evidence-based guidelines for healthy eating (4, 10).

Therefore, with the increasing interest in ketogenic and other types of low-carbohydrate diets, where dietary fiber sources may be limited, there is a need for studies investigating the effects of these types of diets on health-related outcomes, especially to the gut microbiota and short-chain fatty acids (SCFAs). Studies have shown that dietary fiber or nondigestible carbohydrate (NDC) consumption is critical for the maintenance of good health, and also for managing symptoms of metabolic disease (11). If there is a lower abundance of microbiota accessible carbohydrates (MACs) in the diet to support the growth of specialist microbes that produce SCFAs, the SCFA production is reduced and the gastrointestinal microbiota metabolism may start producing

detrimental metabolites which consequently leads to increase in bacteria associated with chronic inflammation and disease (12). There seems to be a knowledge gap in connecting the gut microbiota, and production of SCFAs, to ketogenic and other low carbohydrate diets. Therefore, the aim of this study was to investigate the role of carbohydrate intake on a low-energy diet on gut microbiota and SCFA, and whether a low carbohydrate (CHO) intake has a negative impact on gut microbiota in individuals with obesity.

1.2 Theoretical background

Ketosis, ketone bodies, ketogenic diets, and weight loss

The ketogenic diet (KD), or the Ketogenic Low Carbohydrate Diet (KLCD) has reached a new level of popularity during recent years for its supposed ability to induce a successful WL and at the same time suppress appetite (13). This is thought to help long-term WL maintenance in a more efficient way than other dietary WL interventions (4, 9), although there is still no evidence supporting its superiority above other diets in this context (14). KDs have a long history of being a tool in the treatment of refractory epilepsy (15), but in recent years evidence seems to support its potential therapeutic effect for certain metabolic disorders (16). A ketogenic diet consists of a very low carbohydrate intake, down to around 5-10% of total intake, or below 50 g per day, for the purpose of enhancing ketone production (17). After several days of drastically reducing CHO intake to a level of 5% of total daily energy intake, the body is not able to achieve enough energy from glucose and has to use fats as a primary source of fuel. Since free fatty acids are not capable to cross the blood brain barrier, the energy needs to be provided by ketone bodies (12). The rate of ketone production through the process of ketogenesis, and the rate of utilization in the process of ketolysis, are the processes that determine the levels of ketone bodies in the blood (10). Three types of ketone bodies are formed: Acetoacetate (AcAc), β-hydroxybutyric (BHB) and acetone (10, 13). These are formed in the liver, as a result of the overproduction of acetyl-CoA due to low carbohydrate intake and low insulin (13, 18, 19).

The low and very low energy diets (LEDs and VLEDs) differ from the KLCD in that it does not allow an ad libitum consumption of protein and fat, but remains low in intake of all macronutrients, including carbohydrates, thus limiting CHO intake (4). Total energy consumption in VLEDs is limited to less than 3347 kJ (or 800 kcal) per day, with approximately 40-60g of dietary CHO(4). As a result of the limited CHO intake it is believed that ketosis can be achieved, but researchers have yet to reach a consensus to which amount of CHO restriction is required to induce this state (10). Although studies have shown that both of these diets have the potential to induce a ketotic state, the ketone levels of the KLCD diet is usually several fold higher than with the VLED diet (4). The LED have an energy restriction of between 800-1200 kcal per day (3347-5020 kJ/d) and will consequently have an increased intake in macronutrients, including CHO,

compared to the VLED (20, 21). While VLEDs often uses meal replacements, LEDs can also be food based, and some LEDs are a mix of these (20).

Gut microbiota

The microbiome consists of the community of hundreds of trillions of microorganisms that exist on and in every human, divided into different categories, such as the skin microbiome, urogenital microbiome, and a gastrointestinal microbiome (22). The gastrointestinal microbiome is composed of different types of bacteria, archaea, microeukaryotes, fungi and viruses that live in symbiosis within humans, and the majority is localized in the colon (22, 23). The microbiome itself is thought to have a genome that contains 150 times the number of genes in humans, which gives the gut microbiome a symbolic status of an organ (22, 24). This organ consists of prokaryotic cells and is thought to be cooperating with the eukaryotic cells of the human host to maintain good health. Some of the functions of the gut microbiome that are considered essential to health and development of obesity are vitamin and cofactor production, digestion and breakdown of complex polysaccharides to SCFAs, regulation of gastrointestinal motility, epithelial homeostasis, and the development of adaptive immunity (22, 25). A consideration that the gut microbiota is influenced by factors such as long-term dietary habits has been detected, as different types of diets create different enterotypes of the gut microbiota, working in different ways in the human host. The basis of the colonization of the gut microbiota appears to be set already at birth, but the environmental and nutritional factors can contribute to this colonization (12, 22).

Gut microbiota and link to obesity

The microbiome plays an important role in regulating intestinal transit, and thus the amount of energy absorbed from the diet will be influenced by changes in the microbiome, and especially in the gut microbiota (22). There has been an observed connection between obesity and the shift in the proportion of bacterial flora connected to the Firmicutes and Bacteroidetes phyla, which comprise about 90% of the gut microbiota in humans (22). This was observed in a study conducted with ob/ob mice (26), that had a significant reduction of about 50% in Bacteroidetes, and an increase similar to this in Firmucutes and Archaea in the obese mice. This resulted in increased fermentation of dietary polysaccharides and it also had an effect on the energy level remaining in the feces, after measuring with bomb calorimetry, the energy level of the feces had decreased (26).

In human studies as well, there is evidence pointing towards this shift in the proportion of gut microbiota phyla to be associated with weight. Similar to studies done with mice (26, 27), in a human study with participants with obesity who lost weight, there was an increase in Bacteroidetes over a 12 month period (28). While the concentration of firmicutes gradually

increases with increasing BMI, the bacteroidetes phyla decreases with increasing BMI. The Firmucutes/Becteroidetes (F/B) ratio is also shown to raise with increasing BMI (28-30). Both of these phyla of gut microbiota contain species that produces SCFA from dietary compounds that escape digestion in the small intestine, and this will in turn supply the host with an additional energy source (31).

Short chain fatty acids (SCFAs)

Short chain fatty acids (SCFAs), known as fermentation products from undigested food components from the small intestine, are defined as 1-6 carbon volatile fatty acids and can present in both straight or branched-chained conformation, and they are absorbed in the large bowel (32). SCFAs are the end products from this process of fermentation of NDCs, and they become available to the gut microbiota, and consequently impact human health (33). Some of the physiological effects of the SCFAs include influencing the physiology of the colon, shaping of gut environment and participating in host-signaling mechanisms, and as previously mentioned, they can also be used by host cells and intestinal microbiota as energy sources (31, 32). The typical western diet, with a high amount of fat and sugar and low amount of fibers, has the potential of manipulating gut microbiota composition in a negative direction (34). SCFA production is lowered because of the reduced intake MACs, and there is a shift towards the production of detrimental metabolites in the gastrointestinal microbiota metabolism (12). The three most abundant SCFAs, which comprise about 95% of the total amount of SCFAs, are acetate (C2), propionate (C3) and butyrate (C4) (32, 35, 36). These SCFAs are present in a 60:20:20 ratio, respectively, in the stool and the colon, and this ratio seems to be similar in the proximal and distal regions of the large intestine, even though the total concentration decreases throughout the colon (35).

The effects of low-CHO diets on the gut microbiota and SCFAs, and its association with obesity.

A diverse and rich microbiota has been associated with good health. The gut microbiota is easily altered by external factors, with diet being one of the most important contributors (37). A study showed that gut microbiota adapted quickly in a negative direction to a switch in macronutrient composition from a well-balanced western diet to a similar diet, but with a lower CHO and protein content (38). A healthy microbiota is characterized by containing species that are potentially beneficial, mostly within the Firmicutes and Bacteroidetes phyla (39), but also Bifidobacterial species (40) and a low concentration of the potentially pathogenic species, such as Proteobacteria (39). A reduced CHO intake is of particular interest when it comes to investigating the gut microbiota, because a lower CHO intake consequently lowers the intake of polysaccharides, which will in turn decrease many gut microbiota bacteria that produce their energy from polysaccharides (37). In countries where intake of polysaccharides is favored over fat intake, they reported an overall greater diversity of gut microbiota compared with countries that

consume general western diets (41, 42). A diet containing large amounts of fiber from sources such as vegetables, fruits, whole grains and legumes has shown to increase the number of beneficial bacterial species and increase diversity of the gut microbiota, which in turn affects positively the health of the individual (39).

Although several studies have documented an unfavorable shift in gut microbiota with diets low in CHO (ranging from 4% to 46% of total EI) (14, 40, 43-46), there is still uncertainty about the clinical relevance of these shifts, and also the long-term consequences (14, 46). A study comparing bowel health and function following a low CHO diet versus a high CHO diet, suggested that long-term consumption of a low CHO diet may increase the risk of developing gastrointestinal disorders (46). Although the meaning behind "long-term" in this study is not defined, the statement is based on short-term findings (from baseline to 8 weeks) that the low CHO diet lowered stool weight and had detrimental effects on the concentration and excretion of fecal SCFA compared to the high CHO diet (46). Reduced levels of Bifidobacteria and butyrate were also reported (46), and these are associated with a good gut health (39) and increased energy expenditure (47), respectively.

In contrast to the potential detrimental effects of low CHO diets to microbiota composition, evidence from a study in mice, suggest that a KD will increase levels of beneficial bacteria such as Akkermansia munciniphila and Lactobacillus, because of the lowered blood glucose level, and increase in blood ketones. The same study, however, found a decrease in overall microbial diversity (48). A study analyzing gut microbiota of 10 patients with multiple sclerosis on a ketogenic diet for six months, also showed a decrease in bacterial diversity and concentrations during the first 12 weeks of the study, but after this 12-week mark the bacterial concentration began to recover back to the baseline values, and by weeks 23-24, the bacterial diversity even increased beyond baseline (BL) values (49).

There seems to be some contradictory evidence supporting the hypothesis that that the lower the CHO intake, the more negative effect it has on microbiota abundance. Most studies have been conducted with KLCDs and VLEDs, and there is little evidence supporting the hypothesis when it comes to different types of LEDs specifically. More studies need to be conducted to establish the role of CHO intake within an energy restricted diet (and therefore WL) on gut microbiota.

1.2 Objective and hypothesis

The aim of this study was to investigate the role of CHO intake on a LED on gut microbiota and SCFA, and whether a low CHO intake can have a negative impact on gut microbiota in individuals with obesity. The main hypothesis of this project, as mentioned above, was that the lower the CHO intake, the more negative effect it has on gut microbiota and SCFAs.

2.0 Methods

2.1 Study design and randomization

This master thesis is part of the ASKED Trial, a single-center, single-blinded (participants) randomized controlled trial (RCT) with repeated measurements. This study received ethical approval from the Regional Ethics Committee in Central Norway (Ref. 2016/1297) and has been registered at clinicaltrials.gov (NCT02944253)

Participants were randomized to 3 isocaloric LEDs containing varying amounts of CHO: 70, 100 and 130 g CHO/day respectively, in each group for 8 weeks, followed by 4 weeks of refeeding and weight stabilization. The randomization was computer-generated using a block sampling and stratification approach. This was done to account for potential confounding factors of sex and BMI (50, 51). Throughout the study period, participants were asked to maintain their physical activity (PA) levels.

2.2 Study population

2.2.1 Participants

One hundred healthy adult volunteers from 18 to 65 years old, both men and women, with class I or II obesity (30 kg/m2 < BMI > 40 kg/m2), weight stable (<2 kg variation in weight within the last 3 months), and not currently dieting to lose weight were included in this study.

Participants who were pregnant, breast-feeding, dealing with drug or alcohol abuse within the last two years, currently taking medication known to affect appetite or induce WL, and enrolled in another obesity treatment program were excluded from the study. Those who had a history of psychological disorders were also unable to participate in the study, as well as those who have had bariatric surgery, those with metabolic diseases (such as hypo/hyperthyroidism and diabetes type 1 or 2), eating disorders, lactose intolerance, gastrointestinal (particularly cholelithiasis), kidney-, liver-, lung-, cardiovascular-, rheumatoid arthritis, Crohn's disease and malignancies. Consumption of probiotics over the last 6 months and use of antibiotics over the last 3 months was also an exclusion criterion.

The study only included women who were either postmenopausal, or premenopausal on hormonal contraceptives or with a normal cycle (28±2 days). This exclusion criteria were made to ensure that measurements were taken in the same phase of the menstrual cycle, based on evidence suggesting that both resting metabolic rate (RMR) and appetite (52-54) vary across the menstrual cycle in normally ovulating women, but not in women taking oral contraceptives.

2.2.2 Recruitment

Participants were recruited through newspaper advertisements, Facebook, announcements on the intranet of St. Olavs Hospital and NTNU, and posters and flyers placed in Trondheim. Written consent was obtained from all participants enrolled in the study, after recruitment and fulfillment of the eligibility criteria. Participation in this study was voluntary and participants were able to withdraw from the study at any time. The consent form, including a description of the intervention, that was used can be found in Appendix I.

2.3 Detailed protocol

2.3.1 Diet composition

Participants were randomized into one of three LEDs (1000 kcal/day) with low-, medium- or high-CHO (70, 100 and 130g/day of carbohydrates, respectively). The LED was chosen specifically to meet the macronutrient requirements for this study. The three diets had a constant protein content of approximately 75g/day, and a fat content of at least 20g/day, following the recommendations of the European Food and Safety Authority for adults (55). An adequate amount of dietary fiber (including 7,5g inulin and 1g guargum per day) was included in the total CHO intake in each of the groups to avoid constipation as a potential side effect of the diet. Macronutrient composition of the three isocaloric LEDs is shown in Table 1.

Table 1 Macronutrient composition of the three LEDs

	Low CHO		**Medium CHO**		**High CHO**	
	Grams	**% EI**	**Grams**	**% EI**	**Grams**	**% EI**
CHO	70.0	28.0	100.0	40.0	130.0	52.0
Protein	75.0	30.0	70.0	30.0	75.0	30.0
EI: energy intake; CHO: carbohydrate						

2.3.2 Weight loss phase

Participants followed one of the three LEDs previously described for 8 weeks. Ketones were measured in blood, plasma and in urine weekly throughout this period. All participants were provided instructions on how to follow a LED (see appendix II), with meal replacement products that were specifically made in-house at NTNU to meet the macronutrient requirements of the study. This included milkshakes and soups with different flavors. The raw ingredients were provided by *Food Innovations AS.* Participants were also allowed to consume of up to 100g of vegetables containing low amounts of starch (see appendix III), and non-caloric beverages ad libitum.

2.3.3 Weight-stabilization phase

The WL phase was followed by a 4-week weight maintenance phase. The aim of this phase was to achieve weight stabilization. The participants received an individualized dietary prescription and counseling from a dietician on how to undergo a standardized weight maintenance diet matched to the participants' calculated energy expenditure (EE), to maintain their weight. Energy needs were estimated from resting metabolic rate (RMR) measured at week 9 x PA factor. This standardized diet consisted of 50-60% CHO, 15-20% protein and 20-30% fat, and was based on the Nordic Nutrition Recommendations (56). Participants were re-introduced to whole foods by the end of week 8. At the same time, they gradually withdrew from the liquid meal replacements from the LEDs, and the participants were all instructed to discontinue the consumption of these by the end of week 10. Participants were also asked to limit the intake of dietary fats, fatty meat, sweets, pastries, and desserts, and advised to increase consumption of fruits and vegetables, poultry, fish, and lean meat. The healthy eating guidelines provided in this phase can be found in appendix V.

2.4 Data collection

2.4.1 Compliance

Diet

Participants were followed up weekly through the entire intervention by researchers, research nurses and dieticians at the Regional center of Obesity Research (ObeCe). These weekly visits evaluated dietary compliance by measuring fasting ketone bodies in both urine (AcAc: using Ketostix, Bayer Comp, Elkhart, IN), and blood (βHB: using a capillary blood ketone meter, Freestyle Optium Neo, Abbott Diabetes Care Inc, Alameda, CA), in addition to weighing of the participants. Fasting βHB plasma concentration was also measured using a Ketone body Assay Kit (Mark134, Sigma-Aldrich, St Louis, MO, USA). All participants were asked to complete paper-based food diaries detailing daily food and fluid consumption along with any side effects they

might have experienced and this was discussed at each visit. Weekly food diaries were completed by all participants throughout the entire dietary intervention, but only diaries completed during weeks 2, 5 and 8 of the study were analyzed. A web-based diet planner based on the Norwegian food composition table, *Kostholdsplanleggeren* (Norwegian Directorate of Health and Food Safety Authority, Oslo, Norway) was used to estimate the daily average energy and macronutrient intake reported during the WL phase of the trial.

Physical activity

Participants were all asked to maintain the same PA level throughout the entire 12-week intervention. The PA levels of the participants were monitored with armbands (SenseWear, Pittsburgh, USA), for a 7-day period, at baseline, week 4, week 8 and week 12. The data was considered valid if the participants wore the device for 4 days minimum, including at least 1 weekend day and > 95% of the time (57). Instructions for activity monitors can be found in Appendix IV.

2.4.2 Outcome variables

The following variables were measured at baseline, weeks 9 and 13 in the fasted state.

2.4.2.1 Anthropometric measurements and Resting metabolic rate (RMR)

Height was only done at baseline, without shoes, to the nearest 0.5 cm (using Seca 217 stadiometer, SECA, Hamburg, Germany). Weight was measured at weekly follow ups and test days after emptying bladder, and wearing minimal clothing, to the nearest 0.1 kg (using Seca 877 digital scale, SECA, Hamburg, Germany). Hip and waist circumference were also measured to the nearest 0.1 cm with a metric measuring tape, using the standardized procedures.

Body composition was measured with air-displacement plethysmography (ADP) using BodPod (COSMED, Italy). All participants were tested in a fasting state. Standardized procedures were followed: jewelry and metals removed, tight underwear was used, and a Lycra swim cap. Participants were instructed not to move and to be relaxed during the test. Two repeated measurements were performed for each participant. RMR was measured using indirect calorimetry (Vmax Encore 29N; Care Fusion, Baesweiler, Germany). This was done using standard operating procedures for a minimum of 15 minutes to obtain at least 5 minutes of stable data (58, 59).

2.4.2.2 Fecal SCFA and gut microbiota analyses

In order to conduct SCFA and gut microbiota analysis, stool samples were collected and sent to the Norwegian University of Life Sciences (NMBU), in Ås (Norway) to be analyzed. This was done by the research group led by Professor Knut Rudi. Prior to analysis, all fecal samples were diluted

1:10 in stool DNA stabilizer (PSP Spin Stool DNA Plus Kit, Invitek Molecular) and stored at -80°C. For easier extraction, the fecal samples were homogenized, and pulse centrifuged (1200 rpm for 8 seconds). From the 1:10 diluted samples, 300µl and 100µl aliquots were used for 16S rRNA sequencing and SCFA composition, respectively.

Short-Chain fatty acid analysis

The main fatty acids analyzed were: Acetate, Propionate, Butyrate, Iso-butyrate, Valerate and Iso-valerate. The aliquots were diluted 1:1 with MilliQ-water, and then 1:1 with an internal standard, containing 2% formic acid with 500µM 2-methylvaleric acid. Samples were then centrifuged (at 13 000 rpm for 10 min). The supernatant was filtered with 0.2µM filter columns (VWR, USA) (at 10 000 rpm for 5 min). The eluate was transferred to gas chromatograph (GC) vials (VWR, USA) and applied to the gas chromatograph (Trace 1310 equipped with an autosampler, ThermoFisher Scientific) with ramping temperatures from 90°C to 150°C for 6 minutes and 150°C to 245°C for 1.9 minutes. 0.2µl was applied with a split injection to a Topaz 4.0mm drilled uniliner (Restek), using helium as the carrier gas with 2.5ml/min column flow, 3 ml/min purge flow and 200 ml/min split flow. The column used was a Stabilwax DA 30m, 0.25mm ID, 0.25µM (Restek), with a flame ionization detector analyzing the analytes. The chromatograms were processed with the Chromeleon 7 software.

A standard with 300µM acetic acid, 12µM propionic acid, 8µM isobutyric acid, 12µM butyric acid, 8µM isovaleric acid, 8µM valeric acid, 25µM internal standard and 1% formic acid was applied twice in between every 10^{th} sample to detect shifts or variabilities. All acids used were purchased from Sigma-Aldrich, Germany.

Gut microbiota analysis - 16S rRNA sequencing

For gut microbiota analyses, a selection of primers was used to cover the most important bacteria in the gut. These include: Bacterial species (or groups) that show marked changes, changes in the relations of the phyla Bacteroidetes and Firmicutes (proportion %, ratio), Bacteriodes group, Clostridium clusters (Ruminococcaceae, Lachnospiraceae), Akermansia (and/or its relatives), Roseburia spp. and Eubacterium rectale subgroup, Bifidobacteria, Lactobacilli, Sulfate reducing bacteria, Ruminococcus, Methanobrevibacter (Archaebacteria), Faecalibacterium prausnitzii (and/or its relatives), Prevotella species, Veillonella, Rikenellaceae (Alistipes). Principal component scores were first evaluated to identify which taxonomic groups of the microbiota present would best explain the variation in microbiota present in the samples collected during the ketogenic diet-induced WL intervention.

The 16S rRNA data were analyzed with Quantitative Insights Into Microbial Ecology (QIIME) pipeline. QIIME was used to assemble forward and reverse reads and split them into their respective samples. The reads were checked for chimeras and removed, and OTUs with a 97% or higher 16S rRNA ident were created and assigned taxonomy by the SILVA database. Two sequencing runs were performed resulting in 30 878 312 ssDNA fragments. The cut-off was set at 5 000 dsDNA fragments, resulting in 352 samples with sufficient depth and quality.

Bacterial cells in fecal sample aliquots were disrupted using 0.2g <106µm acid-washed glass beads (Sigma-Aldrich, Germany), 0.2g 425-600µm acid-washed glass beads (Sigma-Aldrich, Germany) and 2×2.5-3.5mm acid-washed glass beads before being processed twice on a FastPrep 96 (MP Biomedicals, USA) at 1800rpm for 40 seconds. The samples were centrifuged at 13 000 rpm for 5 minutes before DNA was extracted using LGC Mag Midi Nucleic acid extraction kit (LGC genomics, UK). The V3 to V4 region of 16S rRNA was amplified using PRK341F and PRK806R primers(60) at 95°C for 15 minutes followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds, before a final step at 72°C for 7 minutes. Cycles were increased to 30 for meconium. Reactions contained 2µl DNA template with 1× HotFirePol Blend Master Mix Ready to Load (Solis BioDyne, Germany) and 0.2µm PRK forward and reverse primers. Samples were purified using 1× Sera Mag beads to the DNA volume, following AMPure's protocol on a Biomek 3000 (Beckman Coulter, USA). Index PCR was performed with a combination of 16 forward and 30 reverse modified PRK primers with Illumina indexes. Samples were amplified at 95°C for 5 minutes followed by 10 cycles of 95°C for 30 seconds, 55°C for 60 seconds, and 72°C for 45 seconds, before a final step of 72°C for 7 minutes. Each reaction consisted of 1× FirePol Master Mix Ready to Load (Solis BioDyne, Germany), 0.2µM forward & reverse primers, nuclease free-water (VWR, USA) and 1µl DNA. The DNA concentration was quantified following Qubit's protocol, normalized and pooled on a Biomek 3000. The pooled sample was split in two for quantification and sequencing. Samples for quantification were first subjected to droplet generation using BioRad QX200™ – Droplet Generator, before being amplified at 95°C for 5 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds before the last two steps at 4°C for 5 minutes and 90°C for 5 minutes before quantification on BioRad QX200 – Droplet Reader. The reactions contained 1× Super mix for EvaGreen (BioRad, USA), 0.2µM Illumina colony forward & reverse primer, 2.4µl DNA template and PCR water. The second part of the sample was diluted to 6 pM DNA with 15% PhiX following Illumina's instructions, except from using nuclease-free water instead of Tris and sequenced on Illumina MiSeq.

2.5 Power calculation

No power calculation was done specifically for this project. This master project is a part of a larger clinical trial, where a simulation-based power calculation was performed to compare the change in hunger (mm) assessed by a visual analogue scale (VAS) between the three CHO groups using a linear mixed model (LMM) with two time points (baseline, week 8). This method has been shown to be useful in designing trials with multiple endpoints and/or correlated outcomes (61). It was estimated that a total of 75 participants was necessary for this three-treatment parallel-design study to obtain at least 80% power to detect differences corresponding to mean changes in hunger (0-20 mm) between the three groups at a significance level of $P<0.05$. Given that a dropout of 25% is often seen in these types of studies, the aim was to recruit 100 participants.

2.6 Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics 27. Statistical significance was set to $P<0.05$, unless specified otherwise. Data are presented as estimated marginal means ± standard error of the mean (SEM) and mean ± SD for BL characteristics. Participants with stool samples collected at a minimum of two timepoints (either BL and W9, or BL and W13) (n=85) were considered completers and included in the analysis. A Shapiro-Wilk test and assessment of normal Q-Q plots were used to check for normality. For normally distributed data, an independent samples t-test or a One-way ANOVA were performed to examine differences between groups for baseline characteristics, and a non-parametric equivalent, either the Mann-Whitney U test or the Kruskal Wallis test, were used if the data was not normally distributed.

For the aim of this thesis, results from participants were analyzed by comparing the randomized groups. Because of the randomization process, and since BL measurements were taken prior to the dietary intervention, any differences between groups would have been due to chance, as the mean BL values of all variables were assumed to be equal using constrained longitudinal data analysis (cLDA) (62). A linear mixed-effects model (LMM) was used to look at differences in gut microbiota and SCFA between groups over time (repeated measurements), with restricted maximum-likelihood estimation, including fixed effects for sex, energy intake, intervention group, time, and intervention group * time, and the participants were set as a random effect. Bonferroni adjustment was used for post-hoc pairwise comparisons. When residuals of the LMM were non-normally distributed, a non-parametric equivalent was performed (i.e., residuals of hip, βHB in plasma, Bacteroides, Blautia, Eubacterium rectale group and Ruminococcaceae). The following residuals were normally distributed after logarithmic (lg10) or square root (sqrt)-transformations:

FFM (kg), propionic acid, isobutyric acid, isovaleric acid, valeric acid, acetic acid, Alistipes and Faecalibacterium.

The LMM was constructed in a way that assumed there to be no differences in BL values between the groups in any of the variables. The groups and timepoints were coded into seven categories, into a new variable called "grouptime", to easier compare the tree LEDs in line with the aim of the study, and to account for differences in total calorie consumption, which turned out to be significantly different between the groups. The new variable was coded like this:

1= BL, all groups
2= low CHO – W9
3= medium CHO – W9
4= high CHO – W9
5= low CHO – W13
6= medium CHO – W13
7= high CHO – W13

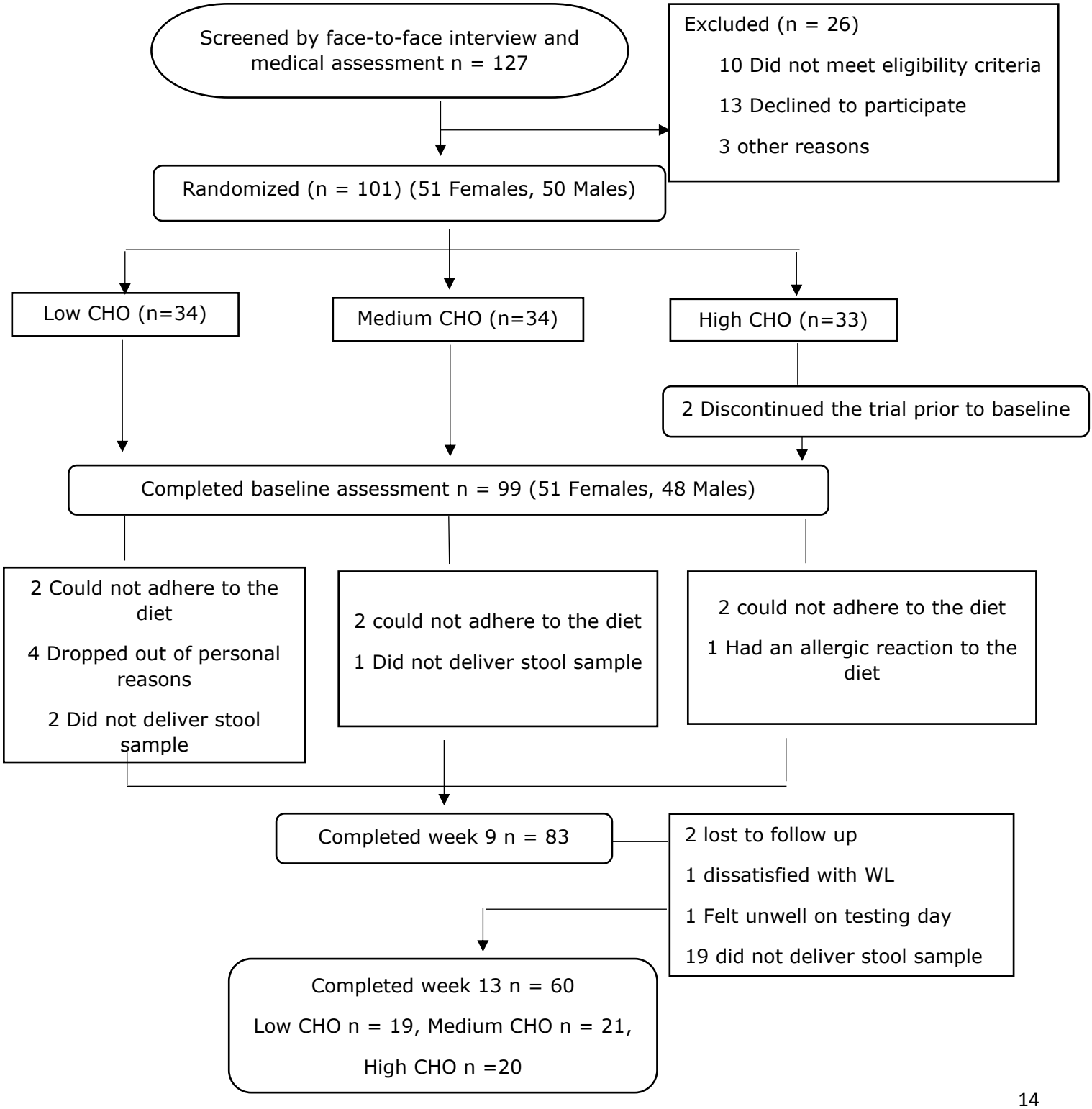
Correlation between CHO intake at 9 weeks, β-hydroxybutyrate concentration at 9 weeks and concentration of gut microbiota and SCFAs at W9 and from BL to W9 was performed using Spearman's correlation as one or both of variables tested were not normally distributed. The correlation was defined as a weak correlation if the coefficient were <0.30, a moderate correlation had a coefficient of 0.30 to <0.50, and a strong correlation was defined as having a correlation coefficient of 0.50 or larger. As the residuals were normally distributed, a multiple linear regression model was used to check for further explanation of the variation in changes over time. Extreme values, defined as any data values which lie more than 3.0 times the interquartile range below the first quartile or above the third quartile, were identified and deleted from the dataset. Adjustments were made for different confounders in the regression analysis (age, sex, FFM and FM loss in kg).

3.0 Results

3.1 Study population

Out of one-hundred-and-one recruited participants, ninety-nine started the LED. Eighty-five participants were included in the final analysis. Reasons for dropouts and exclusions are shown in Figure 1.

Figure 1. Flow chart of the study



Baseline characteristics of the participants are shown in table 2.

Table 2. Baseline characteristics of all participants and each CHO group

	All groups		**Low CHO**	**Medium CHO**	**High CHO**
Characteristic:	Non-completers	Completers			
	(n=14)	(n=85)	(n=26)	(n=31)	(n=28)
Sex, F:M (%F)	7: 7 (50%)	44: 41 (52%)	15: 11 (58%)	15: 16 (48%)	14: 14 (50%)
Age, years	46.3 ± 11.5	44.6 ± 9.4	47.1 ± 9.4	44.9 ± 10.2	41.8 ± 8.1
Height, cm	173.4 ± 8.0	173.7 ± 9.0	173.1 ± 9.2	175.3 ± 8.9	172.5 ± 9.0
Weight, kg	107.3 ± 17.0	104.9 ± 15.9	102.7 ± 12.4	106.6 ± 18.0	105.2 ± 16.5
BMI, kg/m²	35.9 ± 3.4	34.6 ± 3.5	34.2 ± 3.0	34.5 ± 3.9	35.2 ± 3.5
FM, kg	47.0 ± 9.1	43.7 ± 9.2	42.7 ± 7.6	44.8 ± 9.5	43.4 ± 10.2
FM, %	44.0 ± 6.2	41.9 ± 6.3	42.2 ± 6.9	42.2 ± 4.7	41.4 ± 7.2
FFM, kg	60.0 ± 12.2	61.2 ± 11.2	60.1 ± 10.6	61.6 ± 11.0	61.7 ± 12.3
FFM, %	56.1 ± 6.3	58.2 ± 6.1	58.2 ± 6.4	57.9 ± 4.9	58.6 ± 7.2
WC, cm	112.1 ± 12.3	112.3 ± 10.9	111.5 ± 6.4	112.8 ± 13.9	112.4 ± 10.7
HC, cm	117.2 ± 9.0	116.1 ± 8.7	115.4 ± 7.8	115.8 ± 9.4	117.2 ± 8.8
Data presented as mean ± SD. BW: body weight; BMI: body mass index; FM: fat mass; FFM: fat free mass; WC: waist circumference; HP: hip circumference, Low CHO: carbohydrate.					

Participants had an average age of 45 years and a BMI of 35 kg/m². There were no significant differences between completers and non-completers or among the three LED-groups regarding sex distribution or any of the anthropometric variables measured.

3.2 Body composition and anthropometric measurements

Anthropometric measurements over time in all participants and in each group are shown in table 3.

Table 3. Anthropometric measurements over time in all participants and in each CHO group

Measure	**Grouped (n=85)**			**Low CHO (n=26)**		**Medium CHO (n=31)**		**High CHO (n=28)**	
	BL	W9	W13	**W9**	W13	**W9**	W13	**W9**	W13
BW (kg)	104.9 ± 1.72^{a,b}	90.79 ± 1.39^{a}	89.89 ± 1.51^{b}	**87.65 ± 1.90^{a}**	89.15 ± 2.22^{b}	**92.71 ± 2.61^{a}**	91.94 ± 2.92^{b}	**91.59 ± 2.50^{a}**	87.98 ± 2.56^{b}
BMI (kg/m²)	34.6 ± 0.38^{a,b}	30.25 ± 0.34^{a}	30.16 ± 0.39^{b}	**29.41 ± 0.54^{a}**	29.83 ± 0.61^{b}	**30.30 ± 0.61^{a}**	30.47 ± 0.73^{b}	**30.98 ± 0.59^{a}**	30.18 ± 0.69^{b}
Hip (cm)	116.1 ± 0.94^{a,b}	108.04 ± 0.89^{a}	107.26 ± 1.07^{b}	**107.83 ± 1.47^{a}**	107.03 ± 1.64^{b}	**107.21 ± 1.63^{a}**	107.72 ± 2.02^{b}	**109.14 ± 1.52^{a}**	106.90 ± 1.88^{b}
Waist (cm)	112.3 ± 1.18^{a,b,c}	102.15 ± 0.98^{a,c}	100.32 ± 1.19^{b,c}	**100.48 ± 1.26^{a}**	100.03 ± 1.36^{b}	**103.24 ± 1.87^{a}**	101.10 ± 2.45^{b}	**102.49 ± 1.79^{a}**	99.64 ± 2.35^{b}
FM (kg)	43.7 ± 0.99^{a,b}	32.99 ± 0.95^{a}	31.06 ± 1.07^{b}	**31.69 ± 1.65^{a}**	30.65 ± 1.79^{b}	**34.18 ± 1.50^{a}**	32.19 ± 1.87^{b}	**32.87 ± 1.79^{a}**	30.12 ± 1.96^{b}
FM (%)	41.9 ± 0.68^{a,b}	36.26 ± 0.84^{a}	34.52 ± 1.03^{a}	**36.20 ± 1.75^{a}**	34.37 ± 1.80^{b}	**36.71 ± 1.07^{a}**	34.82 ± 1.49^{b}	**35.85 ±1.63^{a}**	34.34 ± 2.22^{b}
FFM kg)	61.2 ± 1.22^{a,b}	57.86 ± 1.13^{a}	58.89 ± 1.32^{a}	**55.93 ± 1.96^{a}**	58.45 ± 2.10^{a}	**58.65 ± 1.84^{a}**	59.84 ± 2.13^{b}	**58.73 ± 2.08^{a}**	58.20 ± 2.92^{b}
FFM (%)	58.2 ± 0.66^{a,b}	63.73 ± 0.84^{a}	65.51 ± 1.03^{a}	**63.80 ± 1.75^{a}**	65.72 ± 1.80^{b}	**63.29 ± 1.07^{a}**	65.18 ± 1.49^{b}	**64.15 ± 1.63^{a}**	65.66 ± 2.22^{b}

Data presented as estimated marginal means ± SEM. BW: body weight. BMI: body mass index. FM: fat mass. FFM: fat free mass. Averages sharing the same superscript letter denote significant changes overtime (a, b, P<0.001; c, d P<0.05) compared to BL values.

A significant main effect of time ($P<0.001$) and a time*group interaction ($P<0.01$) was found for body weight (BW). Participants lost an average of 14 kg (13,5%) of their initial BW at W9 ($P<0.001$ for all), and this was maintained from W9 to W13 for all participants and each group. No significant differences were found between groups in BW overtime.

A significant main effect of time ($P<0.001$) and a time*group interaction ($P<0.001$) was found for BMI. BMI decreased for all participants and all three groups between BL and W9 ($P<0.001$) and was maintained from W9 to W13 for all participants and all groups. There were no significant differences in BMI between groups at any time point.

A significant main effect of time ($P<0.001$) was found for hip circumference (HC). HC (cm) decreased significantly from BL to W9 for all participants ($P<0.001$), and for each group ($P<0.001$ for all). The decrease in HC was maintained between W9 and W13. No difference between groups were found.

A significant main effect of time ($P<0.001$) and group ($P<0.05$) was found for waist circumference (WC). WC (cm) decreased significantly from BL to W9 in all participants and for each group ($P<0.001$ for all) and decreased further from W9 to W13 for all participants ($P<0.05$), but not for each group. There were no significant differences between the groups.

A significant main effect of time ($P<0.001$) was found for FM (kg), FM (%), FFM (kg) and FFM (%). A significant time*group interaction ($P<0.05$) was also found for FFM (kg). FM (kg and %) decreased significantly in all participants, and all groups from BL to W9 ($P<0.001$), and from W9 to W13 for FM (%) for all participants ($P<0.001$), but the difference between W9 and W13 was not significant for the three groups separately. There were no significant differences in FM (kg and %) between groups at any time point. FFM (kg) decreased from BL to W9 for all participants and for all groups ($P<0.001$). In the Low CHO group there was a significant increase in FFM (kg) from W9 to W13 ($P<0.001$), and this significant increase was also seen for all participants combined ($P<0.001$). FFM (%) increased in all participants and in all groups from BL to W9 ($P<0.001$), and from W9 to W13 for all participants ($P<0.001$), but not for each group individually. There were no significant differences in FFM (kg and %) between groups at any time points.

3.3 Diet

Actual energy and macronutrient intake in the tree LED groups is shown in table 4.

Table 4. Actual energy and macronutrient consumption in each CHO group

	Low CHO		**Medium CHO**		**High CHO**	
Energy (kcal/day)	873 ± 6[a] (range: 814-928)		1130 ± 3[a] (range: 1081-1172)		1215 ± 3[a] (range: 1185-1259)	
	grams	**%EI**	**grams**	**%EI**	**grams**	**%EI**
Carbohydrates	58.2 ± 0.5[a] (53-62)	27 (26-27)	100.4 ± 0.7[a] (85-106)	36 (31-37)	146.7 ± 0.6[a] (141-153)	48 (47-49)
Protein	54.4 ± 0.4[a] (50-59)	25	69.4 ± 0.2[a] (67-72)	25 (25-26)	73.1 ± 0.3[a] (71-76)	24 (24-25)
Fat	43.7 ± 0.3[a] (41-46)	45 (45-46)	43.1 ± 0.1[b] (42-44)	35 (35-37)	33.8 ± 0.1[a,b] (33-34)	25 (25-26)
Fiber	14.1 ± 0.2 (11-18)	3 (3-4)	14.6 ± 0.2 (13-17)	4 (3-5)	14.6 ± 0.2 (13-18)	3 (2-3)

Data presented as mean ± SEM and (range); kcal: kilocalories; CHO: carbohydrate; EI: energy intake. Averages sharing the same superscript letter denotes a significant difference between groups: a, b, P≤0.001.

A significant main effect of group was found for energy intake (kcal/day) and intake of CHO, fat and protein ($P<0.001$). Energy intake was higher in the high CHO group compared with the medium and low CHO group, also the medium CHO group had a significantly higher energy intake compared to the low CHO group. This trend was the same for CHO and protein as well, with the highest intake in the high CHO group and the lowest intake in the low CHO group. For fat, the lowest intake was seen in the high CHO group compared with the medium and low CHO groups, with no significant differences between the medium and low CHO groups. Fiber intake was not significantly different between the three groups. This was important to ensure that fiber intake would not be a significant confounder to the results.

β-HB plasma concentrations over time are shown in table 5.

Table 5. β-HB plasma concentration over time in all participants and in each CHO group

Measure	**Grouped (n=85)**			**Low CHO (n=26)**		**Medium CHO (n=31)**		**High CHO (n=28)**	
	BL	W9	W13	**W9**	W13	**W9**	W13	**W9**	W13
β-HB (mmol/l)	0.13 ± 0.01^{a,b}	0.72 ± 0.06^{a,b}	0.12 ± 0.01^{b}	**1.11 ± 0.11$^{a,b*\#}$**	0.12 ± 0.02^{b}	**0.63 ± 0.08^{a,b*}**	0.14 ± 0.02^{b}	**0.46 ± 0.05$^{a,b\#}$**	0.10 ± 0.02^{b}

Data presented as estimated marginal means ± SEM. β-HB: β-hydroxybutyric acid. Averages sharing the same superscript letter denote significant changes overtime (a, b, c P<0.001) compared to BL values and sharing the same superscript symbol denote significant differences between groups (* P<0.01 and # P<0.001) at W9 and W13.

All three groups showed a significant increase in plasma βHB concentration from baseline to week 9 (mean increase across all groups was 0.7 ± 0.1 m), with significantly greater circulating βHB levels in the low CHO group (1.1 ± 0.1 mM) compared to the medium (0.6 ± 0.1 mM, P≤0.01) and high (0.5 ± 0.0 mM, P≤ 0.001) CHO groups. By week 13, β-HB plasma concentrations had returned to baseline levels in all groups, with no significant differences seen between groups.

3.4 Important taxonomic groups

The composition of some important taxonomic groups of gut microbiota over time is shown in table 6.

Table 6. Gut microbiota over time in all participants and in each CHO group

Measure	Grouped (n=85)			Low CHO (n=26)		Medium CHO (n=31)		High CHO (n=28)	
	BL	W9	W13	**W9**	W13	**W9**	W13	**W9**	W13
Bacteroides	0.21 ± 0.01	0.23 ± 0.02	0.23 ± 0.02	**0.27 ± 0.04***	0.25 ± 0.03	**0.17 ± 0.02$^{*\#}$**	0.18 ± 0.03	**0.26 ± 0.03$^{\#}$**	0.26 ± 0.04
Alistipes	0.06 ± 0.01^{a,b,c}	0.09 ± 0.01^{a}	0.08 ± 0.01	**0.10 ± 0.01^{b}**	0.08 ± 0.01^{c}	**0.08 ± 0.01**	0.08 ± 0.01	**0.09 ± 0.01^{c}**	0.07 ± 0.01
Blautia	0.04 ± 0.01^{c}	0.04 ± 0.00	0.03 ± 0.00^{c}	**0.02 ± 0.00$^{\#*}$**	0.02 ± 0.00$^{c*¤}$	**0.04 ± 0.01$^{\#}$**	0.03 ± 0.01*	**0.04 ± 0.01***	0.04 ± 0.01$^{¤}$
Eubacterium rectale group	0.07 ± 0.01^{a,b,c}	0.01 ± 0.00^{a,d}	0.05 ± 0.01^{b,d}	**0.006 ± 0.00^{a,d*}**	0.06 ± 0.01^{d}	**0.009 ± 0.00^{a,d}**	0.04 ± 0.01^{d}	**0.012 ± 0.00^{a,d*}**	0.05 ± 0.01^{c,d}
Faecalibacterium	0.07 ± 0.01^{c}	0.06 ± 0.00	0.07 ± 0.01	**0.06 ± 0.01^{c}**	0.07 ± 0.01	**0.06 ± 0.01**	0.07 ± 0.01	**0.07 ± 0.01**	0.06 ± 0.01
Ruminococcaceae	0.03 ± 0.01^{b,c}	0.04 ± 0.01^{b,e}	0.03 ± 0.01^{e}	**0.03 ± 0.01**	0.03 ± 0.01	**0.06 ± 0.01^{e}**	0.05 ± 0.01^{e}	**0.04 ± 0.01^{c}**	0.03 ± 0.01

Data presented as estimated marginal means ± SEM. Averages sharing the same superscript letter denote significant changes overtime (a, d P<0.001; b, P<0.01; c, e P<0.05) compared to BL values and sharing the same superscript symbol denote significant differences between groups (*, ¤, P<0.05 and #, P<0.01) at W9 and W13.

Overall, there was a significant increase in Alistipes and Ruminococcaceae between BL and W9 ($P<0.001$ and $P<0.01$, respectively), and a significant decrease in the Eubacterium rectale group between the same time points ($P<0.001$). Between BL and W13, there was a significant decrease in the Blautia genera ($P<0.05$), and although the relative proportion of Eubacterium rectale group increased significantly between W9 and W13 ($P<0.001$), values at W13 were still below BL ($P<0.01$). A significant decrease in Ruminococcaceae between W9 and W13 was also seen ($P<0.05$).

In the low CHO group, there was a significant increase in Alistipes ($P<0.05$) between BL and W9, and values at W13 were still above BL ($P<0.05$). There was also a significant decrease in Faecalibacterium ($P<0.05$) between BL and W9, and a significant decrease in Blautia ($P<0.05$) between BL and W13. Eubacterium rectale group decreased significantly in this group between BL and W9 ($P<0.001$), followed by a significant increase between W9 and W13 ($P<0.001$), with values at W13 no longer different from BL.

In the medium CHO group, there was a significant decrease in Eubacterium rectale group ($P<0.001$) between BL and W9, followed by a significant increase from W9 to W13 ($P<0.001$), and at W13 the difference from BL was no longer significant. This group also showed a significant decrease in Ruminococcaceae between W9 and W13 ($P<0.05$).

The high CHO group showed significant increases in Alistipes and Ruminococcaceae genera ($P<0.05$ for both) between BL and W9. There was a significant decrease in Eubacterium rectale group between BL and W9 ($P<0.001$), following by a significant increase from W9 to W13 ($P<0.001$), with values at W13 still bellow BL ($P<0.05$).

At W9, the medium CHO group had significantly lower relative proportion of the Bacteroides genus, than both the low ($P<0.05$) and high ($P<0.01$) CHO groups. For the Blautia genus, the low CHO group had significantly lower values than both the medium ($P<0.01$) and high ($P<0.05$) CHO groups, and at W13 this difference was still significant ($P<0.05$ for both). The high CHO group had a higher proportion of the Eubacterium rectale group at W9 than the low CHO group ($P<0.05$).

3.5 Short-Chain Fatty Acids (SCFAs)

SCFA proportions over time overall and for each group is shown in table 7.

Table 7. SCFAs over time in all participants and in each CHO group

Measure	**Grouped (n=85)**			**Low CHO (n=26)**		**Medium CHO (n=31)**		**High CHO (n=28)**	
	BL	W9	W13	**W9**	W13	**W9**	W13	**W9**	W13
Acetic acid	45285 ± 3745^{a,b,c}	22120 ± 1711^{a,e}	29830 ± 3602^{b,e}	**21070 ± 3383^{a}**	23500 ± 3655^{b*}	**18982 ± 2177^{a}**	23988 ± 3304$^{¤}$	**26533 ± 3307^{c}**	42295 ± 9345$^{*¤}$
Propionic acid	13419 ± 985^{a,b,c,d}	7215 ± 420^{a,e}	9760 ± 1070^{b,e}	**6629 ± 635^{a}**	8093 ± 1035$^{c\#}$	**6759 ± 725^{a}**	7713 ± 894$^{d\&}$	**8244 ± 775^{d,g}**	13575 ± 2815$^{g\#\&}$
Isobutyric acid	2809 ± 118^{c,d}	2509 ± 112^{c}	2417 ± 106^{d}	**2193 ± 150^{d*}**	2161 ± 112*	**2584 ± 201**	2236 ± 127$^{¤}$	**2708 ± 207***	2862 ± 250$^{*¤}$
Butyric acid	19396 ± 1340^{a,c}	8191 ± 566^{a,f}	13754 ± 1269^{c,f}	**6987 ± 701^{a,g}**	12533 ± 1991^{g}	**8636 ± 1085^{a}**	12496 ± 1683	**8795 ± 1025^{a,f}**	16296 ± 2812^{f}
Isovaleric acid	4016 ± 165	3779 ± 156	3656 ± 129	**3411 ± 178**	3393 ± 145	**4047 ± 335**	3676 ± 218	**3824 ± 236**	3900 ± 282
Valeric acid	3593 ± 121^{a,d}	2941 ± 84^{a}	3231 ± 132^{d}	**2806 ± 132^{a}**	3108 ± 117	**3103 ± 160**	3200 ± 133	**2890 ± 136^{d}**	3388 ± 365

Data presented as estimated marginal means ± SEM. Averages sharing the same superscript letter denote significant changes overtime (a, b, e P<0.001; c, f P<0.01; d, g P<0.05) compared to BL values and sharing the same superscript symbol denote significant differences between groups (*, ¤, P<0.05 and #, &, P<0.01) at W9 and W13.

Overall, there was a significant main effect of time for acetic, propionic, isobutyric, butyric and valeric acid ($P<0.001$, $P<0.001$, $P<0.01$, $P<0.001$, $P<0.001$, respectively). There was a significant main effect of group for acetic and propionic acid ($P<0.01$ and $P<0.01$, respectively), and a time*group interaction for acetic and propionic acid ($P<0.01$ and $P<0.05$, respectively).

Overall, there was a significant decrease in proportion of acetic, propionic, butyric, isobutyric and valeric acid from BL to W9 ($P<0.001$, $P<0.001$, $P<0.001$, $P<0.01$ and $P<0.001$, respectively). Proportions then increased from W9 to W13 for acetic, propionic and butyric acid ($P<0.01$, $P<0.01$ and $P<0.001$, respectively), although values at W13 were still below BL values for all ($P<0.001$, $P<0.001$ and $P<0.01$, respectively). Values of isobutyric and valeric acid were also still significantly below BL values at W13 ($P<0.05$ for both).

The low CHO group showed a significant decrease from BL to W9 in the proportions of acetic ($P<0.001$), propionic ($P<0.001$), isobutyric ($P<0.05$), butyric ($P<0.001$) and valeric acids ($P<0.001$). This was then followed by a significant increase in butyric acid ($P<0.05$) between weeks 9 and 13. Proportions of acetic ($P<0.001$) and propionic acids ($P<0.01$) were still below basal at W13.

The medium CHO group showed a significant decrease from BL to W9 in the proportion of acetic ($P<0.001$), propionic ($P<0.001$) and butyric acids ($P<0.001$). No significant changes of any SCFAs was seen from W9 to W13 and only propionic acid ($P<0.05$) was still significantly below BL at W13.

The high CHO group also experienced a significant decrease from BL to W9 in proportion of acetic ($P<0.01$), propionic ($P<0.05$), butyric ($P<0.001$) and valeric acids ($P<0.05$). This was then followed by an increase from W9 to W13 in propionic ($P<0.05$) and butyric acids ($P<0.01$). No significant changes were found between BL and W13 for the proportion of any SCFA in this group.

At W13, acetic acid proportion was significantly higher for the high compared with both the medium and low CHO groups ($P<0.05$ for both). The high CHO group also had significantly higher proportion of propionic acid at W13 compared to both the medium and low CHO groups ($P<0.01$ for both).

Differences between groups at W9 were only significant for Isobutyric acid, with lower proportions in the low CHO compared to the high CHO group ($P<0.05$), and at W13 the difference between these groups was still significant ($P<0.05$).

3.6 Correlation analysis

For the gut microbiota, a weak positive correlation was found between CHO intake and both Blautia (r= 0.259, n= 82, P<0.05) and Eubacterium rectale group (r= 0.230, n= 84, P<0.05) at W9. The greater the intake of CHO, the greater the relative proportion of Blautia and Eubacterium rectale group at W9. After adjusting for FM and FFM loss (in kg), age and sex, CHO intake was no longer a significant predictor of Blautia at W9, but it was still a significant predictor of the Eubacterium rectale group at W9 (P<0.05). The regression analysis showed that CHO intake at W9 explained 7% of the variation in the Eubacterium rectale group at W9. Scatterplots of correlations can be seen in figure 2 and 3.

Figure 2. Scatterplot of correlation between CHO intake and Blautia at W9

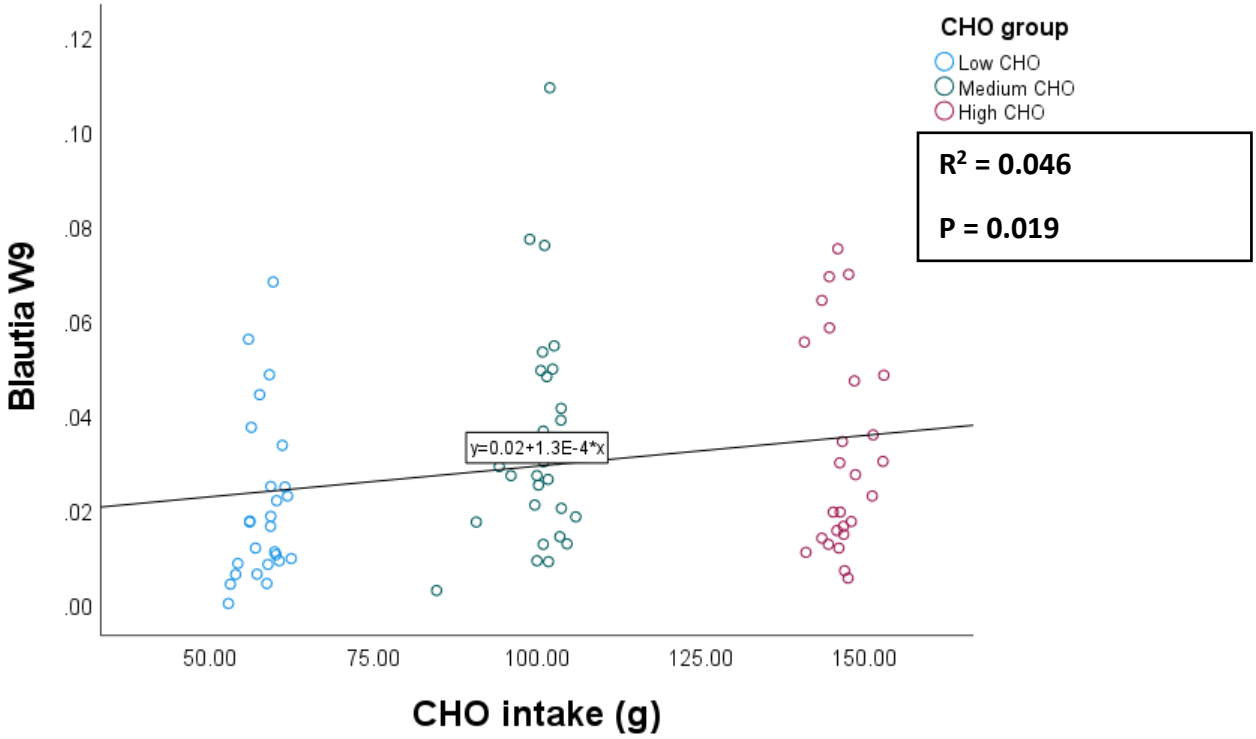
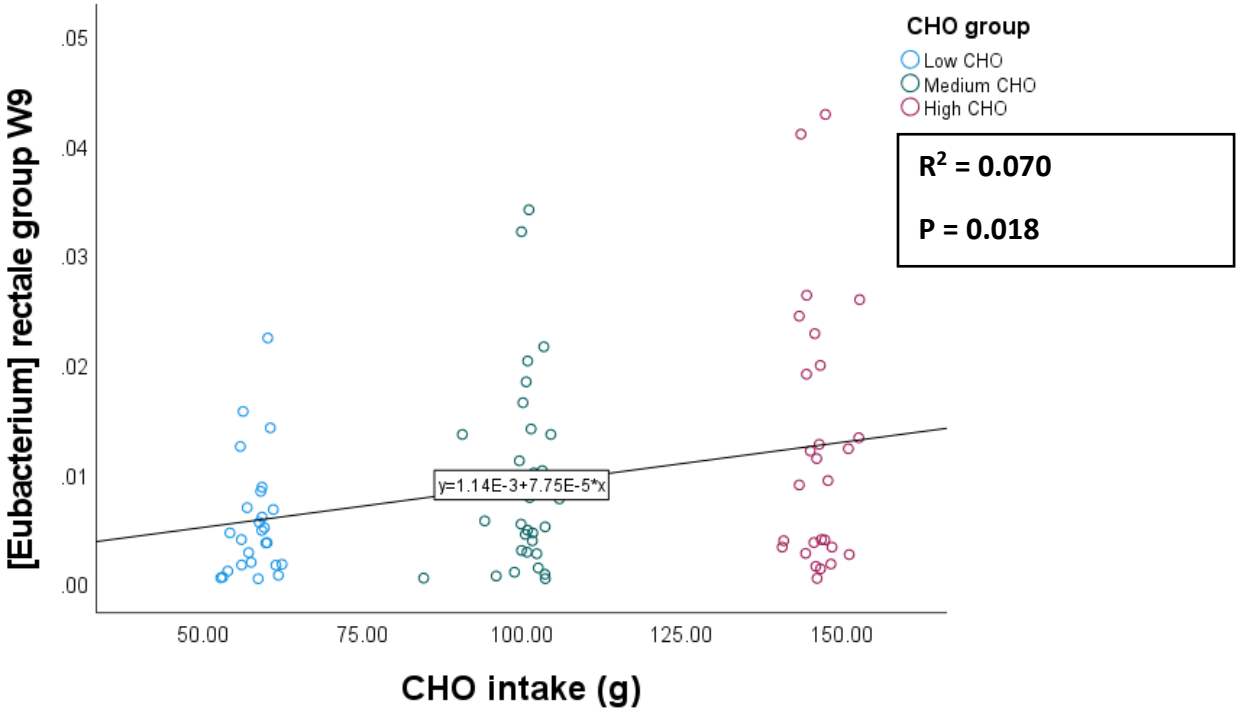


Figure 3. Scatterplot of correlation between CHO intake and Eubacterium rectale group at W9



For the SCFAs, a weak positive correlation was found between CHO intake and isobutyric acid both at W9 (r= 0.217, n= 83, P<0.05) and for changes from BL to W9 (r= 0.221, n= 83, P<0.05). The greater the intake of CHO, the greater the concentration of isobutyrate at W9. However, after adjusting for loss of FM and FFM (kg), age and sex, CHO intake was no longer a significant predictor of isobutyric acid. Scatterplot of correlation for all participants can be seen in figure 4 and 5.

Figure 4. Scatterplot for correlation between CHO intake and isobutyric acid at W9

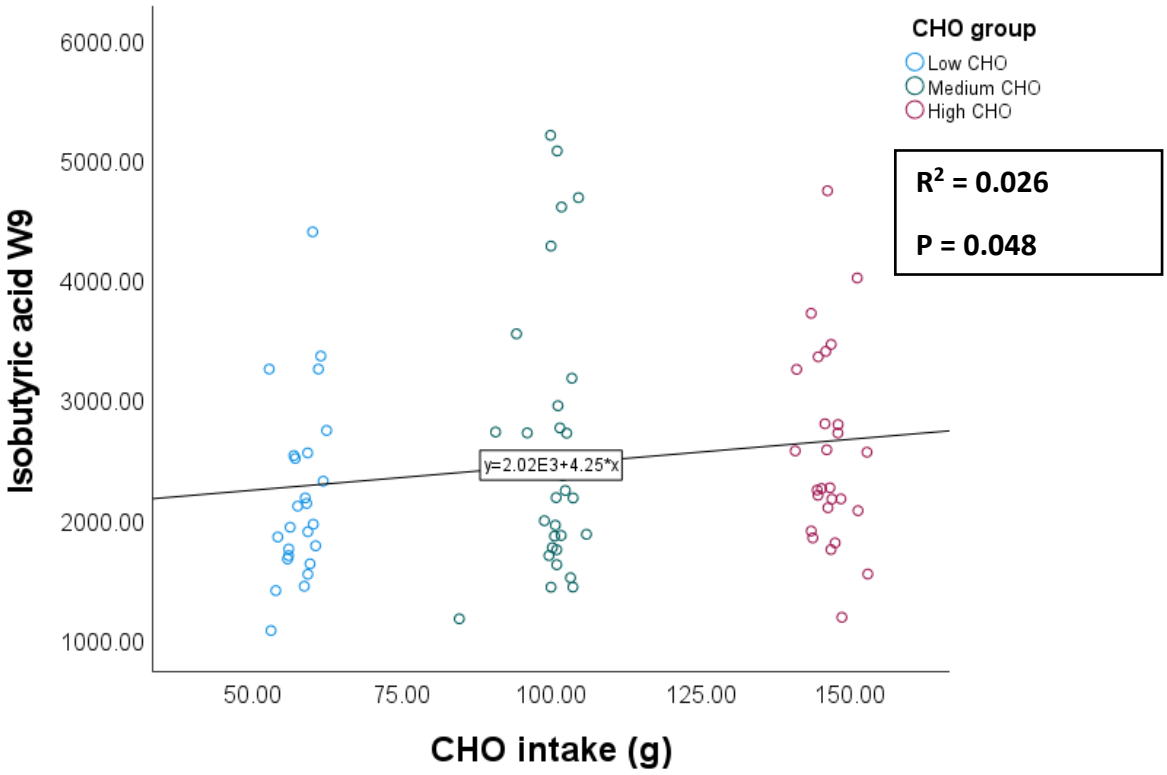
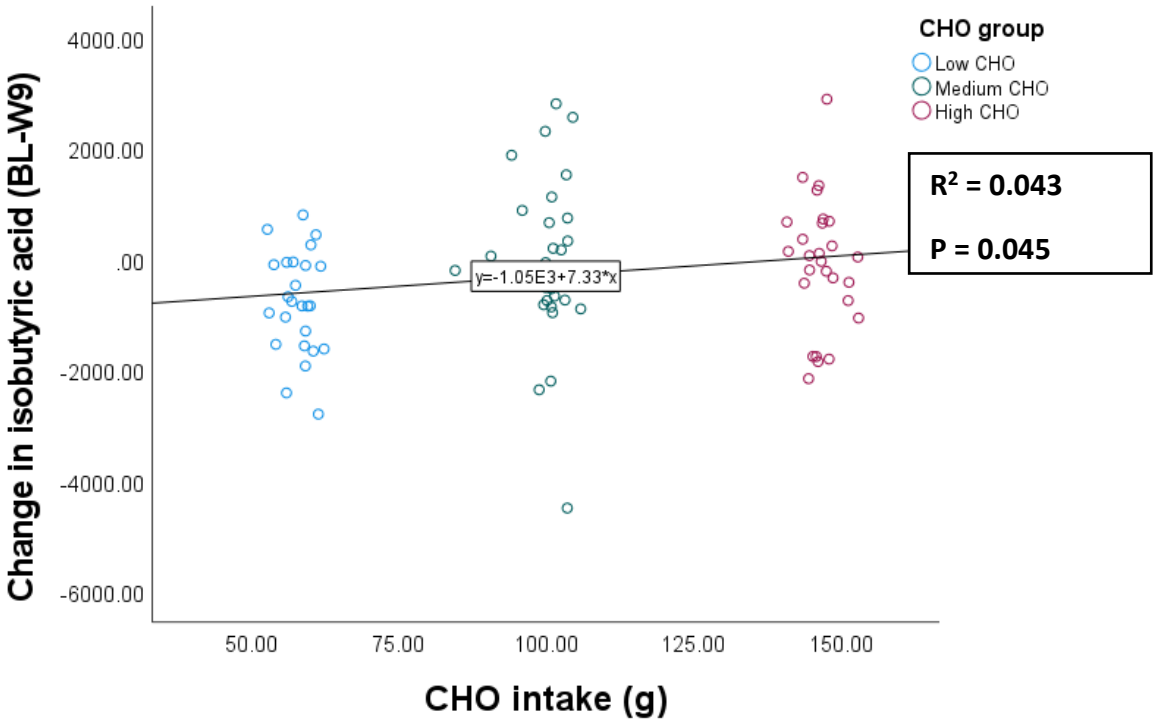
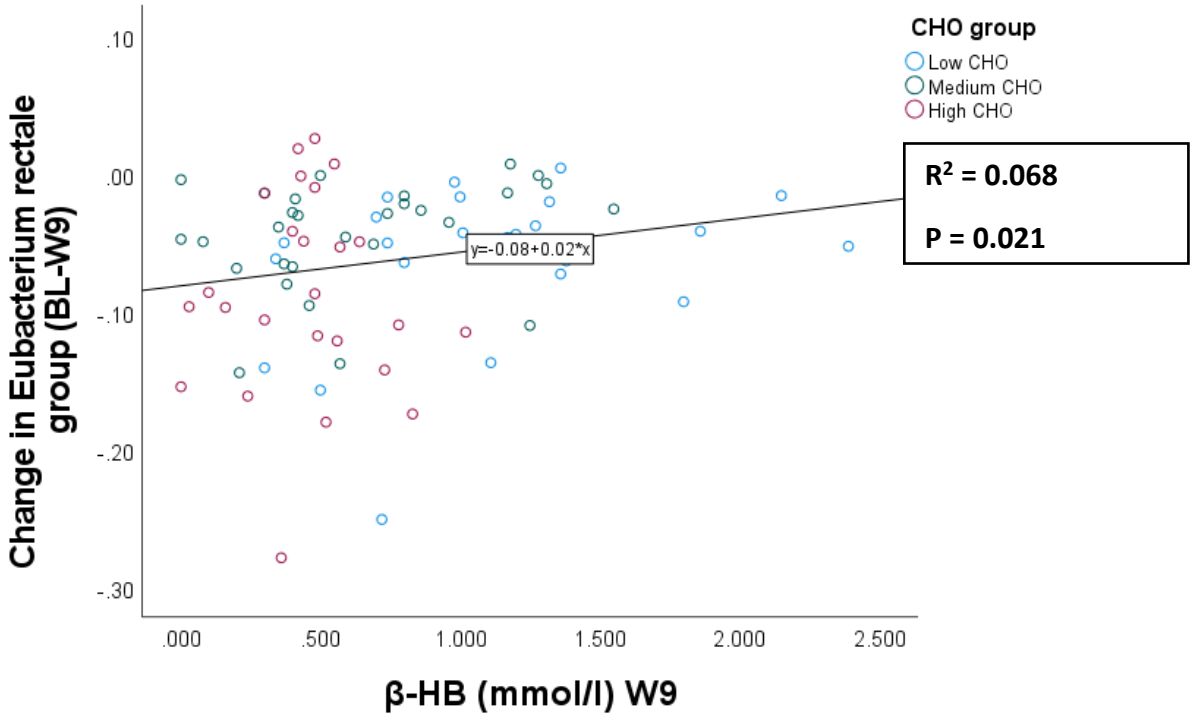


Figure 5. Scatterplot of correlation between CHO intake at W9 and changes in isobutyric acid from BL to W9



A weak positive correlation was also found between BHB concentrations at W9 and the change in relative proportion of Eubacterium rectale group from BL to W9 (r= 0.227, n= 78, P<0.05). After adjusting for loss of FM and FFM, age and sex, regression analysis showed that BHB at W9 explained 7% (P<0.05) of the variation in changes in Eubacterium rectale group between BL and W9. Scatterplot of the correlation can be seen in figure 6.

Figure 6. Scatterplot of correlation between βHB concentration at W9 and changes in Eubacterium rectale group between BL and W9.



4.0 Discussion

The present study aimed to assess the effects of a low CHO intake on gut microbiota and SCFA production following WL induced by a LED, by comparing 3 groups with a low, medium and high CHO intake. The main hypothesis was that the lower the CHO intake, the more negative effect it would have on microbiota abundance and SCFA. This hypothesis requires there to be a linear association between CHO intake and microbiota abundance and SCFA, and that there are significant differences between the three groups with differing amounts of CHO intake after diet intervention.

This study found that the medium CHO group had a lower relative proportion of Bacteroides at W9, compared with both the low and high CHO groups. The low CHO group also presented with lower values of Blautia than both the medium and high CHO groups and the high CHO group had a higher relative proportion of the Eubacterium rectale group than the low CHO group at W9. For the SCFAs, the differences between groups at W9 were only significant for isobutyric acid, with lower proportions in the low CHO compared to the high CHO group. Moreover, a weak positive correlation was found between CHO intake, and BHB concentration at W9, and the relative proportion of Eubacterium rectale group at W9.

Participants lost an average of 14 kg (14%) of their initial BW with the intervention, which is the expected WL for a LED with this energy restriction level (63). The three LEDs lead to similar decreases in BMI, weight, waist and hip circumference and FM, and an increase in FFM, suggesting that these diets all have beneficial effects on body weight and composition over time, despite differing CHO levels. This is consistent with findings from previous studies suggesting that macronutrient composition of the diet does not have a significant impact on WL (64, 65). Nutritional-induced ketosis was seen at W9 in all CHO groups, higher in the low CHO, followed by the medium and high CHO groups, and disappeared with refeeding (W13).

KDs usually result in plasma concentration of β-HB around 0.33-0.72 mmol/L (66). Although there is no agreement as to which levels of CHO are required to achieve ketosis (4), KDs are usually considered to have a very low CHO intake of <50g per day, or 5-10% of daily energy intake (17). There is no established definition of a "low carbohydrate diet", but it is common in research to use the definition of a diet containing <100g or <30% of the energy intake of CHO per day as a low-CHO diet (67, 68). The low CHO group had a CHO intake above 50g/day, but CHO provided <30% of the EI. Based on this, we would not expect nutritional-induced ketosis, especially in the high CHO group. However, 68% of the participants in this group had βHB

concentrations within ketosis levels at W9. The corresponding percentage for the low and medium CHO groups was 89% and 74%, respectively. These results show that it is possible to achieve ketosis at higher levels of CHO intake (in this context: CHO intake >50g or >30% of EI). After the 4-week weight stabilization phase, where a normal eucaloric diet was reintroduced (50-60% CHO, 15-20% protein and 20-30% fat), the participants were no longer in ketosis.

In the present study, taxonomic groups of gut microbiota that belong both to the Firmicutes (Faecalibacterium, Ruminococcaceae, Eubacterium rectale group and Blautia), and to the Bacteroidetes (Bacteriodes and Alistipes) phyla were analyzed. Previous studies have investigated the importance of the F/B ratio in relation to obesity (27-30), but not to the same degree in relation to diet composition or CHO intake. The abundance of the Bacteroidetes at a phylum level is reported to be lower in obese individuals, and studies have found an increase in Bacteroidetes with WL interventions, regardless of diet composition or if the weight loss was induced by diet intervention or bariatric surgery (28, 69, 70). This is consistent with the present finding that the concentration of Alistipes increased after the 8-week WL phase overall, as well as in the low and high CHO groups. Considering that the changes did not have a linear relationship with CHO intake, and that there were no differences between groups at W9, these changes are assumed to be due to WL rather than CHO intake.

For the Firmicutes phylum, there were some contradicting findings to support the F/B ratio theory. A reduction in Eubacterium rectale group was also seen, as expected based on this theory, but there was an increase in the abundance of Ruminococcaceae after the WL phase. Even if the Ruminococcaceae family is a part of the Firmicutes phyla, there has been some evidence suggesting a link to this family of gut microbiota to a lean phenotype (71), which could explain the increase after the WL intervention. However, this theory is still not clinically proven, and more studies are required to determine the significance of this finding. Regardless, considering there were no observed differences between the groups regarding Ruminococcaceae following the diet intervention, the change over time seems to be explained by WL rather than CHO intake.

From the literature, a ratio between the Prevotella/Bacteroides (P/B) genera seems to be of higher importance when it comes to CHO intake than the previously discussed F/B ratio theory. This P/B ratio theory links a western-type diet with high amounts of fat and protein to a Bacteroides-enterotype, while a diet high in CHO and fiber is linked to the Prevotella-enterotype (72, 73). In the present study, the three diets were all composed of a macronutrient composition favoring the Bacteroides-enterotype, with relatively low amounts of carbohydrates. Regardless of this, the concentration of Bacteroides did not change over time, as it was expected based on the

literature. However, this might be explained by the intervention population in the present study, as this study was conducted in a Western population, and the participants are likely already associated with the Bacteroides-enterotype prior to diet intervention. After the diet intervention in the present study, a lower proportion of Bacteroides was found in the medium CHO group, compared with both the low and high CHO groups, but the reasons for this remains uncertain, as there are no clinical studies to our knowledge that has found a similar outcome.

There seems to be limited evidence in the literature investigating the impact of CHO restricted diets on Blautia. One study investigating the impact of high-fat diets on several bacterial taxa, including Blautia, found a negative correlation between CHO intake and Blautia (74). This is inconsistent with the findings from the present study, where a weak positive correlation was found between CHO intake and Blautia, and there were also significant differences in Blautia between groups after the diet intervention, where the low CHO group had a lower abundance than both the medium and high CHO groups. However, after adjusting for WL (FM and FFM loss in kg), the correlation was no longer significant. In contrast to the present study, the participants in the study mentioned above did not have obesity, and the diet reported a sufficient energy intake to keep participants in energy balance. The study was also conducted with a cross-sectional study design (74). Hence, the results of this study were not fully comparable to those of the present study. Nevertheless, there is need for further investigation on the association between CHO intake and Blautia, to establish a possible link between the two.

The abundance of Faecalibacterium did not change over time overall, but it did decrease for the low CHO group at W9. A previous study also found a decrease in Faecalibacterium after consuming a low carbohydrate/high fat (LCHF) – diet (75). However, that study connected this reduction of the relative abundance of Faecalibacterium to the high fat intake of the diet rather than the low CHO intake, with the theory that high fat diets are likely to change the profile and the amount of bile acid secretions that reaches the large intestine, and since Faecalibacterium is considered a "bile sensitive bacterium", this could result in a reduction in the relative abundance (75). This might also explain the lack of a significant correlation between CHO intake and abundance of Faecalibacterium in the present study, as the CHO intake might not be the strongest predictor of this genus. This would also explain why only the low CHO group showed significant reductions over time for this genus, as fat intake was the highest in the low CHO group. Correlation analysis did not support this hypothesis, however, as no significant correlation between fat intake and Faecalibacterium at W9 was found. More studies are necessary to establish such a hypothesis.

Overall, our findings seem to be consistent with an association between F/B ratio and obesity and WL, with Bacteroidetes having an inverse relationship with BMI, and the opposite association for Firmicutes (27, 28, 30). However, there is some contradicting evidence to this association in the literature. Duncan et al. was not able to find this association between bacterial composition at the phyla level and BMI (76). Instead, they suggested that the changes in bacterial composition was linked to diet, especially the type and amount of CHO present. 23 of the participants in the study underwent a WL regime for 8 weeks, where they followed two diets (MC (moderate CHO) and LC (low CHO)) for 4 weeks each, in different diet orders. They found that Roseburia+Eubacterium rectale group decreased with diet order MC to LC, but increased with diet order LC to MC, even though the WL was the same with either diet (76). This is consistent with our findings of the same reduction in the Eubacterium rectale group following diet intervention, and its correlation to CHO intake rather than WL. This significant decrease in Eubacterium rectale group has been detected in several studies and seems to be a common consequence of a lower CHO intake (44, 46, 77-79).

Further, it has been suggested that the production SCFAs, as a result of the changes in microbiota composition, is the key, not gut microbiota composition per se (31, 76). In the present study a decrease in all SCFAs (except iso-valerate) was seen after the 8-week WL phase compared to BL values, for all LED groups. This is for the most part consistent with previous studies in relation to acetate, butyrate, and propionate (44, 46, 77). A study by Brinkworth et al. (46) comparing a very low-carbohydrate, high fat (LC) diet to a conventional high-carbohydrate, low fat (HC) diet, found a decrease in fecal acetate, butyrate and total SCFA after an 8-week diet intervention on the LC diet, but not on the HC diet. At week 8 of this study, they also reported that total SCFA concentration was positively correlated with fiber intake and CHO intake. A measure of plasma ketone bodies revealed that the LC group had elevated levels after the 8-week diet intervention, due to the low CHO intake (20 g, 5% of EI), but a similar elevation was not observed in the HC group (CHO intake of 170g, 46% of EI) (46). Two studies using a crossover design, compared a high protein/moderate CHO (HPMC) to a high protein/low CHO (HPLC) for 4 weeks, and these were both compared with a maintenance diet (containing 360-399g CHO) (44, 77). One of the studies found a decrease in total SCFA concentrations in response to the HPLC diet, that was not seen in the HPMC diet, and a decrease in butyrate and acetate was also found (44). In the other study, a decrease in butyrate was found for the HPLC diet, and a positive correlation between butyrate and CHO intake was also observed (77). While there were no other differences in any of the other SCFAs between the two groups, both the HPMC and HPLC groups showed lower values of acetate, propionate and total SCFA compared to the maintenance diet (77). The remaining SCFAs have not been investigated to the same degree in the literature, as they comprise a very small proportion of the total SCFAs in the colon and stool (32), yet there are some evidence showing decreases in these components as a result of a

low CHO intake as well, which is in coherence with our results (77). The findings of the present study did not reveal any significant correlations between SCFAs and CHO intake, but in contrast to the studies mentioned above, the CHO intake in all groups remained relatively low, while the studies reporting significant correlations with CHO intake all had a much wider range in CHO intake (ranging from 20 to 399 g CHO) (44, 46, 77). This underlines the need for conducting studies in the future with a wide range of CHO intake, to investigate the true association between CHO intake and SCFAs.

The most prominent finding of the present study seems to be the decrease in butyrate, which is most likely due to the decrease in the butyrate-producing bacteria of the Eubacterium rectale group. The decrease in the Eubacterium rectale group was significantly correlated with decreased CHO intake, and studies investigating strategies to increase butyrate production have mentioned the importance of supplying sufficient fermentable CHO from the diet, for this exact reason (77). Increasing the abundance of butyrate would result in health benefits such as a potential decreased risk of colorectal cancer (80, 81). This suggests that a decrease in butyrate, as a result of a low intake of CHO (even if it is only indirectly, via a reduced abundance of butyrate-producing bacteria), would have potentially detrimental effects on health (77). However, the limited duration of the present study cannot account for the long-term health effects associated with the diet intervention. The concentration of butyrate increased significantly after 4 weeks of refeeding, suggesting that the abundance of butyrate will eventually return to BL values, and remain low only during diet intervention.

Even though the connection between gut microbiota, SCFAs and obesity has been explored in the literature (26-29, 31), the connection to CHO intake has not been investigated to the same degree, as many of these studies focus primarily on the WL aspect of diet interventions, rather than the diet composition (82). Total amount of SCFAs are reported to be higher in obese individuals, even when self-reported fiber and caloric intake was the same, indicating that there is a connection between obesity and SCFAs that are not related to CHO intake (30, 31). Still, there is a reason to believe that an association between CHO intake and gut microbiota/SCFAs exists, independently of WL (76, 79, 83, 84). This hypothesis is also substantiated by a previously mentioned study by Murtaza et al. (75), where they compared three different diet interventions in elite race walkers, where all of these diets provided an adequate amount of calories for the participants to remain weight stable during the entire intervention, eliminating any association with WL (75). The LCHF diet was associated with a significant increase in the relative abundance of Bacteroides and Dorea spp., and a reduction in the relative abundance of Faecalibacterium spp., compared to the diets with higher amount of CHO (75), thus

demonstrating that even without the WL-association, the gut microbiota composition changes based on CHO intake.

In the present study, WL was the same for all three groups, hence the changes observed in gut microbiota and SCFA should be a result of differences in CHO intake. Unfortunately, we were not able to assess the relationship between CHO intake and overall microbial diversity of the gut, as we did not conduct calculations of diversity measures. Based on previous studies, where an overall reduced diversity of the gut microbiota has been linked to both a lower CHO intake (44, 46, 77) and obesity (85), it would have been interesting to see if the present study showed similar results. In the present study, no association between CHO intake and taxonomic groups of gut microbiota, or SCFAs, except the Eubacterium rectale group, was seen. However, the association between CHO intake and Eubacterium rectale group was consistent with previous studies (44, 46, 77-79). There may be several reasons for the lack of significant correlations in the present study. First, CHO intake might not have an impact on SCFAs independently of fiber. As previously mentioned, fiber intake was the same for all groups. This was done to evaluate the independent effect of CHO intake, and in this case, we did in fact not find a correlation between CHO intake and SCFA, as we might expect from the results of previous studies (44, 46, 77). It is important to note that contrary to the present study, these studies did not add a set amount of fiber to each group, and we cannot rule out the possibility that the correlation observed in these studies is, in fact, a result of differing fiber intake rather than CHO intake in general. Without adding a set amount of fiber to all CHO groups, maybe differences between groups would be more pronounced. However, this would be contradictory to the aim of the study, where we wanted to investigate the association between gut microbiota, SCFAs and CHO intake, independently of fiber intake. Secondly, all these LEDs, although named throughout the study as "low, medium and high CHO group", all remained low in CHO intake relative to the general population (44, 86). A larger variation in CHO intake between groups may result in more distinct findings on this association, but this remains to be investigated in future research.

High interindividual variability in dietary patterns before a diet intervention is mentioned in several studies to be a contributing factor to the heterogenous evidence connecting the response of gut microbiota composition to diet intervention (87, 88). There are large differences in gut microbiota composition at BL and a large variance in host responsiveness to the diet in any population, mainly because several environmental and genetic factors alter the gut environment, and this makes it harder to draw conclusions from clinical studies, and will also impact the reproducibility among studies (88). This is seen in addition to the challenges comparing studies conducted with different study designs, lengths, and diet compositions.

Strengths and limitations

This study has both strengths and limitations. First, the main strength of this study is its design, a randomized controlled trial, which is considered the "gold standard" within experimental designs. This is due to its ability to minimize bias of different kinds. Second, participants were followed up weekly and compliance was good. Third, to avoid large inter- and intraindividual errors in the repeated measurements of the anthropometrics and the gut microbiota and SCFAs, these measurements were standardized, and performed at BL, at W9 after diet intervention and W13 after the weight maintenance phase. Fourth, all LED groups had the same weight, FM and FFM loss, at both W9 and W13, and similarly, all groups had the same fiber intake. This is also a strength because it allows for the assessment of the independent effect of CHO on the gut microbiota and SCFAs. Fifth, for the statistical analysis, a regression analysis was used to adjust for known confounders, to explore the independent association between CHO intake and taxonomic groups of gut microbiota and SCFAs. Also, Bonferroni adjustment was used for the multiple time comparisons to account for increased risk of type 1 error. Last, different aspects of appetite were measured (objective and subjective) in fasting and after a meal.

This study also suffers from limitations. First, it might not have the statistical power to investigate the association between CHO intake and different markers of gut microbiota, as this is part of a larger study aimed at elucidating the maximum CHO intake still associated with ketosis and appetite suppression. Second, we were not able, from the 16S rRNA sequencing analysis, to identify specific species of gut microbiota that were affected by the diet, only higher-ranking taxonomic groups (mostly genera), which makes it harder to compare results with existing research on the topic, because most studies review specific species rather than genera of gut microbiota. Third, although the three LED groups were divided into a "low", "medium" or "high" CHO intake, all three LEDs remained relatively low in CHO, and a broader range is needed to investigate the aim of this study.

Lastly, this study had an approximately 50:50 ratio of females to males, making it generalizable to both groups. However, this study was conducted in a Caucasian population in Norway, and, as such, cannot be generalized to other ethnic groups. The possibility that different ethnic groups show a different gut microbiota composition, have been investigated, and this might even be true for individuals with different ethnicities living within the same geographical area (89). For example, the association between a lower abundance of gut microbiota and a higher BMI is reported predominantly within Caucasian populations, and the association is not as established among other ethnic groups (89).

Practical implications

For future research, it would be critical to conduct interventions with groups consuming diets containing broader amounts of CHO, especially from dietary fiber sources. The present study investigated the effect of three LEDs with varying amount of CHO intake. Although the CHO intake prescribed between the diets seems to be of a wide range, we know from previous evidence that KDs and KLCDs usually limits the intake of CHO even further. This study provides evidence that CHO intake is correlated with the abundance of at least one butyrate-producing bacteria, further strengthening the hypothesis that CHO intake can modulate gut health of the host in a negative direction.

5.0 Conclusion

The results of this study seem to suggest that a low CHO intake might have a negative impact on gut microbiota and SCFAs production. However, more and higher quality studies are needed in this field, with longer intervention periods, and with a larger range in CHO intake.

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Appendix I – consent form

Forespørsel om deltakelse i et forskningsprosjekt

Hvilken mengde karbohydrat kan man spise og samtidig redusere sult, men øke metthetsfølelse?

Bakgrunn og hensikt
Dette er en forespørsel til deg om å delta i en forskningsstudie med utgangspunkt i en 8-ukers lavkalori diett hvor karbohydrat inntaket vil variere mellom deltakerne etterfulgt av en 4 ukers fase hvor målet er vektstabilisering og 9 måneder oppfølging. Problemsstillingene i studien er:

- Hva er det maksimale inntaket karbohydrater man kan innta og samtidig undertrykke appetitten under en lavkalori diett?
- Hvordan påvirkes appetitt hormonene som regulerer appetitt i diettens aktive fase?
- Hvordan påvirkes blodkomponenter, inflammasjon og immunsystemet
- Hvordan probiotika (melkesyrebakterier som kan ha gunstig helse effekt) påvirke vedlikehold av vekttap

NTNU, Norges teknisk-naturvitenskapelige universitet er ansvarlig for studien.

Hva innebærer studien?
Studien går over en 8-ukers diettperiode hvor inntaket av karbohydrater vil variere mellom deltakerne. Deltakere skal spise et variert utvalg av mat/diett produkter (milkshakes & supper) som tilsvarer et daglig energiinntak på 1000 kcal, fordelt over tre grupper med forskjellig karbohydrat inntak. Vi tar sikte på å oppnå i gjennomsnitt 8-10 % vekttap. Etter diett-perioden gjennomfører alle deltagerne en 4-ukers vekt-stabiliseringsfase, hvor man gradvis går over fra diett-produkter til å spise vanlig mat.

Det vil være ukentlig oppfølging fra forskere ved NTNU som gjennomgår kostdagboken din. Veiing inngår som en del av denne prosessen. Alle deltakerne vil også måtte avgi blod og urinprøver hver uke under diettfasen, og avføringsprøver på begynnelsen av studie (baseline), uke 9 (etter diettfase), uke 13 (etter vektstabiliseringsfase), 6 måneder og 12 måneder.

I uke 13, blir deltakerne randomisert (plassert tilfeldig) til å ta probiotika eller placebo daglig i totalt 9 måneder. Deltakerne skal møte månedlig til oppfølging ved Regionalt senter for fedmeforskning.

Undersøkelsene i studien foregår ved oppstart, uke 8, uke og 12 og ved 6 og 12 måneder. Oppfølgingen omfatter blodprøver, blodtrykksmåling, avføringsprøver, målinger av

energibehov, vekt og livvidde, kroppssammensetning med BodPod (air displacement plethysmography) og BIA (Bioelectrical impedance analysis), bruk av aktivitetsarmbånd, samt utfylling av diverse spørreskjemaer.

Mulige fordeler og ulemper
Fordelen med deltakelse i studien er å oppnå mulig vektreduksjon og vedlikehold av den tapte vekta. I tillegg forbedrer deltakere helsen uten kirurgiske inngrep. Deltakelse kan også gjøre at du blir bedre kjent med mekanismene i kroppen din som påvirker appetitten. Dessuten vil du spare kostnader på mat i studiens diettfase (diettproduktene får du gratis i studien) og får probiota (eller placebo) gratis. Behandlingen anses ikke som risikabel, men siden undersøkelsene innebærer blodprøvetaking, kan noen deltakere oppleve dette som litt ubehagelig.

Hva skjer med prøvene og informasjonen om deg?
Prøvene tatt av deg og informasjonen som registreres om deg skal kun brukes slik som beskrevet i hensikten med studien. Alle opplysningene og prøvene vil bli behandlet uten navn og fødselsnummer eller andre direkte gjenkjennende opplysninger. En kode knytter deg til dine opplysninger og prøver gjennom en navneliste. Det er kun autorisert personell knyttet til prosjektet som har adgang til navnelisten og som kan finne tilbake til deg. Det vil ikke være mulig å identifisere deg i resultatene av studien når disse publiseres.

Frivillig deltakelse
Det er frivillig å delta i studien. Du kan når som helst og uten å oppgi grunn trekke ditt samtykke til å delta i studien. Dette vil ikke få konsekvenser for din videre behandling. Dersom du ønsker å delta, vennligst undertegn samtykkeerklæringen på siste side. Dersom du senere ønsker å trekke deg eller har spørsmål til studien, kan du kontakte studiekoordinator Jessica Røkenes, som nås på telefon 46 77 02 40.

Studien er godkjent av Regional komité for medisinsk og helsefaglig forskningsetikk REK Sør-Øst B.

Ytterligere informasjon om studien finnes i kapittel *A* *– utdypende forklaring av hva studien innebærer.*
Ytterligere informasjon om personvern og forsikring finnes i kapittel B *– Personvern, biobank, økonomi og forsikring.*

Samtykkeerklæring følger etter kapittel B

Kapittel A – Utdypende forklaring av hva studien innebærer

Kriterier for deltakelse
De som kan delta i denne studien må

1. ha BMI mellom 30 og 40 kg/m^2,
2. være mellom 18 og 65 år,
3. ha et ønske om å gå ned i vekt ved hjelp av diett,
4. være relativt vektstabil de siste tre månedene (< 2 kg variasjon),
5. ikke være på diett i de siste tre måneder,
6. være frisk,
7. være inaktiv (ikke trene/mosjonere regelmessig)
8. Ikke har tatt probiotika i løpet av de siste 6 måneder før start av studie
9. ikke har tatt antibiotika i løpet av de siste 3 måneder før start av studie

Kvinner må dessuten enten være over menstruerende alder eller benytte p-piller eller andre hormonell-prevensjonsmetoder.

Bakgrunn for studien
Lavkalori dietter er en relativt sikker metode for å gå ned i vekt og gir også et raskt vekttap. Slike dietter kan gi vekttap på 8-10% i løpet av 8 uker. Dette kan også gi bedring i overvekts relaterte sykdommer og risiko faktorer. Vi vet at lavkalori dietter som er lav på karbohydrater kan indusere ketose, en tilstand som antas å forårsake undertrykkelse av appetitt. Det antas at ketose oppstår når forbruket av karbohydrater er lavt. Det lave forbruket av karbohydrater fører ofte til en begrensning av matvarer som frukt, grønnsaker, melkeprodukter, helkorn/fullkorn og belgfrukter som er gunstig for en persons helse. Den maksimale mengden karbohydrater i en lavkalori diett som er forbundet med ketose er derimot ukjent. Mengden karbohydrater man kan spise før man trigger appetittfølelsen, når man er i ketose, er også midlertidig usikkert. Det er behov for mer kunnskap om hvordan ketose fungerer, og hvordan vi kan innlemme mer karbohydrater i en lavkalori diett må undersøkes videre.
Dessuten vet vi at probiotika kan hjelpe med vekttap, men få studier har sett på vekttap vedlikehold.

Hovedhensikt med denne studien er å sammenligne undertrykkelse av appetitt gjennom en 8-ukers lavkalori diett hos pasienter som deltar i tre diett program med ulik mengde karbohydrat inntak.

Vi vil også se nærmere på hvordan den hormonelle appetitt reguleringen endres i diettens aktive fase. Appetitt er et komplisert samspill av blant annet hormoner som både stimulerer og reduserer matlysten, og vi vil følge utviklingen i disse i løpet av de ukene som dietten varer. Det er hittil gjort lite forskning på dette.

I tillegg skal det også undersøkes hvis daglig inntak av probiotika, sammenlignet med placebo, har en påvirkning på vekttap vedlikehold.

Undersøkelser

Som del av studien vil du måtte møte fastende og gjennomgå ulike undersøkelser før du start studie, slutten av uke 8 og 12 og 1 år oppfølging (totalt vil dette ta cirka 2,5 – 4 timer).

- Veiing og kroppsmassemåling
- Måling av kroppssammensetning med BodPod (Air displacement plethysmography) og BIA (Bioelectrical Impedance Analysis)
- Blodprøver
    - Måling av appetitt hormoner og ketoner i blod (for å måle ketose)
    - Måling av blodkomponenter inklusive inflammatoriske markører og immun funksjon (leukocytt responser)
- Indirekte kalorimetri (måling av energibehov)
- Blodtrykk (systolisk og diastolisk)
- Spørreskjema
- Urinprøver (også ukentlig fram til uke 12) og avføringsprøver (baseline, Uke 9, Uke 13, 6 måneder, 12 måneder)

I enkelte perioder av studien må du gå med et spesielt armbånd som registrerer din fysiske aktivitet. Varighet er en uke. Dette skjer før diett start, uke 4, 8 og 12 og 6 og 12 måneder.

Tidsskjema for intervensjonsperioden (12 uker) - felles for alle

Du vil få utdelt et variert utvalg av mat/diett produkter (milkshakes, supper) tilsvarende et daglig energiinntak på 1000 kcal med forskjellige makro-næringsstoff fordeling. Du skal utelukkende spise disse produktene imens du er i diettens aktive fase (8 uker) (standardisert for alle), men du oppfordres til å drikke rikelig vann (minst 2,5 liter) og eventuelt kalorifri drikke i tillegg. Du vil så få time hos en forsker hver uke for ukentlig oppfølging. Gjennomgang av kostdagbok, veiing og urin-og avføringsprøver er en del av diettfasen. Overgangen fra diett-produkter til normal-kost vil skje gradvis i løpet av studieuke 9 og 10.

Studiedeltakerens ansvar

Det er studiedeltakerens ansvar å møte til avtalt tid, og det er av stor betydning for at kvaliteten på studien skal bli så god som mulig.

Kompensasjon og egenandel

Det gis ingen premiering for å delta i studien, men du vil få diettproduktene i diettens aktive fase og probiotika (eller placebo) gratis. Vi kan dessverre ikke gi kompensasjon for reiseutgifter. Det er viktig å standardisere dietten slik at alle spiser samme mengde energi.

Kapittel B – Personvern, biobank, økonomi og forsikring

Personvern
Ulike opplysninger om deg vil registreres som en del av dette prosjektet. Alle opplysninger som registreres om deg er konfidensielle. Ingen utenforstående forskere vil ha tilgang til dataene.

Vi vil benytte et internettbasert system for å samle inn spørreskjemadata. Dette betinger at du har tilgang til en datamaskin eller iPad. Rapporteringssystemet krypterer svarene dine slik at det ivaretar kravene til personvern.

NTNU ved administrerende direktør er databehandlingsansvarlig.

Biobank
Det biologiske materialet som blir tatt vil bli lagret i den spesifikke forskningsbiobanken "Ketosis study" ved Institutt for Kreftforskning og Molekylær Medisin (NTNU). Materialet vil bli analysert for ulike metabolitter/hormoner som er involvert i appetitt regulering, blodkomponenter, inflammatoriske markører og immunologisk funksjon. Instituttleder Professor Magne Børset er ansvarlig for denne forskningsbiobanken. Hvis du sier ja til å delta i studien, gir du også samtykke til at det biologiske materialet og analyse resultater inngår i biobanken. Det biologiske materialet kan bare brukes etter godkjenning fra Regional komité for medisinsk og helsefaglig forskningsetikk (REK).

Rett til innsyn og sletting av opplysninger om deg og sletting av prøver
Hvis du sier ja til å delta i studien, har du rett til å få innsyn i hvilke opplysninger som er registrert om deg. Du har videre rett til å få korrigert eventuelle feil i de opplysningene vi har registrert. Dersom du trekker deg fra studien, kan du kreve å få slettet innsamlede prøver og opplysninger, med mindre opplysningene allerede er inngått i analyser eller brukt i vitenskapelige publikasjoner.

Økonomi
Studien finansieres av midler fra NTNU.

Forsikring
Studiedeltakerne omfattes av Norsk pasientskadeforsikring, jf. pasientskadelovens §1.

Informasjon om utfallet av studien
Du er berettiget til å motta informasjon om utfallet av studien.

Samtykke til deltakelse i studien

Jeg er villig til å delta i studien

(Signert av prosjektdeltaker, dato)

Jeg bekrefter å ha gitt informasjon om studien

(Signert, rolle i studien, dato)

Appendix II – Instructions for the LED

Veiledning – LED

I de neste 8 ukene skal du følge en LED (lav kalori diett) uten å endre ditt fysiske aktivitetsnivå.

Nedenfor er et eksempel på hvordan du kan legge opp kostholdet ditt de neste 8 ukene:

Måltid	**Mat**
Frokost	**1** shake (sjokolade, jordbær eller vanilje)
Lunsj	**1** suppe (tomat eller kylling) * det er mulighet for å tilsette litt grønnsaker med lavt innhold av karbohydrater til suppen (beskrevet senere)
Mellommåltid	**1** shake (sjokolade, jordbær eller vanilje)
Middag	**1** suppe (tomat eller kylling) * det er mulighet for å tilsette litt grønnsaker med lavt innhold av karbohydrater til suppen (beskrevet senere)
Mellommåltid	**1** shake (sjokolade, jordbær eller vanilje)

Dette er kun ment som et forslag. Du kan planlegge dagene dine som du selv ønsker, men du må få i deg **5 pakker per dag**. (2 supper og 3 shaker)

Viktige faktorer å ta i betraktning:

- Du bør spise all maten som er forskrevet **(5 pakker/dag)**, selv om du ikke føler deg sulten.
- Du behøver ikke å ta noe vitamin eller mineraltilskudd, da dietten inneholder de mikronæringsstoffene som kroppen din trenger.
- Det bør ikke brukes kokende vann i suppene, da suppene klumper seg av dette. Vent litt før du heller i det varme vannet over suppen din.
- Du kan tilføre opptil 50g grønnsaker per suppe per dag. Grønnsakene du har lov til å spise til suppen er de som vokser over bakken (f.eks. blomkål, brokkoli, salat, tomat, agurk, squash, aubergine), og de kan være rå eller kokt.
- Du kan ikke drikke alkohol, brus eller naturlige frukt juicer. Du kan drikke ta, kaffe og andre drikker uten kalorier. Det anbefales at du drikker minst 2,5 liter vann per dag.
- Vær obs på at vann med smak kan inneholde kalorier. Det anbefales derfor at du holder deg til Farris naturell, eller drikker vanlig vann tilsatt sitronskiver (ikke sitronekstrakt). Ved tvil, les innholdet bakpå flasken. Hvis innholdsfortegnelsen viser at drikken inneholder kalorier (energi/kcal/kj per 100 ml) kan denne ikke drikkes.
- Det er normalt og forventet at du de første dagene (i noen tilfeller den første uken) vil føle deg sulten (det er en normal respons fra kroppen), men sultfølelsen forsvinner stort sett etter et par dager. Det er svært viktig at du holder deg til planen.
- Sukkerfri tyggegummi og sukkerfrie pastiller **(maks 3-4 stk/dag)** er tillatt hvis ønskelig. Dette må noteres i kostdagboken.

Appendix III – List of vegetables allowed.

Grønnsaksliste:

Du kan spise 100 gram av disse grønnsakene i tillegg til måltidene. Grønnsakene merket med * kan du kun spise 50 gram av da disse inneholder mere stivelse og energi.

- Agurk
- Alfalfaspire
- Artiskokk
- Asparges
- Aubergine
- Bambusskudd
- Basilikum
- Bladsalat
- Blomkål
- Brokkoli
- Brønnkarse
- Crispisalat
- Endivesalat
- Feltsalat
- Fennikel
- Gressløk
- Grønn paprika
- Grønnkål
- Hjertesalat
* Hodekål
* Ingefærrot
- Isbergsalat
* Løk (alle typer)
- Kinakål
* Koriander
- Raddichio salat
- Rapidsalat
- Reddik
- Romano salat
* Rosmarin
- Ruccola salat
- Sopp (alle typer)
- Spinat
- Squash/ Zucchini
- Stangselleri
- Tomat (Cherry og vanlig)
- Vårløk

Disse grønnsakene er IKKE lov å spise:

- Avokado
- Søtpotet
- Potet
- Bønner
- Erter
- Gresskar
- Kål
- Mais

Hvis det er noen grønnsaker som er ikke på listen som du har lyst til å spise, men er usikker om det er lov, så ta kontakt med oss eller sjekk på matvaretabellen.no om det inneholder mindre enn 3 gram karbohydrater eller ikke.

Appendix IV – User manual for activity monitors

Brukermanual for SenseWare armbånd

1. Armbånd & sensor tåler ikke vann, ta den av når du dusjer, bader (etc)

2. Elektromagnetiske forstyrrelser: skal du i CT-scan eller lignende må armbåndet tas av. Dette informerer som regel helsepersonell om.

3. Armbåndet skal være på minst 7 dager, ta det av etter den 8. dagen.

4. Armbåndet må være på hele døgnet - også når du sover. Tas kun av maks 1 time per dag (f.eks. når du dusjer).

5. Armbåndet skrur seg på når du tar det på, og skrur seg av når du tar det av. Du trenger hverken å trykke på sensoren eller lade den i den perioden du skal bruke båndet.

6. Tørk av synlig skitt eller svette i det tidsrommet du tar av armbåndet (max 1 time av per 24 timer).

7. Ta med deg båndet tilbake til oss neste gang du skal innom, men sørg for at du har brukt det sammenhengende i minst 7 dager før.

8. Bruk armbåndet på din *ikke-dominante* arm, dvs. er du høyrehendt, bruk den på venstre overarm

Appendix V - Healthy eating guidelines for weight stabilization phase

Retningslinjer for vedlikehold av vekten:

Dette heftet er kun en liten hjelp til dere for å se eksempler på hvordan dere kan legge opp måltidene etter at dere er ferdig med pulverkuren. Det anbefales en gradvis nedgang i bruk av produktene. I uke 9 er dette satt til 2 produkter om dagen. I uke 10 er det anbefalt å ta 1 produkt om dagen både for kvinner og menn.

Her har vi laget et eksempel på hvordan dere kan legge opp måltidene i løpet av en dag. Dere trenger ikke følge dette slavisk, da det kun er ment som en liten hjelp frem til dere får eget kostholdsopplegg fra ernæringsfysiolog.

Her er noen generelle kostholdsråd som er greie å tenke på for å legge opp et sunt kosthold:

- Se etter matvare merket med nøkkelhull.

- Sammenliknet med andre matvarer av samme type, oppfyller produkter med nøkkelhull ett eller flere av disse kravene:
  - Mindre og sunnere fett
  - Mindre sukker
  - Mindre salt
  - Mer kostfiber og fullkorn

- Spis minst fem porsjoner grønnsaker, frukt og bær hver dag. Dvs. 2 porsjoner frukt og 3 porsjoner grønnsaker. En porsjon tilsvarer 100 gram. 1 dl juice tilsvarer en av fem om dagen.

Bilde er ett eksempel på hvordan man kan oppfylle kravet om fem om dagen.

- Spis grove kornprodukter hver dag. Brødet bør ha minst tre eller fire kakestykker

- La magre meieriprodukter være en del av det daglige kostholdet. F.eks. ekstra lettmelk, mager cottage cheese, mager kesam, norvegia lett ost.
- Spis fisk til middag to til tre ganger i uken. Bruk også gjerne fisk som pålegg.
- Velg magert kjøtt og magre kjøttprodukter. Begrens mengden bearbeidet kjøtt og rødt kjøtt (f.eks. kjøttdeig, farse)
- Velg matoljer (rapsolje, solsikkeolje, olivenolje), flytende margarin og myk margarin fremfor hard margarin og smør.
- Velg matvarer med lite salt og begrens bruken av salt i matlagingen og på maten. Vær obs på posesupper, sauser, frokostblandinger osv da disse kan inneholde mye salt/sukker.
- Unngå mat og drikke med mye sukker til hverdags
- Velg vann som tørstedrikk
- Ha en god balanse mellom hvor mye energi du får i deg gjennom mat og drikke, og hvor mye du forbruker gjennom aktivitet.

Menyforslag

UKE 9 – valgfritt med 2 produkter om dagen for kvinner (anbefales), og 3 produkter for menn (anbefales).

Frokost:

- Alternativ 1: 1 dl musli eller havregryn (kok gjerne) med ekstra lettmelk/vann
- Alternativ 2: 2 skiver grovt brød eller 3 grove knekkebrød med magert kjøttpålegg, ost eller egg. Litt smør eller lettmargarin. Bruk så mye paprika, agurk og tomat du ønsker.
- Alternativ 3: 1 shake (valgfri type)

Mellommåltid: 1 frukt eller 100 gr grønnsaker (f.eks. sukkerert, gulrot, tomat)

Lunsj:

- Alternativ 1: blandet salat med 125-150gr kylling (1 filet), kjøtt eller fisk med 1 ss oljedressing.
- Alternativ 2: 2 skiver grovt brød eller 3 grove knekkebrød med magert kjøttpålegg, ost eller egg. Tynt lag med lettmargarin. Bruk så mye paprika, agurk og tomat som du ønsker.
- Alternativ 3: 1 shake, **eller** 1 suppe (valgfri type)

Middag:

- Legg opp 125-150gr fisk/kjøtt, 2 små poteter, 50gr fullkornris eller fullkornspasta og grønnsaker etter tallerkenmodellen.

Mellommåltid: 1 frukt eller 100gr grønnsaker (f.eks. sukkererter, gulrot, tomat)

Kveldsmat:

- Alternativ 1: 1 kopp te, 2 grove knekkebrød med mager ost eller skinke, tynt lag med lettmargarin og gjerne tomat, paprika, agurk eller andre grønnsaker.
- Alternativ 2: 1 grovt knekkebrød med magert pålegg (mager ost, skinke, makrell i tomat og lignende), 1 liten yoghurt og 1 glass melk.
- Alternativ 3: 1 shake **eller** 1 suppe (valgfri type)

UKE 10 – valgfritt med 1 produkt om dagen for kvinner og menn (anbefales)

Frokost:

- Alternativ 1: 1 dl müsli eller havregryn (kok gjerne) med ekstra lettmelk.
- Alternativ 2: 2 skiver grovt brød eller 3 grove knekkebrød med magert kjøttpålegg, ost eller egg. Litt smør eller lettmargarin. Bruk så mye paprika, agurk og tomat du ønsker.
- Alternativ 3: 1 shake (valgfri type)

Mellommåltid: 1 frukt eller 150gr grønnsaker (f.eks. sukkererter, gulrot, tomat)

Lunsj:

- Alternativ 1: blandet salat med 125-150gr kylling (1 filet), kjøtt eller fisk med 1 ss oljedressing.
- Alternativ 2: 2 skiver grovt brød eller 3 grove knekkebrød med magert kjøttpålegg, ost eller egg. Tynt lag med lettmargarin. Bruk så mye paprika, agurk og tomat du ønsker.
- Alternativ 3: 1 shake **eller** 1 suppe (hvis denne ikke er tatt tidligere på dagen)

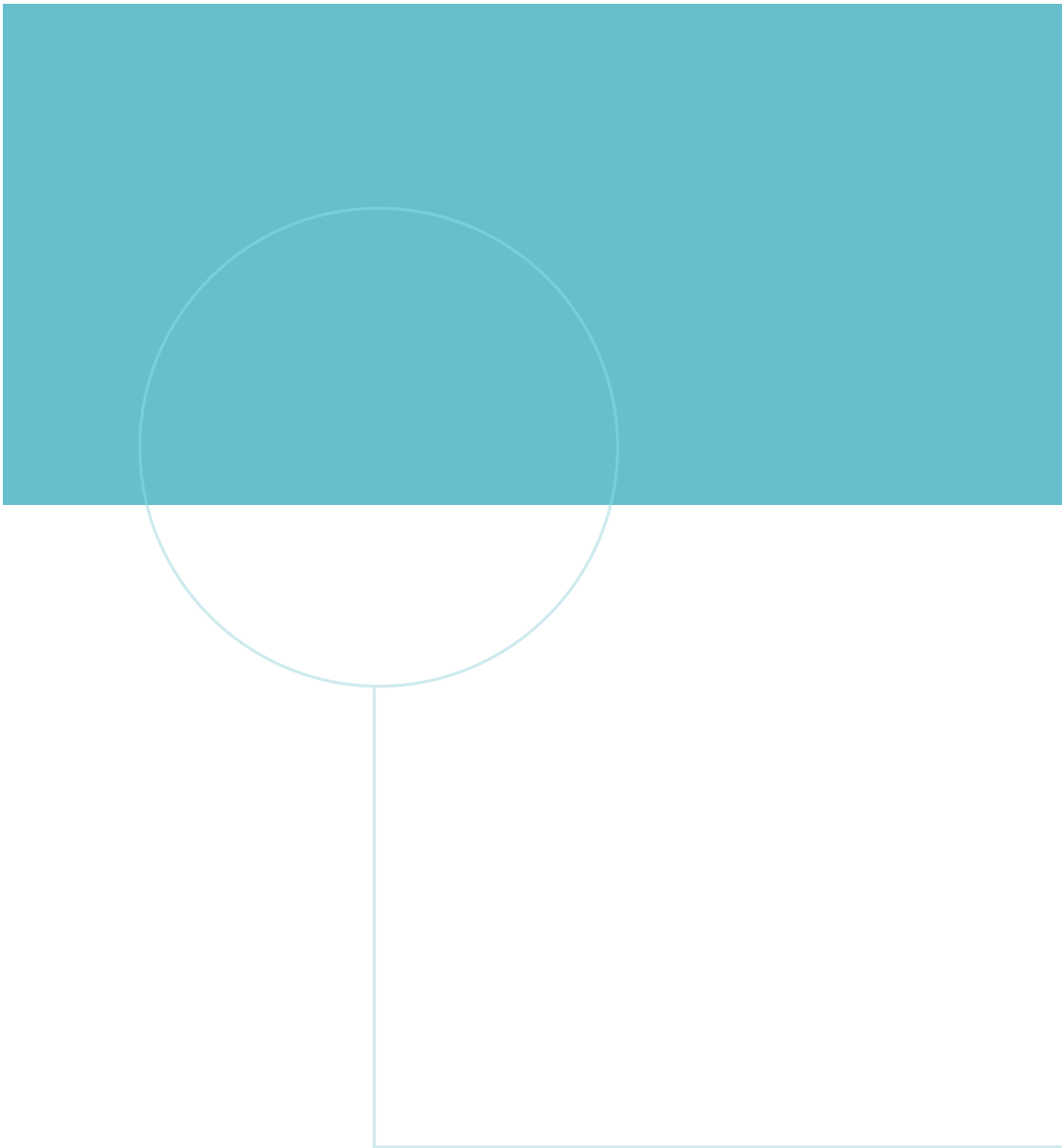
Middag:

- Legg opp 125-150gr fisk/kjøtt, 2 små poteter, 50gr fullkornris eller fullkornspasta og grønnsaker etter tallerkenmodellen.

Mellommåltid: 1 frukt eller 150gr grønnsaker (f.eks. sukkererter, gulrot, tomat)

Kveldsmat:

- Alternativ 1: 1 kopp te, 2 grove knekkebrød med mager ost eller skinke, tynt lag med lettmargarin og gjerne tomat, paprika, agurk eller andre grønnsaker.
- Alternativ 2: 1 grovt knekkebrød med magert pålegg (mager ost, skinke, makrell i tomat og lignende), 1 liten yoghurt og 1 glass melk.
- Alternativ 3: 1 shake **eller** 1 suppe (hvis denne ikke er spist tidligere på dagen)



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