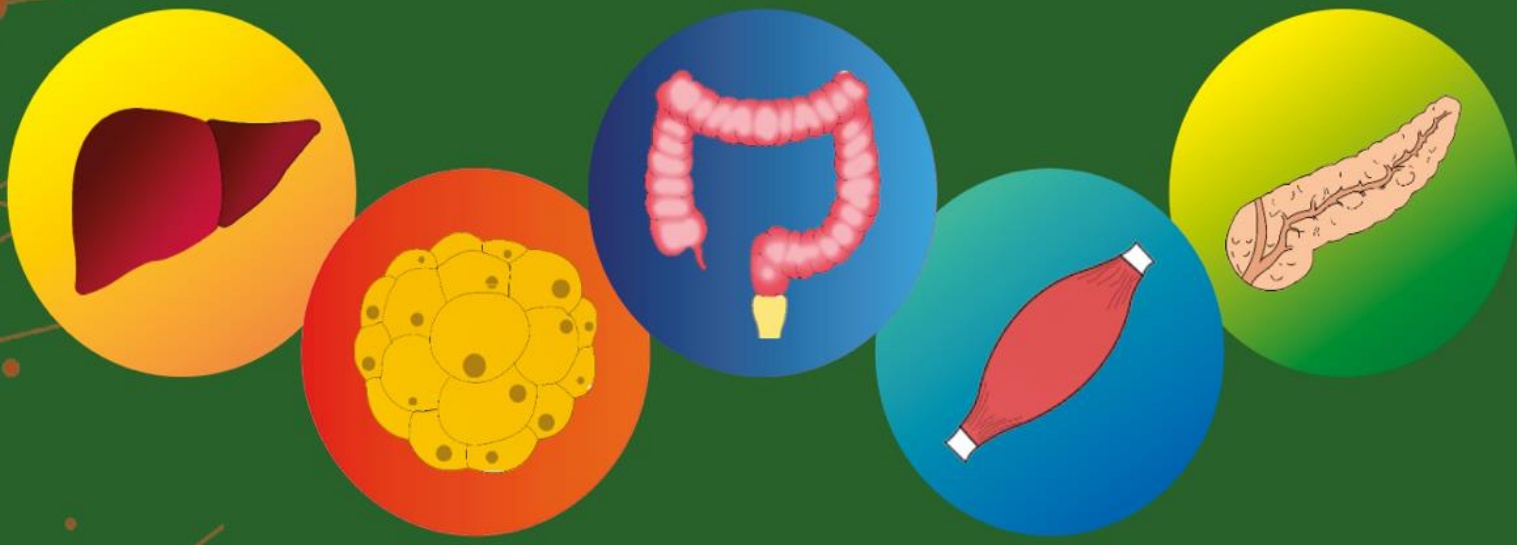
Impact of gut-derived metabollites on substrate metabolism and metabolic health

IMPACT OF GUT-DERIVED METABOLITES ON SUBSTRATE METABOLISM AND METABOLIC HEALTH



Manuel A. González Hernández

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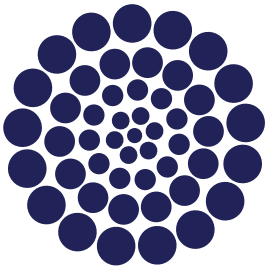

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DISSERTATION

to obtain the degree of Doctor at the Maastricht University, on the authority of the Rector Magnificus, Prof. dr. Rianne M. Letschert, in accordance with the decision of the Board of Deans, to be defended in Public on Tuesday 27th October 2020, at 13:00 hours

by

Manuel A. González Hernández

born on May 12th, 1989 in León Guanajuato, México

Promotor
Prof. dr. E.E. Blaak

Co-promotors

Dr. Emanuel E. Canfora
Dr. Johan W.E. Jocken

Assessment committee

Professor Dr M.A. van Baak, Emeritus Professor of the department of Human Biology – Maastricht University

Professor Dr K. Venema, Professor of Gut Microbiology. Department of Human Biology/ Venlo Campus – Maastricht University

Dr. G.D.A. Hermes, Senior Researcher of the department of Agrotechnology and Food Sciences - Laboratory of Microbiology – Wageningen University

Dr. F.J. Troost, Associate Professor of Food Innovation and Health, Centre for Healthy Eating and Food Innovation, campus Venlo – Maastricht University

Professor Dr. P.C.N. Rensen, Professor of Endocrinology at the Leiden University Medical Center

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CHAPTER 1

General introduction

The global obesity epidemic

In the last decades, the prevalence of overweight adults has increased approximately 2 billion, of which 650 million are obese, according to the wor health organization (WHO) 2016 report [1]. Of note, prediction models estimate that the prevalence of obesity will further rise in the following decades [2]. Excess body weight is mainly classified using the body mass index (BMI) which is defined as body weight (kg) divided by the square of height (m^2). Overweight (>25 kg/m^2) and obesity (>30 kg/m^2) phenotypes are characterized by increased adiposity that may link to adipose tissue dysfunction and the development of insulin resistance, Type 2 diabetes Mellitus (T2DM) and cardiovascular disease (CVD) [1]. However, the BMI as a unique measurement of obesity does not reflect the whole complexity of the disease. The major limitation of BMI is that it cannot distinguish fat from lean body mass nor metabolically benign from metabolically harmful fat at the individual level. Thus, BMI should be interpreted with caution [3]. Importantly, obesity is a risk factor for the development of sleep apnea, stroke, gallbladder disease, hyperuricemia, hypertension, dyslipidemia, gout, osteoarthritis and certain types of cancer among others [4, 5].

Increased physical inactivity together with a relatively high consumption of energy-dense foods are major drivers of the energy imbalance that leads to the obesity epidemic. In addition, other factors may play a role in obesity and comorbidities, including stress, sleep, genetic profile, epigenetic modifications,[6] social determinants [7] as well as microbiota composition and functionality. Nowadays the territories with highest prevalence (between 28-36%) of obesity include the United States of America, Mexico, some parts of Europe and the Middle East [8]. Based on this, there is no question that obesity and its comorbidities pose a risk with potential catastrophic consequences for global health as well as a menace for various nations' economies [9]. Currently, there are three pillars in the therapeutic approaches to combat obesity, including lifestyle modifications (nutritional advice, physical activity), bariatric surgery and pharmacotherapy [10]. Unfortunately, weight is partially or fully regained in most of the individuals (~80%) who undergo a weight loss intervention [11, 12]. In response, vast literature suggests that successful interventions may require continuous monitoring support and behavioral therapy to the obese individuals from the health care providers [13-16]. It has become increasingly clear that intervention success may vary between individuals [17-19] and that addressing the multifactorial etiology of overweight/obesity from different angles (multidisciplinary approach) is needed [20]. Therefore, more targeted or personalized strategies considering individual preferences, genetic, microbial and metabolic phenotypes may possibly increase the effectiveness of a weight loss intervention and increase the improvement in metabolic health. On this matter, the role of the gut microbial community and gut-derived metabolites in the etiology of obesity and cardiometabolic diseases as putative targets for

nutritional interventions have received increasing attention in the past two decades [21-23].

The gut microbiota consists of 10-100 trillion microbial cells [24], and so far ~500-1000 bacterial species have been discovered that ferment dietary components [25]. Mainly in the colon, indigestible carbohydrate (saccharolytic) fermentation by the microbial community produces short chain fatty acids (SCFA) as well as other metabolites (i.e. lactate, succinate) and gases (i.e. methane, carbon dioxide and hydrogen). Major SCFA are acetate, propionate and butyrate, which have been reported to have beneficial effects on metabolic health [26, 27]. Saccharolytic fermentation takes place mainly in the proximal colon and decreases towards the distal colon as observed in sudden death victims [28]. In case of a diet that is low in complex fermentable carbohydrates, the distal portion of the colon specializes more in proteolytic fermentation (residual peptides and proteins). Although proteolytic fermentation may produce SCFA, its contribution to SCFA levels remains largely unknown [25]. In addition, proteolytic fermentation yields the production of a wide variety of metabolites including phenolic compounds, indoles, hydrogen sulfide as well as branched chain fatty acids (BCFA) such as isovalerate and isobutyrate [25, 29, 30]. Of note, many of these proteolytic compounds are of toxic nature and if produced in high quantities may have adverse effects on gut and metabolic health [25, 31]. In turn, more insight into relevant gut microbial metabolites (SCFA/BCFA) is needed to elucidate their effects in host metabolism (saccharolytic/proteolytic balance) and understand their role in the whole-body and tissue specific metabolism in humans.

Microbial community and gut functioning

The gut microbial community is highly interconnected and actively produces various metabolites mainly as a result of saccharolytic or proteolytic fermentation along the colon [25-27, 29, 30]. The gut microbial community is shaped throughout life by various factors including birth delivery mode [32], diet [33-36], antibiotics [37], environment [38, 39], temperature [40] among other factors. A core microbial community consists of various microorganisms, but the most studied group are the gut bacteria, classified by the dominant phyla Bacteroidetes, Firmicutes, Proteobacteria, Verrucomicrobia, and Actinobacteria [41]. It is important to consider various aspects about the core microbial community, such as diversity, ecological robustness, taxonomic classifications (species, phylum level) as well as the functionality of the microbial networks [42, 43]. A high microbial diversity has been linked to a healthy gut as well as to a better insulin sensitivity and metabolic health in various animal and human studies [44-49]. Although healthy gut functioning is not clearly defined, there are various factors that determine gut health and host metabolic health including the maintenance of gut permeability (this is the passage of metabolites from the gut lumen to the

basolateral site which is importantly regulated by tight junction proteins), microbial SCFA production, antimicrobial molecules, gut-derived hormone secretion and maintenance of mucus layer thickness (gut barrier function) [50-52]. Under pathological conditions, a symphony of factors are linked to gut dysbiosis including an increased gut permeability (the so called "leaky gut") causing low-grade inflammation (i.e. liver) [51, 53], a decrease in microbial diversity [54-57], increments in pathogenic bacteria (i.e. proteobacteria) [51, 58] and translocation of pathogenic bacteria to portal vein and peripheral blood [59]. With respect to proteolytic fermentation products (i.e. BCFA), some of these compounds may have detrimental effects on colonic and metabolic health under conditions of a high-protein consumption [25, 60]. In general, gut microbiota composition and functionality as well as gut permeability and gastro-intestinal transit are interlinked and have been connected with the metabolism of various organs including the liver [25, 59, 61], skeletal muscle [62, 63], adipose tissue [23], pancreas [64, 65] and brain [25]; that collectively can affect whole-body substrate and energy metabolism, inflammatory profile and insulin sensitivity [66, 67]. Furthermore, there are compositional characteristics of the gut microbiota (at the genus/species level) that are associated with host metabolic health [68, 69]. Of note, these alterations may improve microbial network functionality through increments in abundance of amongst others bifidobacteria (bifidogenic effect), butyrate-producing bacteria (i.e. *Faecalibacterium, Ruminococcus*) and *Akkermansia muciniphila* (acetate producer) [70, 71].

Under the premise to restore gut and metabolic health, interventions targeting the microbiome (i.e. probiotics or prebiotics) have been performed. In this regard, it is well recognized that prebiotic interventions (i.e. fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), arabinoxylan oligosaccharides (AXOS), inulin) of various durations (1-12 weeks) can have a bifidogenic effect [34, 72-74], however, their effects on metabolic health have been inconsistent, especially data on SCFA production and gut-derived hormone secretion. Currently, considerable attention is given to postbiotics (i.e. SCFA) as they have shown a potential benefit in body weight control and improvements in insulin sensitivity [75]. However, little data are available on the role of SCFA in human substrate and energy metabolism, in particular their role in more metabolically compromised phenotypes which is still not fully understood. Furthermore, little is known about products of proteolytic fermentation, such as BCFA (isovalerate, isobutyrate) in humans.

Short chain fatty acid metabolism

Colonic SCFA production is modulated by various microbial pathways that produce acetate (acetogenesis and carbon fixation pathways) [33], propionate (acrylate pathway) [25] and butyrate (butyrogenic pathways) [76]. These processes, the abundance of specific bacterial species (i.e. *Akkermansia muciniphila*, *Bacteroides thetaiotaomicron*, *Faecalibacterium prausnitzii*) [23,31], bacterial cross-feeding mechanisms (mainly from acetate to butyrate, and to a lower extent from butyrate to propionate) [77] in combination with diet (i.e. fiber characteristics) [78] play a prominent role in SCFA production. In theory, fermentation of 10 g of indigestible carbohydrates can produce approximately 100 mmol of SCFA in the colon [79]. Total production of SCFA has shown a molar ratio of roughly 3:1:1 as observed in fecal levels of acetate, propionate and butyrate, respectively [36]. Moreover, a study in sudden death victims, showed that SCFA concentrations decrease from the ascending, to the transverse, descending and sigmoid colon [28], indicating higher production in the proximal colon (123 mmol/kg, 117 mmol/kg, 80 mmol/kg and 100 mmol/kg, respectively). According to kinetic studies on SCFA metabolism, SCFA follow a preferential order in utilization by colonic epithelial cells, starting with butyrate > propionate > acetate [80, 81]. In particular, butyrate plays an important role as an energy source for colonocytes and colonic mucosa [82] while particularly acetate and to a significant lower extent propionate can reach the portal vein and systemic circulation in relatively high concentrations [63]. In addition, *in vivo* human studies using tracers have reported that colonic SCFA production is nearly matched by liver uptake for butyrate and propionate, whereas acetate can reach to a higher extent the systemic circulation [83, 84]. In general, colonic SCFA absorption is concentration-dependent from the gut lumen to the periphery and circulating levels are impacted by colonic production and absorption rate [85]. Briefly, SCFA absorption is regulated by various mechanisms including hydrogen/sodium coupled monocarboxylate transporters (MCT), exchange with bicarbonate and non-ionic diffusion [86-88]. After colonic absorption, SCFA may reach the systemic circulation in different concentrations (acetate 19-450 μmol/L, propionate 1-13 μmol/L and butyrate 1-12 μmol/L) [36, 89]. Interestingly, a study in men conducted during abdominal surgery reported a higher SCFA release from distal colon compared to proximal colon, indicating that colonic absorption may differ between colonic segments [90]. Acetate was significantly released only from distal colon. Of note, the implications of these findings are that targeting the distal colon with slowly fermentable fibers may increase circulating SCFA and acetate concentrations, which have been previously shown to translate into more pronounced metabolic effects [91, 92]. In the colon, SCFA may modulate gut and host metabolism through G-protein coupled receptors (GPR) 41/43 [93-96], whilst SCFA may also affect metabolism through entry into the systemic circulation affecting thereby

peripheral insulin sensitive tissues that express GPR (liver, skeletal muscle, adipose tissue and pancreatic beta cells) [97-99].

In addition, a study conducted during abdominal surgery also reported a significant liver uptake of all SCFA [90] which highlights the potential significance of SCFA for hepatic metabolic processes (i.e. lipogenesis, gluconeogenesis) [100-102]. With respect to measurements of SCFA concentrations, the majority of human (and murine) studies have measured SCFA levels in fecal material [86, 103-105] as a surrogate marker for SCFA colonic production. In this regard, it is important to consider that gut SCFA absorption is efficient [106, 107], and that fecal levels depend on various factors including microbial community [108], intestinal transit time [109], diet [110, 111], age [112], lifestyle [113] among others. Only a few human studies (mainly with an exploratory focus) have measured SCFA concentrations in the circulation [28, 29, 83, 91, 92, 114]. Therefore, further studies should fully elucidate to what extent fecal and/or plasma SCFA reflect colonic SCFA production and to what extent they relate to metabolic health markers. In addition, mechanistic studies are needed using human derived tissue from biopsies and/or cell lines from relevant tissues (liver, adipose tissue, muscle) to identify tissue specific effects of SCFA in host metabolic health.

Branched chain fatty acid metabolism

The gut microbial BCFA isovalerate, isobutyrate and 2-methylbutyrate are mainly derived from proteolytic fermentation of branched chain amino acids (BCAA, leucine, isoleucine and valine), through the action of specific bacterial genera (i.e. *Bacteroides, Clostridium, Fusobacterium*) [25, 115]. The abundance of BCFA producing bacteria decreases when distal colonic microbes are able to switch to saccharolytic fermentation as a result of an increased carbohydrate availability [116]. In humans following a Western diet, approximately 12 g of amino acids and peptides from both dietary and endogenous origin escape digestion in the small intestine and reach the colon daily [117]. This amount increases proportionally with an increased intake of plant-based protein or an overall increased protein intake from animal sources (such as cow's milk, beef and cheese) [118-120]. In particular, plant-based proteins may be less digestible and more fermentable (as compared to animal-based proteins): for instance brown beans have reported to increase the plasma BCFA isobutyrate concentration in humans [121]. In humans, total colonic BCFA production is estimated around 18.87 mmol/kg of dry matter [25]. As previously explained, the distal colon microbiota specializes more in proteolytic fermentation to produce BCFA [25, 29, 60, 122]. In support, an *in vitro* system using gut contents from distal and proximal human colons, reported that BCFA concentrations were higher in the distal than in the proximal colon (6.3 and 4.5 mmol/L, respectively) [123]. Moreover, a seminal study in sudden death victims showed that BCFA colonic concentrations vary along the different segments of

the large intestine [28]. Isovalerate concentrations were 2.7, 3.4, 3.5 and 3.7 mmol/kg and isobutyrate concentrations were 1.8, 2.6, 2.3 and 1.9 mmol/kg in ascending, transverse, descending and sigmoid colon, respectively [28].

In healthy individuals, fecal concentrations of isobutyrate and isovalerate have been reported 105 ± 67 and 178 ± 126 μg/g of dry weight, respectively [124]. In the colon, isovalerate can activate the olfactory receptor 558 (Olfr558) as observed in a murine colonic organoid system [125]. Of note, OR51E1 the human ortholog of Olfr558 is expressed in human enteroendocrine L cells, thus suggesting a potential effect of BCFA isobutyrate to induce gut hormone secretion [126]. After colonic absorption, BCFA reach the circulation as observed in individuals following emergency surgery, which reported that BCFA concentrations in arterial and portal vein were 43.7, 41.7 μmol/L and 21.1, 15.5 μmol/L for isovalerate/2-methylbutyrate and isobutyrate, respectively, [123]. In the systemic circulation, isovalerate may regulate blood pressure via the Olfr558, which is highly expressed in blood vessels (aorta), heart and kidney. While in other tissues, such as large intestine, brain, lung and liver expression levels of Olfr558 are low, as observed in mice [127]. With respect to isobutyrate, it has been reported to act selectivity towards GPR41 in immune cells [93], and given the broad expression profile of GPR41 [36], it remains a challenge to pinpoint tissue specific functions. Interestingly, an *in vitro* study using rat and human adipocytes showed that both BCFA (isobutyrate and isovalerate) inhibited cytosolic (intracellular) lipolysis and lipogenesis using supraphysiological concentrations (10 mmol/L) [128].

Interestingly, a cross-sectional study in humans showed that total fecal BCFA (isovalerate, isobutyrate and 2 methylbutyrate) concentration was higher in obese individuals (30-37 BMI) compared to individuals with a normal weight [129]. This was attributed to a higher proteolytic fermentation, as suggested by high BCFA together with a tendency for increased ammonium and phenolic concentrations in feces. In summary, little is known on the role of gut microbial-derived BCFA in tissue specific and whole-body metabolism, substrate utilization and energy homeostasis in humans. In the last section, we discuss the available data on the relationship of BCFA with the control of body weight and insulin sensitivity.

Interorgan crosstalk in obesity and insulin resistance

Excessive body weight linked to ectopic fat accumulation increases the risk of developing metabolic disturbances in glucose homeostasis and insulin sensitivity. Increased adiposity is characterized by enlarged (hypertrophic) adipocytes with large lipid droplets (in the form of triacylglycerols) in the white adipose tissue (WAT) [130]. Metabolic disturbances include increased triacylglycerol (TAG) and lipid metabolite (i.e. ceramides, diglycerides, long chain acyl-CoA and acylcarnitines) accumulation in non-adipose tissues (i.e. skeletal muscle, liver) [131] and in the circulation [132, 133], an impaired lipid buffering capacity of adipose tissue and lipid spillover (TAG, free fatty acids) [130].

As indicated, adipose tissue shows an impaired lipid uptake and reduced lipogenesis in obese, insulin resistant conditions when its storage capacity is exceeded [134]. The lipid spillover to the systemic and portal circulation leads to an increase in lipid supply to other tissues such as the liver, skeletal muscle and pancreas. Furthermore, fat oxidation rates in skeletal muscle, liver and adipose tissue fail to match the constant lipid supply, a phenomenon indicated as metabolic inflexibility [135]. According to the adipose tissue expandability hypothesis, its expandability is determined by both genetic and environmental factors [136, 137], and as the adipose tissue expands, immune cell infiltration (i.e. macrophages, T cells) increases and through the secretion of proinflammatory adipokines (e.g tumor necrosis factor alpha, TNFα) insulin signaling is altered [138, 139]. Additionally, the liver and skeletal muscle may secrete TNFα locally [139] as well as several other bioactive molecules (hepatokines and myokines, respectively) with distinct effects on insulin sensitivity. However, the exact mechanisms still need to be fully elucidated [140]. Chronic dyslipidemia may result in a metabolic dysfunction of the liver, known as non-alcoholic fatty liver disease (NAFLD), which may develop into a fibrotic, cirrhotic and insulin resistant liver [141, 142]. Intracellular lipids, in particular bioactive lipid metabolite (i.e. diacylglycerol and ceramides) accumulation in liver and skeletal muscle may alter insulin signaling, glycogen storage and glucose handling [143, 144]. Skeletal muscle is recognized as a relevant organ in glucose disposal, since it can enhance glucose clearance for almost 80% in the postprandial state [145]. In addition, an insulin resistant state may develop in a tissue specific manner (liver, skeletal muscle, adipose tissue) [19]. Alterations in pancreas become apparent when pancreatic beta cells fail to cope with increased insulin demands as a compensation for the progressing insulin resistance. First of all, enhanced insulin secretion compensates for the progressing insulin resistance in order to maintain glucose homeostasis despite pancreatic lipid accumulation and beta cell failure [146].

Secondly, impaired insulin secretion from beta cells and increased whole-body insulin resistance result in altered glucose homeostasis and development of the prediabetic and diabetic state. In addition, the gastrointestinal tract is of major importance for the human substrate and energy metabolism, since it is in first contact with food and its main functions include to digest and absorb food, obtain energy, secrete gut hormones (peptide YY, PYY and glucagon like peptide, GLP-1) and ferment metabolites that escape digestion in the small intestine. Collectively, gut functioning and gut-derived products maintain whole-body homeostasis through their effects on energy and substrate metabolism.

Role of SCFA in the interorgan crosstalk

As explained above, insight has increased on gut-derived metabolites (SCFA) in particular in their role in the interorgan cross talk with the aforementioned tissues.

In the colon, SCFA may modulate various mechanisms that can affect whole body metabolism, including energy harvest [82], intestinal gluconeogenesis (i.e. propionate) [147] and secretion of satiety hormones (GLP-1, PYY) from enteroendocrine cells [148, 149]. Furthermore, SCFA may exert tissue specific effects that can ameliorate an insulin resistant state and aid in whole-body energy homeostasis and weight management. Several studies have reported an antilipolytic effect of acetate in animal adipocytes [128, 150, 151], and on whole body level in animals [150, 152, 153] and humans [154-157]. In contrast, butyrate and propionate have been shown to both enhance [158] and attenuate lipolysis (basal and TNFα stimulated) [150, 159]. In the adipose tissue, SCFA have been proposed to signal through the GPR41/43 receptors and attenuate the phosphorylation of hormone sensitive lipase (HSL at serine 563). Of note, mechanistic studies have been performed in murine adipose cells (which may lack GPR41) [160] with incubations of single SCFA using supraphysiological concentrations (4-10 mmol/L). This calls for future studies that investigate SCFA effects in human adipocyte models using physiological relevant SCFA concentrations. Additionally, SCFA may also improve adipose tissue function and remodeling through increased adipogenesis, expression of browning markers (i.e. Uncoupling protein-1) and alleviation of an inflammatory state through improvements in circulating proinflammatory cytokine profiles [36, 161].

Furthermore, circulating SCFA may directly reach and modulate skeletal muscle metabolism through increases in AMP as previously observed in murine myotubes [162] and *in vivo* in skeletal muscle of animals [63, 163]. For example, according to an *in vitro* study using L6 myotubes, acetic acid increased AMP/ATP ratio and AMPK (threonine 172) phosphorylation in a dose-dependent (0.05-0.5 mmol/L) and time-dependent (0-30 min) manner using physiological acetic acid concentrations [164]. In line, muscle gastrocnemius extracts from butyrate-

supplemented mice (C57BL/6J) showed increments in radiolabeled palmitate oxidation by 200% [162]. In addition, acetate intragastric infusions (6 months) [165], and *in vitro* acetate incubations using L6 myotubes (0.05-0.5 mmol/L) [164], both showed upregulation of glucose transporter 4 (GLUT4). Of note, these findings show the potential of SCFA to increase fat oxidation and/or glucose uptake, which warrants further investigation in humans. In rat hepatocytes, incubations with acetate (0.1-0.2 mmol/L) increased phosphorylation of AMPK (threonine 172) and fat oxidation [166]. The liver, as explained above is in close proximity with the proximal colon and gut-derived SCFA may modulate hepatic processes including lipogenesis and cholesterol biosynthesis (mainly acetate) and gluconeogenesis (propionate) [100-102, 167]. Importantly, SCFA may also promote gut integrity through the upregulation of tight junction proteins [168] and preservation of a healthy mucosa that decreases translocation of proinflammatory molecules (i.e. lipopolysaccharides, LPS) [169] to the liver and the systemic circulation (metabolic endotoxemia).

Circulating SCFA may also modulate pancreatic functioning [170]. In support, SCFA have been shown in animal and *in vitro* studies (using isolated human islets) to modulate glucose-stimulated insulin secretion, however, their role and physiological relevance in pancreatic insulin secretion in humans remains unclear [96, 171, 172]. With respect to the gut-brain axis, acetate can cross the blood brain barrier in animals [173] and humans [174] and together with SCFA-induced secretion of gut-derived hormones (GLP-1, PYY) regulate satiety-inducing effects through central hypothalamic activation (vagal activation) [33]. In summary, colonic SCFA may improve gut health and may reach the circulation in the aforementioned tissues, thereby directly/indirectly modulating substrate and energy metabolism (See Figure 1). In the next section, we discuss the role of SCFA in weight management and insulin sensitivity in humans.

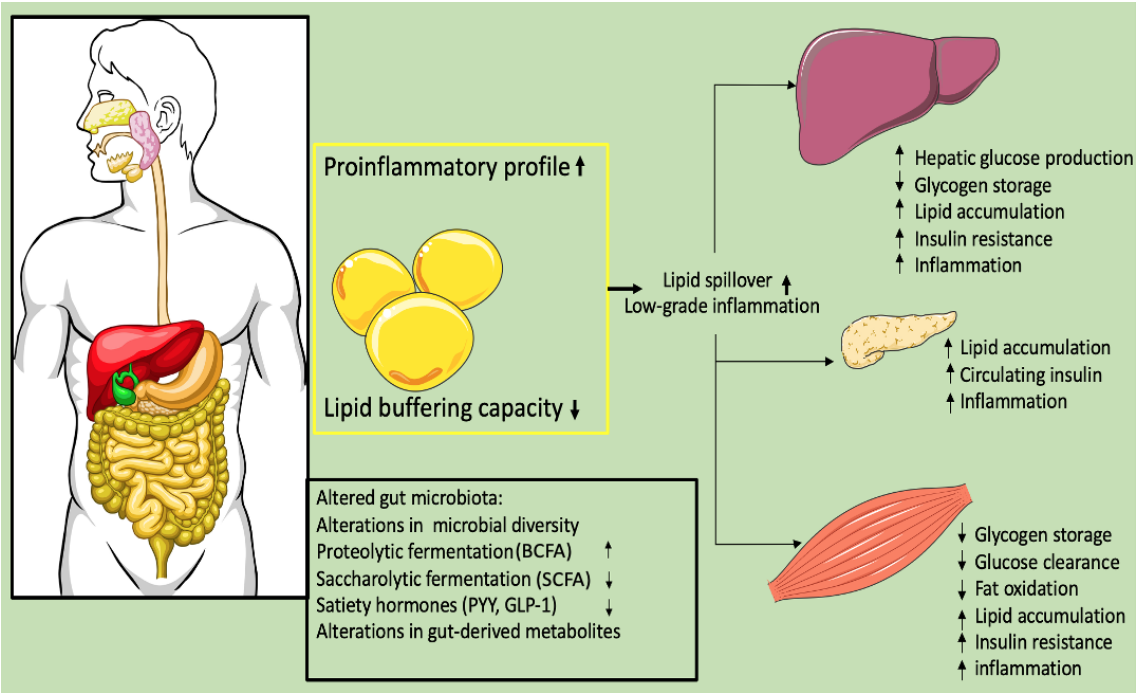

Figure 1. Interorgan crosstalk in an insulin resistant state*. Adiposity impairs the adipocyte lipid buffering capacity, thereby releasing circulating lipids in the circulation (lipid spillover). The resulting lipid accumulation in the liver and skeletal muscle may impair insulin signaling, decrease glycogen storage, increase hepatic glucose output and may result in hyperglycemia. Additionally, adipose tissue dysfunction can lead to proinflammatory adipokine/cytokine secretion, which results in a low-grade inflammation state that disturbs insulin signaling. In the gut, altered microbial composition, diversity and functionality, altered balance between saccharolytic and proteolytic fermentation as well as altered SCFA and BCFA concentrations may impact gut and metabolic health. In light of the current literature, a high fiber diet to promote a high SCFA/BCFA ratio may be preferred to improve gut health, functioning in the aforementioned tissues and whole-body insulin sensitivity. However, more information is needed on plasma SCFA relevance at the tissue level as well as on whole-body substrate metabolism and insulin sensitivity in humans.*

SCFA in control of body weight and insulin sensitivity

Limited human studies have investigated SCFA effects on parameters of body weight control and insulin sensitivity. One study showed that oral administration of sodium propionate increased fasting fat oxidation and resting energy expenditure independent of glucose and insulin levels in healthy subjects [175]. In line, distal colonic administration of SCFA mixtures and acetate alone resulted in increments in fasting fat oxidation, resting energy expenditure as well as in fasting PYY levels in overweight or obese individuals [91, 92]. However, these effects were not observed after proximal colonic acetate infusions.

As described above, a higher distal colonic SCFA release compared to the proximal colon points towards a relevance of distal colonic SCFA production in metabolic health. SCFA may also increase fat oxidation and energy expenditure indirectly through their effects on gut hormone production (i.e. GLP-1 and PYY) as observed in humans [176]. Recently, we showed that long-term supplementation with galacto-oligosaccharides (12-week, 15g/d, GOS), an acetogenic fiber, had no significant effect on peripheral and adipose tissue insulin sensitivity as well as

no effects on fecal and circulating acetate and SCFA despite an observed bifidogenic effect [34]. In view of our above described findings that acute distal colonic SCFA infusions, but not proximal colonic affected parameters of metabolic health [91], we hypothesized that the site of fermentation (distal *versus* proximal) of GOS may have led to an insufficient increase in systemic SCFA to induce pronounced metabolic effects. Thus, increasing dietary fibers that stimulate SCFA production in the distal colon may have the most pronounced effects on parameters of metabolic health. Interestingly, several cohort studies and prebiotic interventions in humans have shown improvements in markers of insulin sensitivity [177-180]. For instance, one study (a 4-week intervention using resistant starch) in healthy subjects showed increments in both skeletal muscle and adipose tissue uptake of SCFA (acetate and propionate) together with improved insulin sensitivity (measured via the hyperinsulinemic-euglycemic clamp) [178, 179], indicating that gut-derived SCFA from saccharolytic fermentation may reach the systemic circulation and insulin sensitive tissues. Notably, a recent study from our group investigated acute metabolic effects of the naturally-occurring soluble fiber inulin and using stable isotope tracer methodology to trace inulin-derived SCFA [35]. Inulin increased fermentation in the early postprandial phase ($^{13}CO_2$), increased circulating acetate in late postprandial phase (3-7 hrs) and fat oxidation in the early postprandial phase (0-3 hrs) in overweight-obese individuals [35].

Furthermore, probiotic supplementation of certain microbial strains (*Akkermansia muciniphila*) may promote a healthy gut functioning as well as improvements in metabolic health [181-184]. For instance, a recent probiotic supplementation for 3-months using living and pasteurized *Akkermansia muciniphila* in overweight-obese insulin resistant volunteers showed improvements in insulin sensitivity (Homeostatic Model Assessment of Insulin Resistance (HOMA-IR)) [185].

Of interest, this long-term supplementation was safe and well tolerated. Although the exact mechanisms were not explored in detail (some effects were reductions in total cholesterol and white blood cells), it is reasonable to hypothesize that *Akkermansia muciniphila*, being an acetate producer, may result in elevated acetate and acetate-mediated metabolic effects in the host. In **chapter 2,** we extensively reviewed the role of acetate in body weight control and insulin sensitivity [33]. In particular acetate, the most abundant SCFA, seems to be of utmost importance in prebiotic/probiotic effects on metabolism, as also evident from positive metabolic effects reported with vinegar (which contains as primary bioactive compound acetic acid/acetate 5-10% per volume) administrations and the above-described distal colonic acetate infusions [91, 92, 186].

Lastly, the effectiveness/outcome of studies focusing on modulating SCFA production or metabolism may depend on the individual metabolic (possibly differing between healthy versus metabolically disturbed individuals) [187] or microbial phenotype [188]. For instance, a study showed that oral supplementation of butyrate improved glucose metabolism in lean, but not in

individuals with characteristics of the metabolic syndrome [189]. In line, vinegar supplementation studies have been shown to beneficially affect glucose homeostasis in healthy individuals, but to a lesser extent in individuals with impaired glucose tolerance [186]. With respect to microbial phenotype, it should be considered that modulation of microbial composition might depend on microbial enterotypes, which are three distinct robust clusters that can stratify microbial community (*Bacteroides, Prevotella, Ruminococcus*) [188, 190, 191]. Furthermore, microbial composition may impact insulin sensitivity in a sex-specific manner, as a study reported an association in men (but not in women) between Bacteroidetes/Firmicutes ratio and peripheral insulin sensitivity [67].

As mentioned above, SCFA may be produced after saccharolytic (i.e. inulin, fructo-oligosaccharides, GOS) fermentation [26, 192, 193] and BCFA after proteolytic (BCAA, leucine, isoleucine and valine) fermentation [25, 29] and linked to beneficial and detrimental effects on metabolic health, respectively. Under this premise, it has been postulated that a balance in carbohydrate/protein fermentation may be of importance for metabolic health [25]. In general, an animal-based diet has higher digestibility and net protein utilization as compared to a plant-based diet [194]. However, little is known about the contribution of a high protein diet varying in animal versus plant-based sources to microbial BCFA production in humans. In various studies with individuals of different phenotypes (BMI 19-51 kg/m^2), high protein diet (mixed sources + casein/soy protein) interventions (1-4 weeks), have shown increments in fecal levels of BCFA (isovalerate and isobutyrate) and reductions in fecal levels of SCFA [195]. Although, the literature is relatively vast on SCFA and scarce on BCFA metabolism, human studies are lacking to fully elucidate their role in metabolic health.

Taken together, *in vivo* human studies investigating SCFA metabolism may need to consider various factors including a) the site of saccharolytic fermentation since this may affect metabolic outcome with more pronounced metabolic effects with distal colonic fermentation, b) the intervention success may depend on metabolic phenotype, as explained above, c) sex-specific effects of gut-derived metabolites since microbial composition relates in a sex-specific manner with peripheral insulin sensitivity. Furthermore, a vast wealth of literature comes from animal data, and studies addressing tissue specific (adipose tissue, skeletal muscle) effects in human cell models are lacking.

In the next section we discuss the role of BCFA in weight management and insulin sensitivity in the interorgan crosstalk.

Role of BCFA in the interorgan crosstalk, body weight control and insulin sensitivity

Most studies have investigated fecal concentrations of BCFA (isovalerate and isobutyrate), and only a few investigated their role in metabolic health and insulin sensitivity. A germ-free mouse model fed a high fat diet showed development of NAFLD and insulin resistance together with high levels of isobutyrate and isovalerate in the cecum [196]. Similarly, individuals with obesity have reported higher fecal BCFA concentrations as compared to healthy individuals [129] as well as association of fecal BCFA with the development of NAFLD in obese individuals [197]. Additionally, fecal BCFA in humans have reported higher abundance in individuals with hypercholesterolemia as compared to healthy controls [198]. Interestingly, isobutyrate and isovalerate in the systemic circulation may have different roles in obesity as a study found that plasma isobutyrate concentrations were lower and isovalerate concentrations higher in individuals with obesity compared to lean individuals (0.33, 1.37 and 0.47, 0.25 μmol/L, respectively) [199]. Of note, a study using a randomized crossover design showed that a high fiber diet (resistant starch type 2 and arabinoxylan fibers) versus a Western diet in individuals with characteristics of metabolic syndrome reduced fecal BCFA (isobutyrate and isovalerate) and increased fecal SCFA (acetate and butyrate) [200]. Although this indicated a modulation in saccharolytic/proteolytic fermentation ratio, further metabolic effects were not explored. In the circulation, differences in artery/portal vein BCFA concentrations, as measured in individuals during emergency surgeries (described above), suggest net production from the gut [123]. Moreover, based on BCFA receptor expression profile in various tissues (explained above), both isobutyrate and isovalerate have the potential to exert pivotal functions in peripheral tissues. In general, little is known about the role of fecal BCFA (isobutyrate and isovalerate) in human obesity and insulin sensitivity. Nevertheless, there are indications that BCFA production and handling may be disturbed and/or contribute to the progression of insulin resistance and NAFLD. Importantly, high fiber diets may modulate saccharolytic/proteolytic fermentation ratio in metabolically disturbed individuals, which might result in benefits for the metabolic profile of the individual.

Aim and outline of the thesis

This doctoral thesis focuses on the role of gut-derived metabolites (SCFA and BCFA) in *in vivo* metabolic health in human studies with overweight and obese individuals as well as the role of these microbial metabolites in human derived adipose and skeletal muscle tissues in *in vitro* models.

In **chapter 2,** we provide a comprehensive review of the current literature on the role of the colonically and systemically most abundant SCFA acetate in body weight control and insulin sensitivity. Here, we discuss acetate effects on satiety and energy expenditure, peripheral tissue metabolism (liver, skeletal muscle, adipose tissue and pancreatic beta cells) and address human intervention studies that modulate acetate ingestion or production (vinegar, pre- and probiotic). Lastly, we describe that the effect of acetate on metabolic health may depend on the colonic site of production or administration (proximal and distal) and metabolic phenotype.

In light of the vast literature on acetate and its beneficial effects on metabolic health (satiety, energy expenditure, substrate utilization and insulin sensitivity) we hypothesized that there is a positive association between acetate and insulin sensitivity. Thus, we investigated in **chapter 3** the associations between sex-specific concentrations of acetate with insulin sensitivity/resistance indices (HOMA-IR, circulating insulin and Matsuda index) in 478 participants (BMI >27 kg/m^2) of the Diet, Obesity and Genes (DiOGenes) Dietary study before and after a low-calorie diet (LCD, 800 kcal/d).

Human studies investigating the role of SCFA in metabolic health have predominantly measured fecal levels as a biomarker of SCFA production while only a few have measured circulating SCFA. Therefore, in **chapter 4**, we investigated the associations between fecal and plasma SCFA (acetate, propionate and butyrate) with metabolic health markers including circulating metabolites, gut hormones, substrate oxidation, inflammatory markers and markers of insulin sensitivity in a well-phenotyped group with a large range in BMI with a normal and an impaired glucose metabolism. In a subgroup, we investigated the fecal and plasma SCFA associations with insulin sensitivity measured via the gold standard hyperinsulinemic-euglycemic clamp technique.

Besides saccharolytic fermentation and SCFA production, proteolytic fermentation may yield various metabolites such as BCFA, which role in human metabolism remains largely unclear. Therefore, in **chapter 5,** we investigated the associations of fecal BCFA (isobutyrate and isovalerate) and other fecal metabolites (valerate, lactate, succinate and caproate) with circulating metabolites, substrate oxidation, and markers of insulin sensitivity (circulating insulin and HOMA-IR) in insulin sensitive and insulin resistant individuals.

A previously conducted *in vivo* study showed that distal colonic infusions of SCFA (rich in acetate) increased whole-body fasting fat oxidation in men with overweight/obesity [91, 92]. To elucidate the underlying mechanisms, in **chapter 6**, we investigated the effects of sodium acetate (SA) on fat oxidation in human primary muscle cells (HSkMC) derived from a healthy insulin sensitive donor. We investigated the dose and time effect of SA on complete and incomplete endogenous and exogenous oxidation of ^{14}C-labelled palmitate as well as SA effect on AMP-activated protein kinase phosphorylation. SCFA have been shown to exert antilipolytic effects in humans, which may reduce lipid overflow ultimately resulting in positive effects on insulin sensitivity. In **chapter 7**, we used the human multipotent adipose-derived stem (hMADS) cell model to investigate the antilipolytic effect of SCFA and to elucidate the underlying mechanisms. We investigated the *in vitro* effects of incubation with SCFA mixtures and single SCFA on fasting and stimulated cytosolic lipolysis. Additionally, we investigated the involvement of GPR41/43 receptors and hormone sensitive lipase. In **chapter 8**, we integrate and discuss the main findings of these studies that investigated the associations/mechanisms of SCFA and BCFA in metabolic health. Finally, we address future perspectives and suggestions for future research.

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CHAPTER 2

The short-chain fatty acid acetate in body weight control and insulin sensitivity

Running title: Acetate in body weight control and insulin sensitivity

Manuel A. González Hernández, Emanuel E. Canfora, Johan W.E. Jocken and Ellen E. Blaak

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Abstract

The interplay of gut microbiota, host metabolism and metabolic health has gained increased attention. Importantly, gut microbiota may play a regulatory role in gastrointestinal health, substrate metabolism and peripheral tissue metabolism including adipose tissue, skeletal muscle, liver, and pancreas via its metabolites short-chain fatty acids (SCFA). Animal and human data demonstrated that, in particular, acetate beneficially affects host energy and substrate metabolism via secretion of the gut hormones like glucagon-like peptide-1 and peptide YY, thereby affecting appetite, via a reduction in whole-body lipolysis, systemic pro-inflammatory cytokine levels and via an increase in energy expenditure and fat oxidation. Thus, potential therapies to increase gut microbial fermentation and acetate production have been under vigorous scientific scrutiny. In this review, the relevance of the colonically and systemically most abundant SCFA acetate and its effects on the aforementioned tissues will be discussed in relation to body weight control and glucose homeostasis. We discuss in detail the differential effects of oral acetate administration (vinegar intake), colonic acetate infusions, acetogenic fiber and acetogenic probiotic administrations as approaches to combat obesity and comorbidities. Notably, human data are scarce, which highlights the necessity for further human research to investigate acetate's role in host physiology, metabolic and cardiovascular health.

1. Introduction

Obesity has reached pandemic proportions worldwide, and its increased prevalence is associated with a plethora of metabolic disturbances [1]. The obese state is characterized by increased adipose tissue mass and disturbed function resulting in systemic lipid spillover and low-grade inflammation, which may contribute to the development of comorbidities such as type 2 diabetes mellitus (T2DM) and cardiovascular disease [2-4]. The crosstalk between various metabolic organs such as the gut, liver, adipose tissue and skeletal muscle plays an important regulatory role in energy and substrate metabolism, thereby impacting metabolic health [5].

In the last decades, the role of the gut microbiota in host energy and substrate metabolism has been under extensive investigation [6-8]. This includes interventions that modify the gut microbiota composition and functionality with antibiotics [9, 10], prebiotics [11, 12], probiotics [13] and postbiotics [14]. Of note, gut microbes are able to ferment indigestible foods, such as dietary fibers, thereby yielding as end products short-chain fatty acids (SCFA) that may confer beneficial metabolic effects [15-17]. In general, saccharolytic fermentation mostly occurs in the distal ileum and proximal colon. The most abundant SCFA are acetate, propionate and butyrate with an approximate molar ratio of 60:20:20, respectively [18-20]. In mice, the cecum has been described as a major site of SCFA production [21]. After colonic absorption and transition to the systemic circulation, the molar ratio changes to approximately 91:5:4, respectively, numbers that are based on findings in sudden death victims [22].

Acetate may act through the binding to the G-protein coupled receptors (GPR), GPR43 (FFAR2) and GPR41 (FFAR3), which are expressed at the mRNA and protein level in human colon [23, 24] with variations along the small and large intestine [25]. Moreover, these receptors have been shown to be expressed at the mRNA level in various insulin sensitive tissues such as the adipose tissue [26], skeletal muscle, liver [27] and pancreatic beta cells [28, 29], thereby illustrating their broad metabolic role. Intracellularly, although acetate may be converted to acetyl-CoA and incorporated in the tricarboxylic acid (TCA) cycle in various peripheral tissues [30-32], it may also impact metabolism through increments in oxidative capacity (e. g. liver and skeletal muscle) via effects on 5'AMP-activated protein kinase (AMPK) phosphorylation [33-35]. Of note, acetate may also increase fatty acid synthesis through epigenetic mechanisms such as histone acetylation [36]. Additionally, acetate may activate other receptors important for blood pressure regulation including olfactory receptors 51E2 (Olfr51E2) and 78 (Olfr78) in renal tissue [37]. In this review, we focus on the metabolic effects of the most abundant (in the colon and systemic circulation) gut-derived metabolite, acetate, which may improve the obese insulin resistant state through various effects in peripheral tissues that collectively improve body weight control and insulin sensitivity.

We provide an overview of recent literature on dietary sources of acetate, gut-derived acetate production/absorption after fiber fermentation and pre- and probiotics that may increase plasma acetate through colonic fermentation. We will

discuss the available literature on the effect of acetate on body weight control (central effects of appetite regulation and satiety hormones and energy expenditure), as well as its role in insulin sensitivity in the context of the metabolic inter-organ cross-talk between skeletal muscle, liver, and adipose tissue metabolism [38-41]. Additionally, acetate effects on insulin secretion will be discussed. With respect to acetate effects on cardiovascular health, data is limited and the role of the gut microbiome on cardiovascular health has been reviewed elsewhere [42, 43]. Lastly, we discuss potential therapeutic approaches to improve insulin sensitivity and metabolic health, including oral acetate administration (vinegar intake), acetogenic fiber and probiotic supplementations.

2. Dietary sources and gut-derived acetate production and absorption

2.1. Acetate from dietary sources

According to the Codex General Standard for Food Additives [44], acetate is present in dietary components as acidity regulator (pH control agent), preservative or sequestrant. For instance, acetate-containing foods include dairy products, dried pastas, bread, liquid eggs, salt substitutes, coffee, coffee substitutes, processed meat and smoked/frozen fish [44]. Other important sources are ethanol [45] and vinegar [46]. Commonly consumed vinegars contain between 4 and 8% of acetic acid, and vinegar ingestion has gained attention because of its acute effects in glucose and lipid metabolism as extensively reviewed by Lim *et al.* [46].

Oral ingestion of vinegar rapidly increases circulating acetate as observed in healthy participants that increased serum acetate levels from 120 μmol/l during placebo conditions up to 350 μmol/l (after 15 min) and 200 μmol/l (after 30 min) after vinegar (100 ml containing 0.75 g acetic acid) and acetic acid capsules (containing 0.75 g of acetic acid) intake, respectively [47]. Acetic acid is a bioactive component with a dominant flavor in different types of vinegars including cider, malt, plum, sherry, tomato and wine vinegar [48]. In addition, vinegars may contain other polyphenol residual components (i.e. gallic acid, catechin) such as in apple cider, grape, sherry and Balsamic vinegar [48]. Therefore, it is important to consider the vinegar type since their composition of phenolic, flavonoid and acetic acid content may differ [49].

In general, various dietary products such as preservatives, acidity regulators, food substitutes, ethanol and vinegar may provide acetate orally. In particular, vinegar may provide rapid increments in plasma acetate levels due to its fast absorption in the upper digestive tract (See Vinegar administrations in humans). However, future vinegar supplementations should specify detailed composition including acetic acid percentage and polyphenols content.

2.2. Microbial-derived acetate production

Microbial-derived acetate production is yielded by the fermentation of indigestible foods in particular of acetogenic fibers (e.g. galacto-oligosaccharides, inulin) [50]. In postprandial conditions, acetogenic fibers can be fermented and may elevate production of acetate in the proximal colon (See acetogenic fibers in human studies) [51]. When acetogenic fibers reach the colon, acetate is mainly generated by the microbial community via two metabolic pathways: acetogenesis and the carbon fixation pathway [52]. Acetogenesis is the production of acetate, mediated by homoacetogenic bacteria or acetogens (found in the digestive tract of humans and ruminants) which are capable to produce acetate from H_2 and carbon dioxide (CO_2) [53]. As part of acetogenesis, the carbon fixation pathway (also known as Wood-Ljungdahl pathway) may also produce acetate from CO_2 as precursor [52].

In addition, acetate may originate from microbial fermentation of residual peptides and fats [54-56]. For instance, HFD-fed rats (60% fat) showed elevated colonic and whole-body acetate turnover together with a shift on the phylum level (increased *Firmicutes*/*Bacteroidetes* ratio) [57]. Additionally, microbiota transplant of HFD-fed to germ-free rats increased acetate turnover [57]. Of note, in a Western diet (low fiber intake) protein fermentation occurs mainly in the distal colon where saccharolytic substrates are depleted [58] and this produces other compounds of toxic nature such as ammonia, amines, phenols and sulfides [59]. Branched chain and aromatic amino acids may be produced and further metabolized via cross-feeding mechanisms and alter gut integrity and impair insulin sensitivity [60]. In summary, gut-derived acetate production is determined by the balance between saccharolytic and proteolytic fermentation, in particular by the presence of acetogenic fibers and it is tightly regulated by the intricate interplay within the microbial community.

Although gut-derived acetate production is expected to be low during states of low presence of fibers such as fasting, some studies suggest a possible contribution of fasting-induced alterations in the gut microbiota to fasting acetate concentrations [61]. This was accompanied by an increase in *Firmicutes*/*Bacteroidetes* ratio and cross-feeding mechanisms as evidenced by an upregulation of pyruvate fermentation pathways to acetate and lactate by *Lactobacillus reuteri* and other unclassified bacteria [61]. In support, human fasting and caloric restriction interventions have described an increase in microbial diversity and *abundance* of important acetate producers, such as *Akkermansia muciniphila (A. muciniphila)* and *Bifidobacterium* [62, 63].

2.3. Colonic and systemic acetate concentrations

Acetate concentrations in the colon start with the highest levels in the caecum (69 mmol/l) and ascending colon (63 mmol/l), followed by a subsequent decrease in the transverse (57.9 mmol/l), descending (43.5 mmol/l) and sigmoid colon (50.1 mmol) (measured by kg of intestinal luminal contents) as observed in sudden death victims [22]. This progressive decline along the colon suggests that

major acetate production and absorption occurs in the proximal colon. In general, microbially produced acetate in the proximal colon may follow a colonic-hepatic-periphery distribution starting with colonic levels in the mmol/l range followed by a significant drop, around 10-fold in the liver and reaching the periphery in the μmol/l range (See Table 1. Circulating acetate in humans) [22].

Table 1. Circulating acetate in humans.

Condition	**Site and average (SEM) concentrations**	**Population**	**Study**
Fasting	Superior mesenteric vein 50.4 ± 11.3 μmol/l Inferior mesenteric vein 102.7 ± 27.2 μmol/l Portal vein 41.4 ± 7.8 μmol/l Hepatic vein 23.6 ± 4.8 μmol/l Radial artery 21.8 ± 7.6 μmol/l	Healthy/Overweight upper abdominal surgery patients (54-75 yr.)	Neis *et al. (2018)* [64]
Fasting	Peripheral vein 44 ± 4.4 μmol/l	Healthy/Ileostomy (56-80 yr.) patients	Scheppach *et al. (1991)* [65]
Fasting	Small intestine 77.6 ± 3.23 mmol/kg Large intestine 53.72 ± 9.87 mmol/kg Portal vein 258 ± 40.13 μmol/l Hepatic vein 115 ± 28.20 μmol/l Peripheral vein 70 ± 18.55 μmol/l	Sudden death victims (16-89 yr.)	Cummings *et al. (1987)* [22]
Fasting	Peripheral vein 53.8 ± 4.44 μmol/l Peripheral artery 125.6 ± 13.4 μmol/l	Healthy patients (19-41 yr.)	Pomare *et al. (1985)* [51]

Abbreviations: SEM, standard error of the mean

Interestingly, a recent study that sampled acetate simultaneously from different colonic sites (proximal, distal), inferior/superior mesenteric veins (IMV/SMV), portal/hepatic vein and radial artery in patients undergoing surgery found acetate release was the highest in the IMV (102.7 ± 27.2 μmol/l) and lowest in the radial artery (21.8 ± 7.6 μmol/l). They reported a correlation of arterial acetate concentrations with those in the IMV (r^2=0.65, $p<0.01$), not with SMV, and much lower concentrations in hepatic and portal veins (23.6 ± 4.8 μmol/l and 41.4 ± 7.8 μmol/l, respectively). Collectively, this suggested a greater release from the distal colon [64]. This highlighted the distal colon as a potential location to promote acetogenic fiber fermentation and/or as the location to test the effectiveness of a fiber supplementation in future studies.

A rodent study identified increments in circulating acetate concentrations after intraperitoneal infusion and oral gavage, but not after acetate administration in drinking water [66]. Nevertheless, some studies that provided acetate in drinking water have reported beneficial effects [67-69]. However, the study [66] suggests that acetate effects/kinetics and clearance may depend on administration site of exogenous acetate. However, it should be mentioned that this study did not measure acetate with the gold standard technique (electron ionization gas

chromatography mass spectroscopy) instead it used a commercially available colorimetric kit that allowed the measurement of multiple time points (each measurement required only ~1 μL of plasma) [66]. In summary, acetate production and colonic acetate release may vary along the colon. Thus, the site of fermentation and acetate production may be an important determinant of circulating concentrations.

2.4. Prebiotics and bacterial acetate producers

2.4.1. Prebiotic *in vitro* studies

First, using an *in vitro* system mimicking colonic digestion [70] researchers reported that fermentation of starch entrapped microspheres, fructo-oligosaccharides (FOS) and Psyllium led to substantial *in vitro* acetate production at 24 h and 48 h (140 and 211, 187 and 231, 178 and 219 μmol/50 mg carbohydrate, respectively). However, corn bran arabinoxylans in the same system showed a higher acetate production after 24 and 48 hours (222, 284 μmol/50 mg carbohydrate, respectively) [71]. Furthermore, using the proximal large intestine *in vitro* TIM-2 model (developed by TNO, Zeist, The Netherlands), the addition of the acetogenic fiber galacto-oligosaccharides (GOS) for 72 h increased total SCFA (231 vs 144 control mmol/l, respectively) and acetate production (+49%) to approximately 500 μmol /50 mg of carbohydrates [72]. Another *in vitro* study, comparing FOS vs. inulin mixtures with various degrees of polymerization (DP) reported that a mixture of inulin (90-94% DP>10 and 6-10% DP=1-2) showed the greatest acetate levels at 24 h (25.1 mmol/l) and a mixture of Oligofructose (>93.2% DP<10 and <6.8% DP=1) at 4 h increased the levels (39.7 mmol/l), however not reaching significance [73]. Similarly, a comparison of inulin (DP 3-60) versus Oligofructose (DP 2-20) with various DP using the Simulator of Human Intestinal Microbial Ecosystem (SHIME) *in vitro* model, reported that inulin with longer chain lengths (i.e. Chicory) showed a slower breakdown, more beneficial changes in microbial community (both proximal and distal), higher acetate production and higher bifidogenic effect in the distal colon than oligofructose [74]. In addition, the glycosidic bond configuration of dietary fibers may affect acetate production [75]. For instance, different glycosidic bonds in the β orientation led to differences in acetate production (diglucose β 1-6 vs diglucose β 1-4, 3.7 ± 0.3 vs 1.9 ± 0.2 mmol/g carbohydrate/day, P= 0.001) but it did not differ between fibers with various glycosidic bond configurations (α and β) [76].

With respect to timing, a recent *in vitro* study concluded that fermentation was delayed more when a mixture (arabinoxylan, chondroitin sulfate, galatomannan, polygalactunoric acid and xyloglucan) rather than when the individual fibers were provided. Of note, they suggested that mixtures of fibers may promote the late fermentation of soluble fibers in the distal colon and prevent deleterious effects of proteolytic activity [77]. Collectively, these studies have demonstrated, by the use of *in vitro* fermentation models, the potential of prebiotic fibers as well as the importance of fiber characteristics (i.e. DP, glycosidic bond

configuration, orientation, chain length) to produce acetate. However, future studies are needed to investigate whether these results on delayed colonic acetate production can be replicated in humans and to what extent acetate is further absorbed and metabolized in humans.

2.4.2. Bacterial acetate producers

Acetate production is widely distributed within the bacterial community, as many bacterial species are acetate producers [78]. However, some species seem to be major acetate producers. For instance, humanized gnotobiotic mice that were co-colonized with *Bacteroides thetaiotaomicron/methanobrevibacter smithii* reported higher serum acetate levels as compared to *Bacteroides thetaiotaomicron* alone [79]. In overweight/obese individuals, a positive association between fecal *A. muciniphila* and serum acetate (r^2=0.36 p=0.01) at baseline was described, however, 6-week caloric restriction resulted in decreased serum acetate levels and disappearance of the associations which indicated sensitivity to diet changes [63]. Of importance, differences in abundances of species along the colon may contribute to differences in acetate production between the proximal and distal colon [80, 81]. According to the SHIME *in vitro* model, *A. muciniphila* colonization preferred high pH (6.6-6.9) and high concentration of mucin, which are more abundantly present in the distal compared to the proximal colon [82]. In support, murine studies have reported higher abundance of *A. muciniphila* in the colon as compared to the ileum [83, 84]. Moreover, *in vitro* models that mimic the human colon reported highest concentration of *A. muciniphila* in the transverse compartment, followed by the descending compartment and no detection in the ascending colon [85]. Further discussion on important acetate microbial producers is continued under therapeutic approaches (See Probiotics, body weight control and insulin sensitivity).

3. Acetate in control of body weight

Acetate administration or microbially-derived acetate may have an impact on body weight control through effects on energy intake as well as energy expenditure. In a 12-week intervention in HFD-fed mice, Den Besten *et al.* [86] found that oral sodium acetate supplementation incorporated into the diet (at 5%, wt./wt. proportion) resulted in a suppression of HFD-induced weight gain (~30%) compared to control mice fed a HFD [86]**.** Similarly, Lu *et al.* [87] showed that 16-weeks of oral sodium acetate supplementation (5%, wt./wt.) suppressed HFD-induced weight gain by 72% ($p<0.05$) in comparison to control mice fed a HFD. In addition, a 6-week intragastric administration of acetic acid (50, 250 mmol/l) to HFD-fed mice reduced weight gain (7, 8%, respectively) and body fat accumulation in comparison to HFD alone [88].

In contrast, Perry *et al.* [57] described that both continuous intragastric acetate infusions (10 days, at a rate of 20 μmol kg^{-1} min^{-1}) and a HFD (3 d and 4 weeks) in rats led to similar increased systemic ghrelin, gastrin concentrations and glucose

stimulated insulin secretion (GSIS) that collectively promoted hyperphagia and energy retention that ultimately caused weight gain. Importantly, this elegant rodent study provided valuable mechanistic insight in acetate role in metabolic health although conflicting with other important rodent studies [86-89]. The differences might be related to the species and phenotype of animals used (i.e. Sprague-Dawley rats versus C57BI/6J mice) or administration site (intragastric versus oral or colonic administration). This further highlights the need to investigate metabolic effects of an increased whole-body acetate turnover in humans. Of note, in contrast to the outcomes of Perry *et al*, a recent human study conducted by the same group failed to corroborate these findings, reporting a higher acetate turnover in lean (~30%) versus obese individuals and no effect of increased circulating acetate levels (intravenous infusion) on ghrelin and GSIS in obese individuals [90]. These important data showed that there are important differences across species and between modes of administrations.

In humans, long-term oral acetate supplementation or intravenous/gastric/colonic infusion studies with weight loss and energy expenditure as the primary outcome are limited (See Acetate effects on energy expenditure). Cross-sectional/cohort analyses have shown inconsistent results with obesity and adiposity. For instance, one study showed that fasting acetate correlated negatively with visceral adipose tissue mass in obese women [91]. In contrast, in obese men and women a positive association (r^2= 0.11, P=0.004) of fasting plasma acetate (0-2 μmol/l range) with degree of adiposity at baseline including total body, visceral and subcutaneous fat (magnetic resonance imaging) was observed independent of age, sex and ethnicity [92].

In addition, baseline fasting plasma acetate positively predicted changes in adiposity (delta BMI per year) after a 2.2 $\pm$ 1.7-year follow-up time independent of baseline BMI, age, sex and ethnicity. Additionally, fasting plasma acetate levels correlated positively with *de novo* fasting hepatic lipogenesis (measured by hepatic palmitate incorporation) [92]. As mentioned above, long-term oral acetate supplementation studies in humans are lacking and data on the role of acetate in body weight management are mainly based on animal data and human cross-sectional data. The above discrepancies highlight that acetate effects on body weight control may depend on mode of administration, metabolic phenotype and species-specific differences in acetate metabolism. In the following paragraphs, we discuss the putative mechanisms of how acetate might influence body weight control via central nervous system mechanisms and gut-derived hormones as well as via effects on energy expenditure.

3.1. Acetate and central effects on appetite regulation

Appetite regulation is coordinated by nutrients and microbial metabolites through the central nervous system circuitry and circulating hormones from peripheral tissues [93]. Of note, acetate has been reported to cross the blood-brain barrier in both mice [89] and humans [94]. Additionally, acetate has been detected in the cerebrospinal fluid, thus suggesting a central homeostatic mechanistic role [95]. In support, Frost *et al.* [89] described an association of elevated colonic acetate and a direct role of acetate on appetite regulation in HFD-fed mice supplemented for 8 weeks with acetogenic oligofructose-enriched inulin. Acetate accumulation in the hypothalamus, was shown to affect appetite regulation through the glutamate-glutamine transcellular cycle which resulted in increments in lactate and gamma aminobutyric acid (GABA) production, after both intraperitoneal acetate injections and after colonic fermentation of ^{13}C-labelled carbohydrate [89]. Interestingly, mice showed a peak in serum acetate levels (350 μmol/l) at 10 min after an intraperitoneal acetate injection (500 mg/kg^{-1}), that was associated with changes in the expression of neuropeptides (AMPK and acetyl CoA carboxylase (ACC)) that regulate appetite suppression [89]. Hypothalamic acetate administration showed inactivation of AMPK and activation of ACC (via decreased phosphorylation), suggesting that acute administration of acetate may increase hypothalamic ACC activity. ACC activation may increase malonyl-CoA, which may lead to a reduction in food intake through the expression of orexigenic and anorexigenic neuropeptides in the hypothalamus via two mechanisms: (i) via the interaction of malonyl-CoA with signaling proteins or (ii) via inhibition of carnitine/palmitoyl-CoA transferase that prevents the entry of the long-chain fatty acids to the mitochondrion [96, 97]. Of note, these data are of associational nature.

Therefore, to assess a causal role of these pathways and to further study the role of acetate on appetite and satiety regulation animal models (i.e. MC4R-/- mice) might be used.

Interestingly, acetate signaling in the brain may follow a hepatic-portal-vagal route. However, vagal activation and effects on body weight control may depend on site of administration and use of different animal models [98]. For instance, Perry *et al.* [57] reported that both a HFD and continuous intragastric infusions of acetate increased central vagal activation, including a vagal-induced release of ghrelin from the stomach that led to a metabolic syndrome phenotype in rats as explained above. In mice, an intraperitoneal acetate injection (6 mmol/kg) significantly reduced food intake (0.5 and 1 hour after administration) in a vagal dependent manner since vagotomy attenuated the effect [99]. Although these studies may imply that vagal activation in response to acetate are contradictory, it is important to note that the site of administration and use of different animal models may explain the difference in vagal activation. In addition, energy intake may also regulate vagal activation by gut hormones (GLP-1, glucagon like peptide 1 and

peptide YY, PYY), as vagotomized humans showed impaired effects of exogenous GLP-1 on food intake [100].

In summary, acetate may regulate appetite possibly through central hypothalamic mechanisms and satiety through acetate-induced or gut hormone-induced vagal activation. However, due to the lack of human evidence and the inconsistency in animal data, further research should elucidate the exact underlying mechanism.

3.2. Gut-derived satiety hormones

As indicated above, gut-derived satiety hormones can be secreted from enteroendocrine cells located in the gut [101]. Enteroendocrine cells, in particular the L-cells, secrete GLP-1 and PYY hormones that seem to play an important role in gut health and in the connection of the gut-brain axis [102]. Notably, both acetate receptors GPR43 [23] and GPR41 [24] are expressed at mRNA and protein level in enteroendocrine cells in the human colonic mucosa, thus potentially indicating an acetate-mediated effect in their secretion [103]. In support, *in vitro* culturing of enteroendocrine cells (SCT-1 cell line) with acetate (3 and 30 mmol/l) for 24 h showed increased expression of proglucagon (GLP-1 precursor) in a dose-dependent manner [104]. In a mice study, inulin supplementation protected from HFD-induced obesity, which was dependent on an increased PYY secretion in a GPR43 dependent manner [105]. In addition, dietary fiber supplementation in rats with resistant starch increased plasma levels of GLP-1 and PYY in the short term (24-hour period) [104]. Similarly, rats supplemented with resistant starch (30-day period) increased plasma levels of GLP-1 and PYY and increased caecal acetate [106]. Using inulin type fructans, Cani *et al.* [107] demonstrated that a 3-week oral supplementation in rats increased GLP-1 and decreased ghrelin plasma levels, possibly via colonic SCFA production, in particular of acetate. In addition, feeding of dogs with a mixture of high fermentable fibers for 14 days increased plasma GLP-1 concentration 15 min after an oral glucose load, however, systemic acetate was not measured [108].

Importantly, GPR41 has been reported in other enteroendocrine cell types (secretin and neurotensins types) in the duodenum and proximal colon of mice, respectively [25]. However, whether acetate can induce a GPR41-dependent secretion of these appetite-suppressing hormones needs further investigation. In humans, production of gut-derived satiety hormones in response to prebiotic supplementation is scarce and inconclusive. For instance, Rahat-Rozenbloom *et al.* [109] reported an increase in colonic acetate but no increase in serum levels of satiety hormones (GLP1, PYY) in overweight individuals after inulin ingestion (single dose 24 g) in comparison to glucose as control. In contrast, oligofructose supplementation (21 g/day) for 12 weeks in overweight/obese individuals increased the area under the curve (AUC) of PYY, and decreased AUC of ghrelin secretion.

This coincided with a reduction in self-reported caloric intake and was associated with a significant weight loss (1.03 $\pm$ 0.43 kg) as compared to maltodextrin [110]. In addition, oligofructose (35 g/d) in lean individuals, increased

postprandial PYY concentrations [111]. Importantly, these human prebiotic interventions did not measure fecal/systemic acetate. Another approach to investigate secretion of gut-derived hormones is through colonic acetate infusions.

Interestingly, infusions in humans in different sites may elicit differential effects on gut-derived satiety hormones. For example, rectal (60 mmol/l) sodium acetate administrations increased PYY and GLP-1 significantly as compared to intravenous infusions (acetate 20 mmol/l and saline) [112]. Similarly, distal (not proximal) colonic infusions of sodium acetate (180 mmol/l) increased PYY in overweight/obese men and only 180 mmol/l (not 100 mmol/l), elicited these effects [39]. Moreover, rectal/intravenous infusions showed the potential to induce gut-derived hormone secretion (GLP-1 and PYY) in a metabolically disturbed phenotype [113]. Together, these distal colonic infusions studies suggest the relevance of the site of administration or fermentation (distal versus proximal colon) to modulate gut hormone secretion. Of note, a higher density of PYY producing cells in the distal colon in rodent studies [114, 115] may explain the differential effect on hormonal secretion dependent on site of fermentation.

Acetate may also increase the secretion of leptin from the adipose tissue. For instance, in a rodent study, acetate (860 μmol/l) increased transcription of the leptin gene, and propionate (78 μmol/l) showed even stronger effects [116]. In bovine adipocytes, acetate (1 mmol/l) increased leptin expression by ~60%, which was inhibited by pertussis toxin, indicating GPR dependence [117]. Further studies have to show the relevance of this mechanism under *in vivo* conditions. In summary, animal and *in vitro* data studies have shown the potential of acetate to increase gut hormone secretion, however, dietary fiber supplementation in humans have shown inconsistent results. Future human trials may aim to target distal colonic fermentation since distal colonic acetate infusions have shown pronounced increments in gut satiety hormones.

3.3. Acetate effects on energy expenditure

A few studies have investigated the direct effects of acetate on energy expenditure as a primary outcome. Of note, acetate has been related to increments in energy expenditure through various mechanisms in peripheral tissues [41] (See Acetate inter-organ crosstalk and insulin sensitivity in peripheral tissues). Here we discuss the effects of acetate infusions in humans and also in the form of vinegar through oral administrations in the context of body weight control.

3.3.1. Acetate infusions in humans and energy expenditure

In humans, a lipid lowering effect of acetate after rectal [118] and intragastric [119] infusions in healthy subjects almost three decades ago paved the way for future human acetate infusions (See Table 2). Recently, acute colonic infusions showed increments in fasting fat oxidation and energy expenditure in humans [39, 120]. First, distal colonic infusions in normoglycemic overweight/obese individuals increased fasting fat oxidation (1.78 $\pm$ 0.28 vs -0.78 $\pm$ 0.89 g fat 2 h^{-1}, P=0.015), however no

effects on energy expenditure [39]. Another study in the same phenotype, using SCFA mixtures (rich in acetate) reported positive associations of fasting acetate with fasting fat oxidation (r=0.328 P=0.0228) and with resting energy expenditure (r=0.349 P=0.0149) [120]. In contrast, a study in healthy and T2DM subjects, acetate intravenous infusions (2.5 mmol per min for 1 h) did not increase energy expenditure, which was partly explained by the fact that acetate might replace long chain fatty acids as preferred oxidation fuel [121]. Collectively, these studies suggested an acetate-mediated beneficial role in substrate utilization and energy expenditure in humans, however, results are inconclusive and further research is needed. In addition, the beneficial effects after distal colonic (not proximal) acetate infusions [39], together with the higher acetate release in distal colon [64] strengthened the notion that targeting distal colonic site might increase the metabolic health effects.

Table 2. Effects of sodium acetate infusions in humans at different administration sites

Dose	Primary outcome	Subjects	Administration site	Effects on lipid/glucose metabolism	Study
SCFA infusions mixtures rich in acetate (24 mmol/l acetate, 8 mmol/l propionate and 8 mmol/l butyrate) and propionate (18 mmol/l acetate, 14 mmol/l propionate and 8 mmol/l butyrate)	Fat oxidation and Energy expenditure	Overweight/ obese men (n=12)	Colonic infusions	Attenuation of whole-body lipolysis ↑ Fat oxidative capacity Fat oxidation and energy expenditure related to increments in fasting acetate ↑ Fasting and postprandial PYY No effects on insulin and glucose	Canfora *et al.* 2017 [120]
180 mmol/l sodium acetate	Fat oxidation and energy expenditure	Overweight/ obese men (n=6)	Proximal and Distal colonic	↑ Fasting fat oxidation ↑ Postprandial glucose and insulin Tendency to decrease TNF-α Fasting peptide YY	Van der Beek *et al. (2016)* [39]
140 mmol/l in 90 min sodium acetate	Peripheral uptake	Overweight normoglycemic and hyperglycemic subjects (n=9 vs 9)	IV	No difference in acetate clearance between individuals with normal (NI) and high (HI) insulin levels. ↑ FFA rebound in NI than HI.	Fernandes *et al.* (2012) [113]
60 mmol/l (rectal), 20 mmol/l (intravenous) Sodium acetate	Gut-derived hormone secretion	Hyperinsulinaemic females (n=6)	Rectally and IV	↑ in PYY/GLP-1 after rectal infusions and decrease in TNF	Freeland *et al.* (2010) [112]
12 mmol/l per hour	Hepatic glucose production	Healthy subjects (n=6)	IG	↓ Circulating FFA No effect on hepatic glucose production	Laurent *et al. (1995)* [119]
800 ml rectal infusions with 180 mmol/l	Glucose homeostasis	Healthy subjects (n=6)	Rectal infusion	No effects on insulin and glucose ↓ Circulating FFA	Wolever *et al.* (1989) [118]

Abbreviations: SCFA, short chain fatty acids; PYY, peptide YY; GLP-1, glucagon like-peptide 1; TNF-α, Tumor necrosis factor; FFA, free fatty acid; IV, Intravenous; IG, Intragastric.

3.3.2. Vinegar administrations in humans

Studies related to vinegar effects on body weight and energy expenditure in humans are limited. Nevertheless, a few studies have reported effects on body weight. For instance, a study in individuals with obesity, a 12-week vinegar intervention significantly lowered body weight with low (0.75 gr) and high (1.5 gr) acetate doses versus placebo (0 gr), in a dose-dependent manner [122].

In addition, a crossover study in overweight-obese subjects, the consumption of Kimchi (fermented Korean dish, unclear % of acetic acid) vs unfermented dish reduced body fat (~1%), body weight (~ 1.5 kg) and BMI (0.6 kg/m^2) after 2 weeks [123]. In a similar crossover study, in overweight women, fermented Kimchi (unclear % of acetic acid) decreased *Firmicutes*/*Bacteroidetes* ratio [124] which has been associated with weight loss [125]. In HFD-fed mice, supplementation of synthetic acetic acid (4%) and high dose of Nipa vinegar (unclear % of acetic acid) reduced lipid deposition, inflammation and improved serum lipid profiles in comparison to control. Both vinegars decreased *Firmicutes*/*Bacteroid*etes ratio and increased the relative abundances of various bacterial species including *A. muciniphila* and *Lactobacillus* among other potential acetate producers [126]. With respect to differences in acetate infusions (sodium acetate) versus vinegar (acetic acid) administrations, both the route and absorption may differ. Saunders *et al.* [127], reported that oral acetic acid administration was more rapidly absorbed in the stomach compared with sodium acetate administrations, possibly through a pH-dependent mechanism, as acetic acid (unionized acetate) absorption increased when gastric pH decreased.

Although, in previous human and rodent studies the effects were attributed to acetic acid, either its content (%) or the presence of other bioactive components is unclear. In addition, fermented dishes (e.g. Kimchi) may have other volatile and non-volatile compounds and overall composition can vary depending on fermentation time and storage room temperature [128]. In general, acetate may modulate body weight control through different mechanisms that can affect central appetite regulation, gut-satiety hormones as well as improvements in lipid metabolism and energy expenditure. Of note, human evidence is accumulating that acetate may prevent body weight gain and adiposity through increments in energy expenditure as observed in following acute colonic distal infusion studies. Importantly, there is a lack of longer-term human studies that investigate acetate effects on energy expenditure as primary outcome. In the next section, we discuss acetate effects in the peripheral tissues that collectively may improve insulin sensitivity (See Table 3).

4. Acetate and the inter-organ crosstalk and insulin sensitivity in peripheral tissues

Adipose tissue is the main organ for triacylglycerol storage in the human body and an active endocrine regulator of energy homeostasis, therefore metabolic derangements in adipose tissue function contribute to pathophysiology and dysregulation of glucose homeostasis and whole-body insulin sensitivity [129].

4.1. Acetate and vinegar studies and insulin sensitivity

Of note, human acute acetate infusions have shown inhibitory roles in whole-body lipolysis, increase in gut-hormone release and increase in fat oxidation and energy expenditure among other effects (See Table 2). Collectively, these effects may improve adipose tissue lipid buffering capacity, satiety regulation, oxidative capacity and in turn improve whole-body insulin sensitivity and peripheral tissue functioning. In addition, vinegar administrations have reported improvements in glucose homeostasis and insulinemic profiles (See Table 3). Moreover, we discuss vinegar effects on glucose homeostasis and insulinemic profiles with potential T2DM treatment applications [46].

As mentioned above, oral vinegar (4-8% acetic acid) administrations may rapidly increase circulating acetate and its co-ingestion with carbohydrates (50-75 gr) seems more effective for glucose lowering and insulinemic responses. In contrast to colonic sodium acetate infusions, oral vinegar administrations have shown improvements in glucose homeostasis and insulin profiles in healthy subjects [46, 130, 131]. For instance, supplementation of acetic acid (unspecified vinegar) in healthy subjects together with a test meal resulted in reduced postprandial glucose concentration (~35%, during 30-70 min), putatively through a delayed gastric emptying [130]. Similarly, white vinegar (6% acetic acid) administrations (18, 23 and 28 mmol/l) in combination with white wheat bread (50 gr) in healthy subjects lowered glycemic (highest dose at 30-45 min) and insulinemic (highest dose at 15-30 min) postprandial responses [132]. In addition, acetic acid lowered the glycemic index (GI) and increased the satiety score postprandially at 30, 90 and 120 min using a subjective rating scale [132]. Another study in healthy subjects, reported that a vinaigrette (28 g white vinegar, 6% acetic acid) on a potato meal reduced GI and insulinemic index (43 and 31%, respectively) [133].

Furthermore, vinegar administration studies in individuals with metabolic alterations (i.e. impaired glucose tolerance IGT, T2DM) have been performed [46]. For instance, a study in individuals with IGT showed that wine vinegar (6% acetic acid) administration decreased arterial plasma insulin (by 33%) and increased muscle glucose uptake (by 35%) after a meal test as compared to placebo (50 ml water) [134]. Additionally, in T2DM individuals, oral wine vinegar administration (1.2 g acetic acid) decreased $iAUC_{120min}$ of glucose (41%) only after a high GI meal test (mashed potatoes and low-fat milk) but not after a low GI meal test [135].

However, these reported beneficial effects of vinegar on glucose homeostasis in metabolically compromised individuals have not been confirmed in all studies. A study in T2DM individuals using white vinegar (1 g acetic acid) did not show any effect on postprandial glucose levels after an oral glucose load (75 g) [136]. In summary, acetate infusions and vinegar administrations have reported beneficial effects on glucose homeostasis and potentially on insulin sensitivity. However, inconsistencies exist and differences between phenotypes require further research.

Table 3. Vinegar administrations in humans.

Dose, vinegar type	**Primary outcome**	**Subjects**	**Effects**	**Study**
Unspecified vinegar	G&IR	Healthy (22-51 yr.), 7 females (n=10)	↓ 35% postprandial glucose Delayed gastric emptying	Björck *et al.* 2005 [130].
White vinegar 6% acetic acid	G&IR	Healthy (19-27 yr.), 10 females (n=12)	↓ Glucose and Insulin	Dimitriadis *et al.* (2015) [132].
White vinegar 6% acetic acid	G&IR	Healthy (19-32 yr.), 10 females (n=13)	↓ Glycemic index Insulinemic index (43 and 31%, respectively)	Diakoumoupolou *et al.* (2010) [133].
Wine vinegar 6% acetic acid	Muscle glucose metabolism Circulating lipids endothelial function	Individuals with impaired glucose tolerance (26-66 yr.), 4 females (n=8)	↑ Muscle blood flow 33% ↑ Muscle glucose uptake 35%	van Loon *et al.* (2012) [134].
Wine vinegar 1.2 g acetic acid vs placebo	G&IR	TD2M individuals (n=8 vs 8) (40-78 yr.), 4 females	↓ $iAUC_{120}$ Glucose 41%	Crovetti *et al.* (1995) [135].
White vinegar 1 g acetic acid	G&IR	TD2M male individuals (n=12) (63-67 yr.)	No effect	Haldar *et al.* (2016) [136].

Abbreviations: T2DM, type 2 diabetes mellitus; iAUC, incremental area under the curve; G&IR, glycemic and insulinemic response.

4.2. Adipose tissue metabolism

4.2.1. Lipolysis

Acetate administration has been shown to affect whole-body as well as intracellular lipolysis in adipocytes in *in vitro*, and *in vivo* animal and human studies. Ge *et al.* [137] reported that acetate-mediated activation of GPR43 in 3T3-L1 adipocytes was accompanied by an inhibition of the lipolytic response within a physiological range of 100-300 μmol/l [137]. Moreover, a supraphysiological concentration of sodium acetate (4 mmol/l) induced an antilipolytic effect in murine 3T3-L1 adipocytes, via a decrease in phosphorylation of the cytosolic lipase HSL (hormone sensitive lipase) at serine residue 563 (equivalent to Ser 552 in humans) [138]. Similarly, Heimann *et al.* [139] described an antilipolytic effect of acetate, possibly mediated by a decrease in phosphorylation of HSL (at Ser 563) in rat and human primary (at Ser 552) adipocytes with a supraphysiological (10 mmol/l) concentration of acetate [139]. Recently, an *in vitro* study using differentiated human multipotent adipose-derived stem cells (hMADS) showed that acetate (1 μmol/l – 1 mmol/l) decreased basal and isoprenaline-stimulated lipolysis via attenuation of HSL Ser 650 phosphorylation (equivalent to Ser 660 in rats) in a GPR-dependent manner [140].

In mice, an antilipolytic effect of acetate (30% reduction of plasma free fatty acids) was observed after intraperitoneal infusions of sodium acetate (500 mg/kg), which coincided with a rise in plasma acetate after 15 min of infusion (circulating levels reached a range of 0.2-1.0 mmol/l) [137]. In HFD-fed mice, using a nanoparticle delivery system, acetate decreased lipolysis and circulating FFA after intraperitoneal injection [141]. In addition, adipose tissue mRNA expression of adipose triglyceride lipase (ATGL) was reduced.

In the human *in vivo* situation, a handful of studies demonstrated effects on lipolysis on whole-body level. First, Crouse *et al.* [142] showed that orally administered sodium acetate (given in two doses 143 mg/kg initially and 71 mg/kg 30 min later) increased plasma acetate by 3 to 4-fold and decreased FFA plasma concentrations by 25% in healthy humans within 20 min after ingestion. Second, in healthy young subjects, an intragastric infusion of sodium acetate (12 mmol/l) significantly decreased the total AUC of circulating FFA in comparison to saline infusions during five hours after infusion [119]. Third, rectal infusion of high dose sodium acetate (180 mmol/l) decreased serum FFA in comparison to saline infusions in healthy subjects during 2 hours after infusion [118]. Fourth, one-hour intravenous infusion of sodium acetate in healthy individuals increased plasma acetate from 0.19 to 0.99 mmol/l and inhibited whole-body lipolysis as evidenced by the decrease in plasma glycerol and FFA concentrations [143].

Thus, both animal and human data show, an antilipolytic effect of acetate that may decrease lipid overflow to peripheral insulin-sensitive tissues (e.g. skeletal muscle) thereby possibly improving insulin sensitivity and decreasing hypothalamic inflammation.

4.2.2. Adipogenesis and browning of adipose tissue

Acetate may affect the proliferation and differentiation of adipocytes, thereby contributing to adipose tissue morphology, browning and function, which can induce high thermogenic activity with the potential to enhance oxidative capacity [141]. Hereby, we discuss adipogenic/browning effects of acetate mainly derived from *in vitro* and animal data. First, a 7-day acetate incubation (0.1 μmol/l) of 3T3-L1 pre-adipocytes increased mRNA levels of GPR43 and peroxisome proliferator-activated receptor gamma (PPARγ), which is a master regulator of adipogenesis, suggesting a modulatory effect in adipocyte differentiation [144, 145]. In support, incubation of immortalized mice brown adipose tissue cells with a supraphysiological concentration of acetate (10 mmol/l) enhanced mRNA expression levels of *PPARγ*, and the browning markers peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (*PGC-1α*) and uncoupling protein 1 (*UCP1)* [146]. In another *in vitro* study in 3T3-L1 pre-adipocytes, acetate (1 mmol/l) modulated the gene expression profile with an increase in the mRNA levels of *UCP1, PPARα*, and *PPARγ*, PR domain containing 16 protein (PRDM16) and cell death-inducing DNA fragmentation factor-a-like effector (CIDEA) as well as in markers of beige adipocytes including transmembrane protein 26 (TMEM26) and T-box protein 1 (TBX1) [147]. Importantly, *in vitro* effects were replicated in KK-Ay mice after a 16-week oral administration of acetate (0.12 g/day), however only in epididymal adipose tissue and not in inguinal or brown adipose tissues [147].

In line, several animal studies reported comparable effects. For instance, a 16-week oral acetate supplementation (5% w/w diet) in HFD-fed mice increased the expressions of several key genes in epididymal adipose tissue possibly mediated via increased *GPR41*/*GPR43* expression. The genes were involved in mitochondrial biogenesis (PGC-1α, nuclear respiratory factor 1, mitochondrial transcription factor a, beta-F1-ATPase, nuclear-encoded subunit IV and cytochrome complex), which were all reduced by the HFD alone **[87]**. Interestingly, in HFD-fed mice, acetate (injected intraperitoneally in a nanoparticle) increased PRDM16 mRNA expression in white adipose tissue (WAT) and increased thermogenesis **[141]**.

Recently, intermittent fasting treated mice (every other day fasting, EODF) significantly induced the expression of browning markers (*UCP1)* in subcutaneous inguinal WAT which was proposed to occur through gut-derived acetate since browning depended on gut microbiota depletion and transplantation [61]. In support, both colonic and serum acetate levels were significantly increased after both short-term and long-term intermittent fasting (3 and 15 cycles of 24 hours, respectively), suggesting an acetate-mediated browning effect [61]. Although human data are scarce, a recent cross-sectional study in morbidly obese individuals reported that elevated circulating acetate levels were positively correlated with mRNA expression of the browning marker PRDM16 in abdominal subcutaneous adipose tissue, and improved insulin sensitivity (2-h euglycemic hyperinsulinemic clamp) and changes in gut microbiota composition (i.e. increased *Firmicutes* abundance) **[148]**. In summary, although the human evidence is limited, acetate seems to play a role

in adipogenesis and browning of WAT and together with the antilipolytic effects, acetate may restructure adipose tissue morphology and improve adipose tissue functioning and energy metabolism and thereby overall metabolic health.

4.2.3. Adipose tissue inflammation

Adipose tissue has been recognized as an important modulator of local and systemic low-grade inflammation, which is often associated to the pathophysiology of obesity and development of insulin resistance [149]. Therefore, metabolic cues from the adipose tissue can increase the recruitment, infiltration and activation of immune cells that can promote a proinflammatory secretory profile (low-grade inflammation) [150]. A pro-inflammatory secretory profile is partly characterized by increased pro inflammatory macrophages (M1), less anti-inflammatory macrophages (M2) and T-cell polarization [151]. Here, we discuss acetate-mediated effects on gut-derived metabolic endotoxemia and local adipose tissue inflammation.

Under different pathological conditions, a leaky gut may occur and result in metabolic endotoxemia, characterized by high circulating lipopolysaccharide (LPS) levels which can potentially lead to chronic low-grade inflammation, as often observed in obesity and insulin resistance [152]. Gut-derived acetate may affect gut health via an improvement in intestinal barrier function through cross-feeding mechanisms (i.e. increased butyrate concentration) [153]. In an *in vitro* study, acetate (30 mmol/l) decreased LPS-stimulated secretion of tumor necrosis factor (TNF-α) from human neutrophils by ~ 33% ($P<0.01$) [154].

Furthermore, microbially-derived acetate from specific bifidobacteria protected mice against a lethal injection of *Escherichia coli* O157:H7 that can potentially increase LPS [155]. In a 3-week oral treatment in mice, acetate added to drinking water (150 mmol/l) increased the number and function of colonic anti-inflammatory Treg cells in a GPR43 dependent manner [156], suggesting a protective and anti-inflammatory role of colonic acetate. Additionally, *in vitro* data show that acetate has the potential to decrease immune cell infiltration in the adipose tissue. First, incubation of T cells from mice with supraphysiological concentration of sodium acetate (5-20 mmol/l) promoted T cell differentiation towards an anti-inflammatory T cell polarization (both Th1 and Th17), capable to produce anti-inflammatory cytokines such as IL-17, interferon γ and interleukin 10 (IL-10) [157]. Authors indicated that acetate can bypass the cell surface and regulate cells that have low expression of GPR41/GPR43 [157]. These effects were putatively regulated by the activation of mTOR-S6K pathway independently of GPR41/GPR43, since both receptors are not expressed in T cells [68].

In other relevant immune cells, acetate (10 mmol/l) induced TNF-α expression/secretion in M2 but not in M1 macrophages, which showed that acetate may have a different role through the GPR43 receptor [158]. Acetate may also affect CD8 T cells, which play a regulatory role in the initiation of adipose tissue

inflammation, putatively through macrophage differentiation, activation and migration [159]. In mice, abundance of systemic acetate in response to bacterial infections was linked to an optimal function of memory CD8 T cells through an enhanced glycolytic rate and acetylation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [160]. In summary, acetate may have a potential role to counteract a leaky gut by preserving gut integrity and health and through immunomodulating pro-inflammatory mechanisms. Furthermore, these putative acetate mediated effects on immune cells (M2 macrophages and T cells) may improve adipose tissue remodeling/functioning and consequently its function towards homeostasis.

4.3. Acetate and skeletal muscle metabolism

Obesity is characterized by ectopic fat deposition and an altered skeletal muscle glucose and lipid metabolism, which may exacerbate insulin resistance and lead to the development of T2DM [161]. In this section, we discuss the role of acetate on muscle lipid and glucose metabolism in the context of obesity-associated derangements originated by excessive lipid overflow, a systemic low-grade inflammation and ectopic deposition that may increase insulin resistance in the skeletal muscle.

As mentioned above, GPR41/43 are expressed in the human skeletal muscle tissue [27]. Moreover, acetate uptake has been reported in skeletal muscle tissue in rodents [34] and humans [11]. In animals, acetate uptake increased together with increments in the AMP/ATP ratio (~ 2 min) in muscle tissue after oral injection of 10.5 mg/kg of BW, however, the absorption machinery remains unclear [34]. Intracellularly, acetate may be rapidly assimilated and metabolized through the TCA cycle in the mitochondrial matrix [162], contribute to acetyl-CoA pool [163] and/or modulate signaling mechanisms involved in muscle fat oxidation. For instance, an acetate intragastric infusion (5 ml/kg BW) versus water infusion during 6 months in obese rats, revealed an increase in AMPK activity [34]. The putative mechanism is possibly through the catalytic activity of acetyl-CoA synthase that produces acetyl-CoA and increases AMP in the cytosol. Subsequently, this increases AMP/ATP ratio thereby resulting in an increment of AMPK phosphorylation. In addition, myoglobin and glucose transporter 4 (GLUT4) and lipolytic gene expression (LCACD, 3KACT, and PPAR) were upregulated in both the abdominal and the foreleg muscle tissue [34]. Although, no actual substrate utilization was measured, oxygen consumption rate measurements (using metabolic cage) showed 7% higher rates in acetate-treated rats, indicating possible increase in whole-body metabolic rate following intragastric acetate infusion.

Furthermore, in an *in vitro* study, using rat skeletal muscle (L6) cells, acetic acid increased the AMP/ATP ratio and the phosphorylation of AMPK in a dose (0.05-0.5 mmol/l) and time (0-30 min) dependent manner using physiological concentrations [33]. With respect to glucose metabolism, acetate supplementation (0.2 g acetic acid/100 g diet for 2 hours) increased glycogen storage (1.1-fold) and

decreased glycolysis in the gastrocnemius as compared to control (no acetate) in Sprague-Dawley rats [164]. In support, as mentioned above, increments in the protein expression of GLUT4 [34] may suggest a modulatory role in glucose homeostasis. In summary, acetate may modulate skeletal muscle lipid and glucose metabolism possibly through activation (phosphorylation) of AMPK. Whether this affects endogenous intramyocellular triacylglycerol (IMTG) and/or exogenous (dietary) fat oxidation and glucose homeostasis in human muscle remains unclear.

4.4. Acetate and liver metabolism

Hepatic steatosis, if untreated, may progress towards non-alcoholic steatohepatitis (NASH) and aggravate pathophysiology in obesity and comorbidities. Therefore, adequate treatment to tackle hepatic fat accumulation is of utmost importance. As mentioned above, the liver plays a central role in acetate metabolism, since important endogenous production occurs here. Furthermore, acetate may be rapidly metabolized and used as a carbon donor for intracellular pathways including cholesterol biosynthesis, acetylation processes [165] and hepatic palmitate formation [92]. Importantly, the liver is the first organ in direct contact with microbially-produced acetate coming from the ileum and proximal colon.

From a mechanistic perspective both *in vitro* and animal studies have shed light on the role of acetate in liver substrate metabolism. In Fao cells (rat hepatoma cell line), 1-hour incubation with physiological concentrations of sodium acetate (100-200 μmol/l) increased AMPK phosphorylation (pThr 172) by 40% compared to control treated cells [35]. Similarly, after 2-hour oral acetate administration (16.7 mmol/l at 10 ml/kg body weight) in ICR (albino strain) mice, pAMPK and pAMPK/AMPK ratio increased [35]. In the same study, a basic chow diet with acetic acid added to a final concentration of 0.3% for 8 weeks in KK-A (y) mice showed a hypoglycemic effect, lowering of TAG and increased glycogen content in the liver, whilst AMPK phosphorylation was not significantly increased [35]. In HFD-fed mice, supplementation with low (0.3%) and high (1.5%) acetic acid for 6 weeks decreased hepatic lipid accumulation, liver lipids and increments in expression of hepatic genes associated with fatty acid oxidation (UCP2, PPARα, CPT1 and ACOX) [88].

Furthermore, in HepG2 cells, these effects were ablated when cells were depleted of AMPK using siRNA, suggesting an AMPK mediated mechanism [88]. In line with modulatory role of acetate, intraperitoneal injections in rats (dose of 20 mmol/kg body weight) showed an increase in the AMP/ATP ratio in liver extracts after 15 min [166].

Additionally, in HFD-fed mice, intraperitoneal injection of acetate (nanoparticle delivery method), decreased hepatic lipid accumulation, improved hepatic function and increased mitochondrial efficiency [141].

Acetate supplementations may also increase hepatic glycogen synthesis in the muscle as reported in rats [167, 168]. Of note, the acetate:propionate ratio may be of importance for hepatic lipid biosynthesis since propionate may favor odd chain fatty acids while acetate may favor palmitate formation [169]. Moreover, odd chain fatty acids have been linked to improvements in insulin sensitivity [170]. Moreover, in

men it has been suggested that propionate may reduce acetate utilization for liver lipid biosynthesis (fatty acid and cholesterol) [171]. However, acetate has shown antilipolytic effects at the whole-body and adipose tissue level and increases in whole-body fat oxidation (See Adipose tissue metabolism). For instance, a 3-hour intragastric infusion of acetate (equivalent to the fermentation of 30 g of dietary fibers) in healthy subjects decreased plasma FFA, thus possibly improving lipid profiles [119]. Importantly, SCFA reach the liver in different ratios, thus single SCFA may not reflect physiological effects in the lipid profile. In general, *in vitro* and animal studies have provided mechanistic insight into the role of acetate in the liver, where it may increase the AMP/ATP ratio, subsequently increasing AMPK phosphorylation/activity and thereby affecting hepatic lipid (FA oxidation) and glucose (glycogen) metabolism.

4.5. Acetate and insulin secretion in beta cells

Glucose homeostasis is intricately regulated via insulin which is secreted from the beta cells in the pancreas [172]. Therefore, there is no question that modulation of glucose stimulated insulin secretion (GSIS) has an impact on glucose homeostasis [173]. From a mechanistic perspective, murine and human beta cells express GPR (GPR41 and GPR43) [29], via which acetate has reported to regulate insulin secretion [28]. In support to this G protein-mediated signaling, GPR43 deficient mice show glucose intolerance, and reduced beta cell mass and function [174]. Importantly, a study reported that GPR43 signaling pathway may be mediated by divergent G protein pathways that can selectively potentiate ($G\alpha_{q/11}$ signaling can lead to Ca^{2+} mobilization and enhance GSIS) or inhibit ($G\alpha_{i/o}$ signaling can lead to cAMP inhibition and diminish GSIS) insulin secretion in rodents [29]. In addition, acetate at physiological concentrations (70-170 μmol/l) [175] only increased *in vitro* insulin secretion in a murine beta cell line but not in human islets and suggested species-specific differences [29]. However, the model to induce insulin exocytosis via Ca^{2+} mobilization inside the beta cells has been a consensus for a long time, other acetate mediated signals may indirectly contribute to insulin secretion, such as via gut-derived GLP-1 and vagal activation of the parasympathetic nervous system [176]. Of note, GLP-1 may exert a direct effect on beta cells by closing K^+_{ATP} channel in a glucose dependent mechanism to stimulate insulin exocytosis [177-179]. However, whether acetate enhances gut satiety hormones remains elusive as discussed above. Furthermore, vagal activation increased GSIS in a rat study (see acetate and central effects on appetite regulation) associated with an increased acetate turnover [40]. However, inconsistent results have been reported, since an *in vitro* study using isolated rat pancreatic islets, showed that acetate (1 mmol/l) strongly decreased GSIS [180]. Acetate may potentially modulate circulating levels of insulin, however, whether acetate directly affects insulin secretion via G protein-mediated signaling, or indirectly via vagal/parasympathetic activation or gut-derived hormones remains to be elucidated. With respect to alpha cells only very few studies have investigated this, with no clear effects of acetate on glucagon release [180, 181].

Overall both human acetate colonic infusions and vinegar administrations have reported effects to improve insulin sensitivity and glucose homeostasis. From a mechanistic perspective acetate may modulate improvements in adipose tissue functioning, as well as through an increase in oxidative capacity (i.e. muscle, liver) and modulation of GSIS in the pancreas. Collectively, these tissue specific effects may synergistically decrease lipid ectopic deposition and contribute to body weight control and glucose homeostasis. In the next sections, we discuss the pre- and probiotic human administrations with potential to increase acetate production.

5. Prebiotic and probiotic administrations, body weight control and insulin sensitivity

In this section, we discuss pre- and probiotic administrations to increase microbial-derived acetate and its effects in metabolic health. First, we discuss the effect of acetogenic fiber supplementation in humans. Second, we discuss the potential of probiotic interventions to increase microbial-derived acetate production in the gut and its effects on human metabolic health.

5.1. Acetogenic fibers in human studies

As described above, acetogenic fibers in the diet may be fermented in the colon by the microbial community and thereby increase colonic acetate production [182-184]. Here we show that acetogenic fibers may increase circulating acetate levels and a few studies have even been linked to improvements in whole-body and peripheral insulin sensitivity (See Table 4). For instance, in healthy individuals, a drink containing lactulose (0-20 g) showed a dose effect to increase acetate concentrations with the highest dose reaching >200 μmol/l in plasma after 2.5 hours and even 6 hours after ingestion (>100 μmol/l), however, no metabolic outcome was measured [51]. In line, other acute lactulose studies in healthy individuals reported an increase in whole-body acetate turnover [185, 186]. In addition, young healthy individuals consuming bread overnight (evening of previous day) supplemented with 18.4 g arabinoxylan oligosaccharides reported increased plasma acetate levels (245 μmol/l) after 10.5 h fasting and 2 hours after a standardized breakfast (215 μmol/l) [187].

This was accompanied by improved glucose tolerance and insulin sensitivity possibly via increased gut fermentation (as evidenced by increased circulating SCFA and breath hydrogen levels) [187]. In overweight individuals, single oral administration of 24g inulin in overnight fasted overweight individuals, increased serum acetate and possibly reduced ghrelin in comparison to glucose and resistant starch as control [109]. A study in overweight subjects, using a single administration of lactulose (30 g) significantly increased plasma acetate (333 μmol/l) in comparison to saline administration (197 μmol/l) 6 h after ingestion. Moreover, acetate turnover correlated negatively with glycerol levels ($r = -0.78$, $p<0.02$), and a decrease in free fatty acids (35 %) was observed 2 h after lactulose

ingestion [188]. In hypercholesterolemic men, oat bran consumption for 3 weeks showed a total cholesterol lowering effect (12.8%) putatively through a greater serum acetate production as compared to wheat bran [189].

Recently, a high fiber diet (including traditional Chinese medicinal foods and prebiotics) for 12 weeks in T2DM subjects, showed higher improvement in glycemic control (HbA1c <7%) than the control diet (89% vs 50%, respectively) [190]. Of note, in this study an active SCFA producer (ASP) index, based on the abundance and diversity of the 15 high-fiber promoted microbial SCFA producers, was increased much more in the high fiber diet as compared to the control diet [190]. Another relevant human study, showed that a resistant starch (30 g RS/d) intervention improved insulin sensitivity (measured with hyperinsulinaemic-euglycemic clamp), lowered circulating lipids (non-esterified fatty acids and glycerol), increased fasting ghrelin and improved insulin sensitivity during a meal tolerance test, which was accompanied by higher AUC for acetate [11]. Despite the beneficial effects of RS, the increments in ghrelin seemed counterintuitive considering the expected higher satiety after RS. Nevertheless, some studies have linked ghrelin with increased insulin sensitivity [191, 192]. Although Robertson *et al.* [11], found beneficial effects on insulin sensitivity in healthy individuals using the gold standard measurement, the studied population is relatively small (n=10) and other RCT with acetogenic fibers within a larger population did not find beneficial effects (See Table 4) [193, 194]. Moreover, a study conducted by Zhao *et al.* [190] differed in duration compared to Robertson *et al.* and according to Zhao *et al.* results, T2DM subjects may need an integrative intervention, since they reported beneficial effects in the group treated with a fiber rich diet combined with lifestyle intervention and medication (acarbose).

Although the previous studies indicate that acetate may increase in response to acetogenic fiber supplementation and improve certain metabolites, other studies reported no effects of acetogenic fibers on metabolic profile. For instance, a 12-week GOS supplementation (15 g/day) to the regular diet in obese prediabetic individuals showed an increase in fecal *Bifidobacterium* but no effects on acetate levels (fecal and plasma), insulin sensitivity and energy metabolism [193].

Similarly, another 12-week GOS supplementation (5.5 g/day) in overweight/prediabetic adults improved gut microbial community (increased *Bifidobacterium* and lowered *Bacteroides*) and reduced inflammatory markers (including C-reactive protein and fecal calprotectin), however, acetate was not measured [195]. Taken together, the discrepancies of effects between studies may suggest the importance of the type and dose of dietary fiber as well as the metabolic phenotype studied. In the next section, we discuss the potential of probiotics to improve body weight and insulin sensitivity in humans.

Table 4. Acetogenic fiber administrations with effects on glucose homeostasis and metabolic health.

Participants	**Non-digestible carbohydrate**	**Design**	**Effects**	**Study**
T2DM individuals (n=43)	High fiber diet (n=27) Control diet (n=16)	12 weeks RCT	Higher improvement in glycemic control (HbA1c <7%) in treatment (89% vs 50%, respectively) ↑ abundance in SCFA microbiota producers in treatment	Zhao *et al.* (2018) [190]
Healthy adults (n=10)	20 g resistant starch 10 g (3 times/day)	4 weeks, placebo/controlled (20 g digestible starch)	Improved whole-body insulin sensitivity (euglycemic-hyperinsulinaemic clamp) by 13% (P<0.05)	Robertson *et al.* (2005) [11]
Hypercholesterolemic men (n=20)	Oat bran (47.4 g/day) wheat bran (control) (41.8 g/day)	RCT 3 weeks	↓ Total cholesterol (12.8%) Linked to high acetate in plasma ↑ Higher acetate in treatment	Bridges *et al.* (1992) [189]
Healthy individuals (n=14)	0, 10 and 20 gr lactulose Control (water)	Single dose	No effects in glucose homeostasis investigated Dose effect in acetate levels ↑ fermentation after 6 hours	Pomare *et al.* (1985) [51]
Healthy individuals (n=19)	Arabynoxylan oligosaccharides (AXOS) (8.9 gr) High AXOS (18.4 gr)	Randomized cross-over Overnight administration	↑ Improvement in glucose tolerance ↑ improved insulin sensitivity index with High AXOS Dose-effect increase in plasma acetate (>200 μmol/l)	Boli *et al.* (2016) [187]
Overweight obese individuals (n=53)	Pea fiber (15 g/day) Control (no fiber)	RCT 12 weeks	No effects in glucose homeostasis ↑ Fecal acetate No effects on plasma acetate	Mayengbang *et al.* (2017) [194]
Lean/overweight individuals (n=12, 13 respectively)	Inulin (24 gr) Control (glucose)	Cross-over Single dose	↑ Acetate in plasma Possibly linked to ghrelin reduction	Rahat-Rozenbloom *et al.* (2016) [109]
Overweight individuals (n=8)	Lactulose (30 gr)	Single dose	↑ Acetate in plasma Correlation of acetate and in lipolysis (glycerol turnover)	Ferchaud-Roucher et al. (2005) [188]

Abbreviations: T2DM, type 2 diabetes mellitus; RCT, randomized controlled trial; HbA1c, glycated hemoglobin; SCFA, short chain fatty acids.

5.2. Probiotic body weight control and insulin sensitivity

Targeting the gut microbiota with probiotics has gained interest as a therapeutic approach to combat obesity and its comorbidities. Interestingly, in rodents at the genus level, *Bifidobacterium* and *Lactobacillus* have been associated with reduced weight gain and markers of adiposity [196]. Here we discuss both animal and human studies on probiotic interventions/associations with putatively acetate-mediated metabolic health effects.

First, a probiotic intervention in mice, (*Bifidobacterium animalis* ssp. *lactis* GCL2505) modified microbial community (increased *Bifidobacterium* and *Lactobacillus)*, increased acetate (caecal and plasma) levels and improved glucose tolerance which was accompanied by a reduction of adipocyte cell size [13]. Second, mice colonized with *Bifidobacterium* strains (*Bifidobacterium longum* JCM 1217, *B. infantis* 157F, or *B. longum* NCC 2705) were protected against a lethal infection of *Escherichia coli* O157:H7 through an augmented acetate production and *Bifidobacterium* that promoted gut barrier integrity and reduced epithelial cell death [197]. Furthermore, Wrsozek *et al.* [198] proposed that two species, including *Bacteroides thetaiotaomicron (*acetate producer*)* and *Faecalibacterium prausnitzii (*acetate consumer*)*, might aid in maintaining epithelial homeostasis and gut health in rats.

Another important bacterium, *A. muciniphila,* has been associated with improvements in adiposity in both rodents and humans [196, 199] and loss of *A. muciniphila* has been reported to impair gut integrity and increase insulin resistance in rodents [200]. *A. muciniphila* has been reported to correlate with serum acetate levels in humans, thus, suggesting a putative role of *A. muciniphila* in acetate production and metabolic health [63]. Recently, a RCT reported that pasteurized *A. muciniphila* supplementation (3 months) in overweight/obese insulin resistant individuals improved insulin sensitivity (HOMA-IR, +28.62 + 7.02%, P=0.002), decreased body weight (-2.27 + 0.92 kg, P=0.091), fat mass (-1.37 + 0.82, P=0.092) among other relevant blood markers [201].

Similarly, a 3 month supplementation (*L.casei* Shirota) improved insulin sensitivity index in metabolic syndrome individuals compared to baseline, but not different with control [202]. Although, these studies showed that the interventions were safe and provided benefits on metabolic health, an in-depth study of the microbial-derived metabolites is needed to identify the metabolic regulators. In addition, as Anhe *et al.* [203] suggested, the identification of these metabolites may provide a safer therapeutic option and overcome the limitations of probiotic supplementations. In addition, a metanalysis of randomized controlled trials reported that probiotic supplementation (i.e. Lactobacillus reuteri, Lactobacillus gasseri) can significantly reduce body weight ((95% CI) -0.60 [-1.19, -0.01] kg), BMI ((95% CI) -0.27 [-0.45, -0.08] kg/m^2) and fat percentage ((95% CI) -0.60 [-1.20, -0.01] %) in overweight/obese individuals, however, the effect sizes were small [204]. *L.*

plantarum reduced glucose and homocysteine levels significantly after 12 weeks in premenopausal insulin resistant women [205]. In addition, combination of probiotics with prebiotics may provide a synergistic effect. For instance, a synbiotic administration (*Lactobacillus* and fructo-oligosaccharides*)* in insulin resistant individuals showed improvements in fasting levels of glucose and HOMA-IR in comparison to placebo after a 28-week treatment [206].

Future probiotic studies should identify the mechanisms, factors that ensure its efficacy as well as which synbiotic mixtures may have a better synergistic effect [207, 208]. Although these studies report benefits on insulin sensitivity (HOMA-IR) and glucose levels with potential to improve metabolic health following probiotic interventions, it remains unknown whether these effects are acetate-mediated. Nevertheless, effects were mediated by specific species such as *Bacteroides thetaiotaomicron* and *A. muciniphila*, which are important acetate producers.

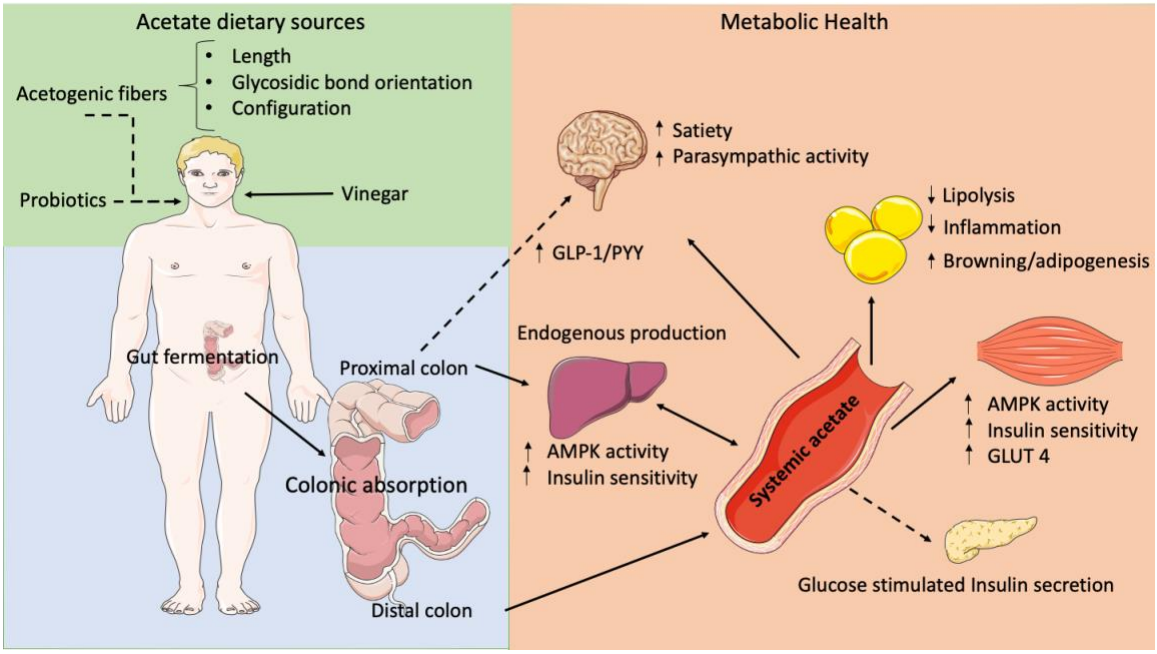

Figure 1: Acetate sources and acetate-mediated effects in metabolic health. *Exogenous acetate production includes vinegar as well as the supplementation of acetogenic fiber and probiotics. Acetogenic fiber characteristics (i.e. length, glycosidic bond configuration) may determine acetate production. Endogenous acetate production occurs in all tissues but predominantly in the liver. Microbial acetate is mainly produced in the colon. Colonic absorption and acetate systemic concentrations may differ between colonic production sites (proximal/distal). Importantly, acetate may increase GLP-1 and PYY secretion in the colon. Systemic acetate may improve metabolic health via improvements in adipose tissue functioning (antilipolytic/anti-inflammatory effects) insulin sensitivity and oxidative capacity (i.e. liver, skeletal muscle) increments in satiety (central nervous system) and modulation of insulin secretion (pancreas). Solid lines indicate well-studied effects of acetate, dashed line indicate more inconsistent findings. Abbreviations: GLP-1 glucagon-like peptide 1; PYY peptide YY; AMPK AMP-activated protein kinase; GLUT glucose transporter.*

6. Conclusions and perspectives

From a mechanistic perspective, a vast wealth of animal data suggests that acetate has an important regulatory role in body weight control, and insulin sensitivity through effects on lipid metabolism and glucose homeostasis (see Figure 1). Current evidence of acetate-mediated effects on metabolism emphasizes the need for well-controlled human intervention studies that ensure an efficient administration of acetate (considering location and concentration) in a physiological and appealing manner. Under this premise, prebiotic supplementations have been conducted, however with inconsistent results to increase colonic/systemic acetate production. Nevertheless, a few human studies have shown the capacity to improve markers of whole-body insulin sensitivity [11, 187, 190]. Similarly, a few probiotic human studies, with the potential to increase acetate production, have reported improvements in whole-body insulin sensitivity [202, 206]. While the effect of changes in acetate levels on the observed metabolic phenotype in these studies remains unclear, in light of the evidence presented in this report, it is reasonable to hypothesize that acetate acts as a direct mediator of these effects. With respect to oral acetate administrations (vinegar), improvements on glucose homeostasis have been reported, and attributed to acetic acid. Of importance, future vinegar supplementations should specify its composition (i.e. acetic acid % percentage, polyphenols). Moreover, future prebiotic/probiotic/vinegar studies should consider that responses may differ between healthy individuals and prediabetic individuals, as evidently shown in vinegar administrations.

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CHAPTER 3

Relation between circulating acetate and human insulin resistance before and after weight loss in the DiOGenes study

Running title: Acetate and insulin resistance

Manuel A. González Hernández, Emanuel E. Canfora, Kenneth Pasmans, Arne Astrup, Wim H. M. Saris and Ellen E. Blaak.

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Abstract

Microbially-produced acetate has been reported to beneficially affect metabolic health through effects on satiety, energy expenditure, insulin sensitivity and substrate utilization. Here, we investigated the association between sex-specific concentrations of acetate and insulin sensitivity/resistance indices (HOMA-IR, circulating insulin and Matsuda Index) in the DiOGenes study at baseline and after a low-calorie diet (LCD, 800 kcal/d). In this analysis 478 subjects (BMI >27 kg/m^2) were included who underwent a LCD for 8 weeks. Linear mixed models were performed, which were adjusted for mean acetate concentration, center (random factor), age, weight loss, and fat-free mass (FFM). At baseline no associations between plasma acetate and insulin sensitivity/resistance indices were found. We found a slight positive association between changes in acetate and changes in HOMA-IR (stdβ 0.130, P = 0.033) in women, but not in men (stdβ -0.072, P = 0.310) independently of age, weight loss and FFM. We were not able to confirm previously reported associations between acetate and insulin sensitivity in this large European cohort. The mechanisms behind the sex-specific relationship between LCD-induced changes in acetate and insulin sensitivity require further study.

1. Introduction

Microbially-derived acetate has been shown to play an important role in substrate and energy metabolism [1]. In rodents fed high-fat diets, beneficial effects on insulin sensitivity [2] and increased white adipose tissue browning were reported [3]. However, rodent data are not entirely consistent since intragastric acetate infusions in rats resulted in hyperphagia and energy retention [4].

In humans, rectal acetate infusions have been shown to increase plasma peptide YY (PYY) and glucagon-like peptide-1 in hyperinsulinaemic overweight women [5]. Furthermore, acute distal colonic acetate infusions and SCFA mixtures high in acetate decreased whole-body lipolysis, increased fasting fat oxidation, resting energy expenditure and PYY secretion in overweight men [6, 7]. Additionally, circulating acetate was positively associated with increased fat oxidation and energy expenditure [7]. Recently, a study reported that acetate produced in the distal colon contributes significantly to systemic acetate concentrations in men [8]. In line, cross-sectional analyses in patients with obesity have shown positive associations between high circulating acetate and insulin sensitivity indices [9, 10]. In women, plasma acetate correlated negatively with fasting and postprandial insulin concentrations [9]. In morbidly obese subjects, acetate correlated negatively with insulin resistance (HOMA-IR) [10]. Notably, a resistant starch supplementation (30 g/d for 4 weeks) in healthy subjects increased acetate uptake in muscle and adipose tissue and improved insulin sensitivity (as measured by hyperinsulinemic-euglycemic clamp) [11].

Here, we assessed the association of (changes in) circulating acetate and insulin sensitivity/resistance indices (HOMA-IR, circulating insulin and Matsuda Index) at baseline and after an eight weeks low calorie diet (LCD) in male and female overweight/obese subjects of the European Diet, Obesity and Genes (DiOGenes) study. Due to the fact that microbial composition and functionality as well as their impact on insulin sensitivity may differ in overweight males and females, we stratified our analysis for sex [12].

2. Materials and methods

2.1. Study Design

The DiOGenes project was a multicenter, randomized, controlled dietary intervention study in 8 European countries. In total, 938 overweight or obese, nondiabetic adults free of cardiovascular disease [age 18-65 years, body mass index (BMI) 27-45 kg/m^2 and fasting blood glucose concentrations <6.1 mmol/L] were recruited. Subjects using prescription

medication or suffering from diseases or conditions that might influence the outcome of the study were excluded.

Of special interest were diseases that influenced body weight regulation (malabsorption, untreated hypo/hyperthyroidism, eating disorders, systemic use of steroids, etc.) and obesity-related cardiovascular risk factors (heart disease, systolic and diastolic blood pressures ≥ 160/100 mmHg, blood glucose > 6.1 mmol L^{-1}, blood cholesterol > 7 mmol L^{-1}, blood triglycerides > 3 mmol L^{-1}). More details on recruitment, inclusion and exclusion criteria, and study design are described elsewhere [13]. The Medical Ethical Committees of the respective countries approved the study protocol, which followed the Helsinki II regulations. The study was registered with ClinicalTrials.gov, number NCT00390637. Succinctly, the study consisted of two phases. In this report, we focus on the baseline characterization of the overweight/obese individuals as well as the weight-loss phase in which individuals with BMI >27 kg/m^2 were assigned to lose weight following a LCD for 8 weeks. The LCD provided 3.3 MJ/d (800 kcal/d) using Modifast products (Nutrition et Santé, France), with a macronutrient composition of 15-20% of total energy from fat, 35-40% from protein and 45-50% from carbohydrate. In addition, subjects could also eat up to 400 g of vegetables, providing a total of 3.3 to 4.2 MJ/d (800 to 1000 kcal/d).

2.2. Study Population

We included 478 volunteers (303 women, 175 men) from whom plasma metabolomics (including acetate) were available before and after the LCD. Volunteers with BMI >27 kg/m^2 were recruited in eight different research centers across Europe: Maastricht (the Netherlands), Copenhagen (Denmark), Cambridge (United Kingdom), Heraklion (Greece), Potsdam (Germany), Pamplona (Spain), Sofia (Bulgaria) and Prague (the Czech Republic). All subjects provided written informed consent before enrollment into the study.

2.3. Anthropometry and blood sampling

Anthropometry and blood samples were collected after fasting (10 hours) at baseline (clinical investigation day 1, CID1) and after weight loss at the end of the LCD period (CID2). In this report, we included the following parameters: anthropometry (body weight, BMI and fat-free mass (FFM)). FFM was assessed by multifrequency bioimpedance (QuadScan 4000, Bodystat, Douglas, United Kingdom) [13]. Additionally, glucose, free fatty acids (FFA) (automatic spectrophotometric enzymatic techniques) and insulin were measured from fasting samples [13]. Insulin concentrations were

measured by a colorimetric assay (Ortho-Clinical Diagnostics, Johnson & Johnson, Birkerød, Denmark) [14].

Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) and insulin sensitivity (Matsuda index) were calculated. HOMA-IR was calculated as follows: HOMA-IR = [glucose (mmol/L) * insulin (μU/mL)/22.5], using fasting values [15]. Matsuda index was calculated as follows: (10,000/square root of (fasting glucose x fasting insulin) x (mean glucose x mean insulin during Oral glucose tolerance test (OGTT))) [16].

BMI was calculated by dividing the mass in kg by squared height. FFM was obtained from Bioimpedance analysis. Acetate, acetoacetate and 3-OH-butyrate were quantified in serum from nuclear magnetic resonance (NMR) spectra, as reported previously in detail [17]. Physical activity was measured before and after LCD by means of the Baecke questionnaire [13].

2.4. Statistical analysis

Normality of data was assessed with the Kolmogorov-Smirnov procedure and histogram and variables that were not normally distributed were Ln-transformed. Repeated measures ANOVA with Bonferroni correction was conducted to investigate sex, time and sex-time effects as a result of LCD phase. A Linear mixed model (LMM) was used to determine the relationship between (changes in) acetate and (changes in) insulin sensitivity/resistance parameters at baseline and after LCD. In the first model besides (changes in) acetate and (changes in) fasting insulin, HOMA-IR or Matsuda index, mean acetate were added. Subsequently, age (Model 2), (change in) weight (Model 3), and (change in) FFM (Model 4) were added as covariates. In all models, center was included as a random factor.

3. Results

3.1. Cross-sectional analysis at baseline

Acetate showed significantly higher levels in men as compared to women at baseline (1.35 ± 1.39 vs. 1.13 ± 0.97 mmol/L, respectively, See Table 1). There were no differences in BMI between males and females. Furthermore, fasting insulin, HOMA-IR, fasting glucose, body weight and FFM were higher in males, whilst the Matsuda index, FFA, and ketone bodies, were lower (See Table 1). There were no relationships between acetate and fasting insulin, HOMA-IR, and Matsuda Index in males as well as in females.

3.2. Weight loss by LCD

In response to the LCD, acetate levels increased in both men and women (1.36 ± 1.54 to 1.41 ± 1.25 and 1.12 ± 1.03 to 1.18 ± 1.04 mmol/L, respectively, Table 1), but did not differ between sexes ($P = 0.672$). Weight reduction increased insulin sensitivity as assessed by Matsuda ($P = 0.002$) and decreased HOMA-IR ($P = 0.004$) and fasting insulin ($P = 0.001$), with more pronounced improvements in males as compared to females. Additionally, FFA slightly increased as a result of weight loss with a more pronounced increase in males as compared to females (Table 1). Ketone bodies (acetoacetate and 3-OH-butyrate) showed an increase while acetate/3-OH-butyrate and acetate/acetoacetate ratios decreased after LCD, however sex-time interactions were not significant. Physical activity (sports and work indexes) did not change after LCD. Leisure activity differed (sex and time), however, sex-time interaction was not significant.

We observed a slight positive relationship between LCD-induced changes in acetate concentrations and changes in HOMA-IR (stdß 0.130, $P = 0.033$, Table 2) and with changes in fasting insulin concentrations (stdß 0.119, $P = 0.051$, Table 3) in women, but not in men (stdß -0.072, $P = 0.310$ and stdß -0.066, $P = 0.359$, respectively; data not shown). Subsequently, the relationship between (changes in) acetate and HOMA-IR did not change after adjustment for age and changes in body weight as well as changes in FFM (Table 2). A similar result was seen for (changes in) fasting insulin (Table 3). The relationship between changes in acetate and Matsuda did not reach statistical significance (data not shown).

Table 1. Characteristics of participants before and after LCD intervention.

	Men		Women		Sex	Time	Time x sex
	Baseline	After LCD	Baseline	After LCD			
N	175	175	303	303			
Age (yr)	42 ± 6	42 ± 6	41 ± 6	41 ± 6	0.000		1
Acetate (mmol/L)	1.36 ± 1.54	1.41 ± 1.25	1.12 ± 1.03	1.18 ± 1.04	0.000	0.016	0.672
HOMA index	3.86 ± 2.35	2.18 ± 2.23	2.93 ± 3.0	2.00 ± 2.53	0.000	0.000	0.004
Matsuda index	4.10 ± 2.50	7.16 ± 3.8	5.67 ± 3.50	7.55 ± 3.70	0.000	0.000	0.002
Insulin (μU/mL)	13.8 ± 7.6	8.1 ± 5.3	11.14 ± 11.14	8.18 ± 9.32	0.000	0.000	0.001
Glucose (mmol/L)	5.3 ± 0.6	5.0 ± 0.5	5.0 ± 0.6	4.8 ± 0.3	0.000	0.000	0.457
FFA (micromol/L)	528 ± 190	634 ± 204	675 ± 263	746 ± 213	0.000	0.000	0.176
Acetoacetate (mmol/L)	0.06 ± 0.04	0.23 ± 0.30	0.09 ± 0.07	0.25 ± 0.30	0.000	0.000	0.376
3-OH-butyrate (mmol/L)	0.35 ± 0.30	1.52 ± 2.00	0.56 ± 0.52	1.69 ± 1.94	0.000	0.000	0.133
Acetate/3-OH-butyrate ratio	5.80 ± 8	2.08 ± 3	3.50 ± 4.90	1.40 ± 2.04	0.000	0.000	0.415
Acetate/Acetoacetate ratio	26 ± 29	12 ± 16	18 ± 21	9 ± 10	0.000	0.000	0.787
Body weight (kg)	111.8 ± 17.5	99.0 ± 15.9	96.8 ± 16.5	86.6 ± 15	0.000	0.000	0.702
BMI (kg/m²)	35 ± 4.8	31 ± 4.4	35.2 ± 5.1	31.4 ± 4.6	0.259	0.000	0.657
Fat-free mass (kg)	73.4 ± 9.9	69.8 ± 8.7	53.4 ± 8.1	51 ± 8	0.000	0.000	0.990
Baecke Questionnaire scores							
Leisure index	2.6 ± 0.7	2.8 ± 0.6	2.7 ± 0.7	3.0 ± 0.6	0.000	0.001	0.569
Sports index	2.6 ± 0.4	2.6 ± 0.4	2.6 ± 0.5	2.7 ± 0.4	0.620	0.675	0.310
Work index	2.7 ± 0.4	2.8 ± 0.4	2.7 ± 0.4	2.7 ± 0.3	0.087	0.670	0.980

Repeated measures ANOVA. 1st P-value reported corresponds to sex effect. 2nd P-value corresponds to time effect and 3rd P-value corresponds to the sex-time effect. Data expressed as mean and standard deviation. Abbreviations: Low-calorie diet (LCD), Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), BMI (Body Mass Index)

Table 2. Determinants of changes in HOMA-IR in participants during weight loss.

		Females		Males	
Model	**Parameter**	**Std ß, confidence interval**	**P-value**	**Std ß, confidence interval**	**P-value**
1	Δ-Acetate Mean acetate	0.111 (0.013 to 0.209)	**0.027**	-0.098 (-0.224 to 0.027)	0.123
2	Model 1+ Age	0.125 (0.027 to 0.223)	**0.013**	-0.101 (-0.227 to 0.025)	0.115
3	Model 2+ Δ-Weight	0.120 (0.022 to 0.218)	**0.017**	-0.069 (-0.189 to 0.051)	0.259
4	Model 3+ Δ-Fat-free mass	0.130 (0.010 to 0.249)	**0.033**	-0.072 (-0.211 to 0.068)	0.310

Linear Mixed model **was adjusted for age, weight, and fat-free-mass. All models were adjusted for center as a random factor (coefficients not shown). Acetate concentration (independent factor) and HOMA-IR as dependent factor. Statistically significant P-values are in bold. Valid cases N= 302 females, 175 males.**

Table 3. Determinants of changes in fasting Insulin in participants during weight loss.

		Females		Males	
Model	**Parameter**	**Std ß, confidence interval**	**P-value**	**Std ß, confidence interval**	**P-value**
1	Δ-Acetate Mean acetate	0.132 (0.034 to 0.231)	**0.009**	-0.085 (-0.212 to 0.042)	0.190
2	Model 1+ Age	0.140 (0.042 to 0.238)	**0.005**	-0.089 (-0.217 to 0.038)	0.168
3	Model 2 + Δ-Weight	0.132 (0.035 to 0.229)	**0.008**	-0.064 (-0.188 to 0.059)	0.306
4	Model 3 + Δ-Fat-free mass	0.119 (-0.001 to 0.238)	0.051	-0.066 (-0.207 to 0.075)	0.359

Linear Mixed model **was adjusted for age, weight, and fat-free-mass. All models were adjusted for center as a random factor (coefficients not shown). Acetate concentration (independent factor) and HOMA-IR as dependent factor. Statistically significant P values are in bold. Valid cases N= 295 females, 175 males.**

4. Discussion

In the present study we could not confirm previously reported positive associations between acetate and insulin sensitivity and metabolic health parameters in a cross-sectional analysis at baseline. LCD-induced weight loss increased insulin sensitivity more in men than in women. Surprisingly, we found a slight positive association between LCD-induced changes in acetate and changes in HOMA-IR and fasting insulin concentrations in overweight/obese women, but not in men, which was independent of age and changes in body composition.

As previously described, cross-sectional studies showed negative associations between acetate and fasting insulin in obese women [9]. Similarly, in morbidly obese subjects a negative association with HOMA-IR and a positive association with insulin sensitivity were observed [10]. Furthermore, acute distal colonic acetate infusions increased fat oxidation and improved metabolic profile in overweight/obese men [6, 7]. Surprisingly, we observed a slight positive relationship between the LCD-induced change in circulating acetate and change in HOMA-IR and fasting insulin in females, but not in males. In line with these results on differential associations between males and females, a previous DiOGenes analysis by Stroeve *et al.* [17], suggested a sex-specific modulatory role of baseline acetate in energy metabolism, since they reported a negative association with LCD-induced BMI change in morbidly obese females (stdß -0.23), but not in males.

Furthermore, a previous analysis in the DiOGenes study showed that despite the fact that hepatic insulin resistance was lower in females as compared to males, there was a more pronounced worsening of blood lipid profile with the progression of hepatic insulin resistance in females, but not in males [18].

In interpreting our results, it is important to note that acetate kinetics (acetate absorption in the colon, hepatic uptake and acetate clearance), and turnover (production/utilization) are intricate processes that are influenced by the interplay of gut microbial fermentation, lipogenesis, fat oxidation and ketogenesis [19]. With respect to insulinemic profile, hyperinsulinemia may affect acetate metabolism, possibly via an alteration in endogenous and exogenous acetate metabolism [20]. In addition, a caloric restriction may augment hepatic ketogenesis via an overproduction of acetyl-coA coming from increased lipid utilization [21] and an increased ketogenesis may also elevate acetate production [22]. The exact mechanisms behind the sex-specific relationship of acetate and HOMA-IR may relate to sex hormones affecting processes such as microbial metabolism, hepatic lipid metabolism and ketogenesis. Notably, in our study most women were in the premenopausal state, however data on the hormone status was not available. Ketone bodies increased after the LCD with no differences between males and females. In addition, acetate/3-OH-butyrate and acetate/acetoacetate ratios decreased after LCD, which may suggest that the contribution of acetate from sources other than hepatic ketogenesis has probably not increased after LCD [23].

Thus, the discrepancy of our findings on the relationship of acetate and insulin resistance as well as the sexual dimorphism in this relationship remains to be elucidated taking into account the complex acetate kinetics. Finally, other SCFA (propionate and butyrate) may be of importance for insulin sensitivity as we and other publications have reported [24-28]. Unfortunately, these metabolites were not included in the NMR measurements and we focused on acetate, the most abundant SCFA in the colonic lumen and systemic circulation.

The major strength of our study is the availability of HOMA-IR and a 5-point Oral glucose tolerance test (including insulin concentrations for calculation of the Matsuda index) in a large and well-characterized cohort, which made additional adjustment for confounding factors feasible. A limitation of our study is the lack of information on acetate kinetics and colonic acetate that may contribute to circulating acetate concentrations. Of note, in the current study we measured plasma acetate concentrations using NMR methodology, which validation has been previously described [17, 29, 30]. Although, the absolute concentrations of acetate may differ as compared to previous studies measured with LC-MS or GC-MS [6-9, 25, 31, 32], high

correlations have been reported between metabolites measured with both LC-MS and NMR [33].

In conclusion, the results of this large pan-European study (478 volunteers) showed no relationship between acetate and markers of insulin sensitivity. Furthermore, a small positive association between LCD-induced changes in acetate and HOMA-IR and fasting insulin (explaining 1-2% of variance in HOMA-IR or fasting insulin) was shown in females and not in males. Future studies should aim to elucidate the underlying mechanisms and physiological significance.

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CHAPTER 4

Circulating but not fecal short-chain fatty acids are related to insulin sensitivity, lipolysis and GLP-1 concentrations in humans

Running title: Circulating and fecal SCFA in metabolic health

Mattea Müller[#]; Manuel A. González Hernández[#]; Gijs H. Goossens, Dorien Reijnders, Jens J. Holst; Johan W.E. Jocken, Hans van Eijk, Emanuel E. Canfora; and Ellen E. Blaak

#These authors contributed equally to the work

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Abstract

Microbial-derived short-chain fatty acids (SCFA) acetate, propionate and butyrate may provide a link between gut microbiota and whole-body insulin sensitivity (IS). In this cross-sectional study (160 participants, 66.3% male, BMI: 19.2-41.0 $kg/m^{2,}$ normal or impaired glucose metabolism), associations between SCFA (fecal and fasting circulating) and circulating metabolites, substrate oxidation and IS were investigated. In a subgroup (n=93), IS was determined using a hyperinsulinemic-euglycemic clamp. Data were analyzed using multiple linear regression analysis adjusted for sex, age and BMI. Fasting circulating acetate, propionate and butyrate concentrations were positively associated with fasting GLP-1 levels. Additionally, circulating SCFA were negatively related to whole-body lipolysis (glycerol), triacylglycerols and free fatty acids levels (standardized (std) β adjusted (adj) -0.190, P=0.023; std β adj -0.202, P=0.010; std β adj -0.306, P=0.001, respectively). Circulating acetate and propionate were, respectively, negatively and positively correlated with IS (M-value: std β adj -0.294, P<0.001; std β adj 0.161, P=0.033, respectively). We show that circulating rather than fecal SCFA were associated with GLP-1 concentrations, whole-body lipolysis and peripheral IS in humans. Therefore, circulating SCFA are more directly linked to metabolic heath, which indicates the need to measure circulating SCFA in human prebiotic/probiotic intervention studies as a biomarker/mediator of effects on host metabolism.

Introduction

In obesity and type 2 diabetes mellitus (T2DM), alterations in the gut microbiota composition and functionality may contribute to disease aetiology. The gut microbiota ferments indigestible carbohydrates (i.e. dietary fibres) and major end-products thereof are the short- chain fatty acids (SCFA) acetate, propionate and butyrate [1]. Acetate, propionate and butyrate are present in the colon in a ratio of approximately 3:1:1, respectively [2, 3]. Most butyrate is utilized by colonocytes as energy source [4]. Via the portal vein, SCFA reach the liver where acetate and propionate are metabolized and partly oxidised or used as substrate in gluconeogenesis and lipogenesis [5]. Consequently, a small proportion of microbial-derived SCFA enters the peripheral circulation whereby acetate reaches the highest concentrations compared to propionate and butyrate [6, 7]. SCFA are ligands to G-protein coupled receptors (GPR) 41 and 43, which are expressed on intestinal, adipose, skeletal muscle, liver and pancreatic tissues [8-10], indicating their important role in the crosstalk between the gut and peripheral tissues. Several rodent studies showed that oral, intravenous and colonic infusion of SCFA as well as microbial-derived SCFA beneficially affect the functioning and metabolism of the aforementioned tissues and consequently improve insulin sensitivity, substrate metabolism and body weight regulation [11]. In humans, distal colonic acetate infusions increased fasting fat oxidation, energy expenditure, and PYY secretion whilst whole-body lipolysis was decreased [12, 13]. Other acute studies show decreased circulating free fatty acid (FFA) concentration after rectal SCFA infusion in healthy participants [14, 15]. In addition, 24 weeks of 10 g/day inulin propionate ester protected against body weight gain as compared to inulin only in overweight individuals [16]. Potential mechanisms may include a SCFA-induced inhibition of energy intake possibly mediated via the stimulation of glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) secretion, increased intestinal gluconeogenesis, increased skeletal muscle fat oxidation and improved lipid buffering capacity of adipose tissue [11].

However, increased microbial-derived acetate formation has been associated with increased body weight gain and insulin resistance in diet-induced obese rats [17]. Additionally, increased fecal SCFA have been reported in overweight and obese compared to lean participants [2, 3, 8, 18, 19], yet it is difficult to interpret the latter data since fecal SCFA reflect the net result of colonic production and absorption [20, 21]. Even though fecal SCFA are commonly used as an indicator of microbial fermentation, fecal SCFA may not accurately reflect *in vivo* colonic fermentation since approximately 95% of colonic SCFA are absorbed and only the remaining 5% are excreted in feces [22-25].

To obtain more information on the validity of fecal SCFA as biomarker for metabolic health effects, the associations between fecal and circulating SCFA concentrations and parameters of metabolic health were studied in a relatively large cohort of 160 participants with a wide range of body mass indices (BMI) and glucometabolic status. Using multiple regression analysis, we analysed the relationship between fecal and fasting circulating SCFA with fasting glucose, insulin, circulating lipids (free fatty acids (FFA), triacylglycerols (TAG), glycerol), insulin resistance index (homeostasis model assessment of insulin resistance (HOMA-IR)), gut hormone concentrations (PYY, GLP-1), fasting substrate utilization and inflammation markers including lipopolysaccharide-binding protein (LBP), tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and interleukin 8 (IL-8). We further investigated the relationship between fecal and fasting circulating SCFA profiles and peripheral insulin sensitivity index (M-value) as measured via the gold standard hyperinsulinaemic-euglycemic clamp technique in a subset of individuals.

Methods

Study participants

This cross-sectional analysis included 160 Caucasian men and women aged 20–70 years with a BMI between 19.2 and 41.0 kg/m^2 from the general population in the vicinity of Maastricht, The Netherlands during August 2013 and December 2016. Individuals had normoglycemia, impaired fasting glucose (IFG, ≥ 5.6 mmol/L) and/or impaired glucose tolerance (IGT, 2 hour plasma glucose of 7.8 – 11 mmol/L after 75g oral glucose challenge) according to the diagnostic criteria of the American Diabetes Association, 2010 [26]. Eligibility of the participants was assessed via a general health questionnaire, medical history and anthropometry during an initial screening visit. Exclusion criteria were as follows: use of antibiotics, prebiotics, or probiotics 3 months before the study, diagnosis of T2DM, gastrointestinal or cardiovascular diseases, abdominal surgery, participants with life expectancy shorter than 5 years and participants following a hypocaloric diet. Participants did not use β-blockers, lipid- or glucose-lowering drugs, anti-oxidants, or chronic corticosteroids. All protocols were reviewed and approved by the local Medical Ethical Committee (MUMC+) and conducted in accordance with the Declaration of Helsinki (revised version, October 2008, Seoul, South Korea). Written informed consent was obtained from all participants.

Study Design

This cross-sectional analysis included metabolic parameters as well as fecal and fasting circulating SCFA concentrations of previously performed intervention studies [12, 13, 27-29]. In the present study, we collated and analyzed study data at baseline and thus prior to the respective interventions.

In all studies, sample collection was performed after an overnight fast, and measurements were conducted according to the same standard operating procedures. Two days prior to the baseline investigation day, participants were asked to refrain from intense physical activity and alcohol consumption, and to collect a fecal sample. In the evening before the investigation day, the participants consumed a standardized low-fiber meal.

Used data sets

The data set included baseline data from the following intervention human *in vivo* studies. These include an intervention study in prediabetic, overweight-obese individuals on the effect of antibiotics on insulin sensitivity (Clinical trial No. NCT02241421) [28], an intervention study in prediabetic, overweight-obese individuals on the effect of dietary fiber (galacto-oligosaccharides) on insulin sensitivity (Clinical trial No. NCT02271776) [27], an intervention study in normoglycemic, normal to overweight individuals on the effect of dietary fibers on gastrointestinal transit (Clinical trial No. NCT02491125) [29], and lastly two acute studies investigating the effect of different mixtures of SCFA in normoglycemic, overweight to obese individuals on human substrate and energy metabolism (Clinical trial No. NCT01826162 [12], Clinical trial No. NCT01983046 [13]).

Baseline investigation day

After an overnight fast (>10 h), participants came to the laboratory by car or public transport. Anthropometry was measured including height, weight and waist to hip ratio. After inserting a cannula into the antecubital vein, blood samples were taken to measure plasma metabolites, hormones and inflammatory markers in the fasted state. After the blood sampling, participants were in a resting, half-supine position and fasting substrate oxidation was measured for 30 min using an open circuit ventilated hood system (Omnical, MUMC+, Maastricht, the Netherlands). Fat and carbohydrate oxidation were calculated according to the equations of Weir and Frayn [30, 31], assuming that protein oxidation accounted for 15% of total energy expenditure.

Hyperinsulinaemic-euglycaemic clamp

Peripheral insulin sensitivity was determined in a subgroup of overweight/obese, prediabetic individuals via hyperinsulinaemic-euglycemic clamps as previously described [27, 28]. In short, a cannula was inserted into an antecubital vein for infusion of glucose and insulin. To measure blood glucose, a second cannula was inserted into a superficial dorsal hand vein, which was arterialized by placing the hand into a hotbox (~50 °C).

A priming dose of insulin infusion (Actrapid, Novo Nordisk, Gentofte, Denmark) was administered during the first ten min (t0 – t10 min) and insulin infusion was thereafter continued at 40 mU/m^2/min for 2 h (t10 – t120 min). By variable infusion of a 20% glucose solution, plasma concentrations were maintained at 5.0 mmol/L. Peripheral insulin sensitivity (M-value, mg*(kg*min)$^{-1}$ was calculated from the mean glucose infusion rate during the steady-state of the clamp (last 30min, stable blood glucose concentration at 5.0 mmol/L) [32]. A high M-value represents high insulin sensitivity (i.e., more glucose needs to be infused to maintain euglycemia during insulin infusion).

Analysis of fecal and circulating SCFA

Fecal samples were collected at home and stored in the subjects' freezer at -20 °C maximum of two days before the baseline investigation day, transported on dry ice, and stored on arrival at the university at -80 °C. Fecal acetate, propionate, and butyrate were measured by gas chromatography-mass spectrometry (Dr. Stein and Colleague Medical Laboratory, Mönchengladbach, Germany) as previously described [33]. Plasma sample preparation for circulating SCFA analysis were performed as reported previously [34]. In short, deproteinization was performed by mixing 1 part plasma (v/v) with 2 parts methanol acidified with 1.5 mmol/l hydrochloric acid. Subsequently, samples were vortex-mixed vigorously and immediately centrifuged at 50000xg in a model Biofuge Stratos (Hereaus, Dijkstra Vereenigde, Lelystad, the Netherlands) for 15 min at 4°C. Then, 100 µl aliquots of the clear plasma supernatant were transferred into glass micro-insert vials and stored in the Combi-Pal until analysis. Samples were calibrated against external standards. The reversed phase separation was performed on a X-select ODS 2.5 µm column (150mm x 2.1mm I.D., Waters, Breda, the Netherlands), mounted in a Mistral Spark column oven (Separations, H.I. Ambacht, the Netherlands), set to 45°C. Samples were completely separated from other components into the individual SCFA in a 25 min. gradient cycle between an aqueous 1 mmol/l solution of sulfuric acid and ethanol.

Post-column, the solvent pH was enhanced to about 9, by mixing with 150 mmol/l ammonia in ethanol to maximize negative ionization. Samples were processed using a Combi-Pal sample processor (Interscience, Breda, the Netherlands) with Peltier chilled sample storage compartments set to 10°C. The system was equipped with a 50 µl sample loop. Separated SCFA were detected using a model LTQ XL linear ion trap mass spectrometer (Thermo Fisher Scientific, Breda, the Netherlands), equipped with an ion-max electrospray probe. The MS was operated in MS-MS full scan negative mode."

Blood collection and biochemical analysis

Blood was collected in pre-chilled EDTA tubes (0.2 mol/L EDTA; Sigma, Dorset, UK) for SCFA, insulin, glucose, FFA, TAG, free glycerol, LBP, GLP-1, TNF-α, IL-6 and IL-8 analyses during fasting conditions. For GLP-1 and PYY analysis, 20 µl of dipeptidyl peptidase-IV inhibitor (Milipore Merck, Billerica, MA, USA) was added to EDTA and Aprotinin (Becton Dickinson, Eysins, Switzerland) tubes, respectively. Samples were centrifuged at 3500 g, 4 °C for 10 minutes; plasma was aliquoted and directly snap-frozen in liquid nitrogen and stored at -80 °C until analysis. Plasma glucose concentrations were determined using commercially available reagent kit (Glucose Hexokinase CP, Horiba ABX Pentra, Montpellier, France) involving a two-step enzymatic reaction with hexokinase followed by Glucose-6-phosphate-dehydrogenase resulting in D-gluconate-6-phosphate. The colorimetric reaction was measured using an automated spectrophotometer (ABX Pentra 400 autoanalyzer, Horiba ABX Pentra). Plasma FFA concentrations were measured using a commercially available kit (NEFA-HR(2) assay, Wako, Sopachem BV, Ochten, the Netherlands) with a two-step enzymatic reaction involving acylation of Coenzyme(Co) A followed by acyl-CoA oxidase resulting in the production of hydrogen peroxide as substrate that in the presence of peroxidase yields a blue purple pigment, measured with a colorimetric reaction measured using an automated spectrophotometer (ABX Pentra 400 autonalyzer, Horiba ABX Pentra). Plasma TAG were determined using a commercially available kit (Triglycerides CP, Horiba ABX Pentra) based on enzymatic reactions involving lipoprotein lipase, glycerolkinase and glycerol-3-phosphate oxidase resulting in the production of hydrogen peroxide as substrate of a colorimetric reaction measured using the automated spectrophotometer (ABX Pentra 400 autonalyzer, Horiba ABX Pentra). Plasma glycerol was measured after precipitation with an enzymatic assay (Enzytec TM Glycerol, Roche Biopharm, Basel, Switzerland) involving phosphorylation of glycerol to L-glycerol-3-phosphate by glycerokinase and the colorimetric reaction is measured

using an automated spectrophotometer (Cobas Fara, Roche Diagnostics, Basel, Switzerland). Plasma insulin was determined with a commercially available radioimmunoassay (RIA) kit (HI-14K Human Insulin specific RIA, Millipore Merck) according to the manufacture's protocol. Plasma IL 6, IL-8 and TNF-α were determined with an commercialy available enzyme-linked immunosorbent assay (ELISA) kit (Human Proinflammatory II 4-Plex Ultra-Sensitive kit, Meso Scale Diagnostics, MD, USA). Plasma samples were assayed for total GLP-1 immunoreactivity using an antiserum that reacts equally with intact GLP-1 and the primary (N-terminally truncated) metabolite as previously described [35].

PYY concentrations were determined using a commercially available radio-immunoassay (RIA) kits (Human PYY (3-36) Specific RIA, Millipore Merck). Plasma LBP was measured as previously described [36]. In short, plates (Greiner Mocrolon 600 high binding; Sigma Aldrich, St. Louis, MO) were coated with polyclonal anti-human LBP antibodies. Diluted plasma samples (1:5000) and a standard dilution series with recombinant LBP were added to the plate. Detection occurred with a biotinylated polyclonal rabbit anti-human LBP IgG, followed by peroxidase-conjugated streptavidin and substrate. The detection limit for the LBP assay was 200 pg/ml.

Statistical analysis

Normality of data was assessed with the Gaussian distribution and Kolmogorov-Smirnov procedure, and ln or Z-score transformation was used if assumption of normality was not met. HOMA-IR was calculated as previously described [37]. In case of missing data, the participant was excluded from the analysis. Multicollinearity was checked using variance inflation factor index < 10. First, we used simple linear regression to investigate the associations between fecal and circulating concentrations of acetate, propionate and butyrate (as dependent variables) and metabolic parameters (as independent variables) i.e. insulin sensitivity (M-value), insulin resistance (HOMA-IR), circulating glucose, insulin, circulating lipids (TAG, FFA and glycerol), circulating inflammatory markers (IL-6, IL-8, TNF-α and LBP) and fasting substrate oxidation. Subsequently, we used multiple linear regression to test whether the associations between fecal and circulating SCFA and the aforementioned metabolic parameters were independent of the covariates sex, age and BMI. All data were analysed using SPSS 22.0 (IBM, Armok, U.S.) with significance set at $P < 0.05$.

Results

Mean age of the participants was 49.6 ± 14.7 years and 66.3 % of participants were male with a mean BMI of 29.8 ± 4.4 kg/m^2, a mean fasting glucose of 5.6 ± 0.6 mmol/L and a mean HOMA-IR of 3.7 ± 1.5 (Table 1). In the subgroup, peripheral insulin sensitivity (M-value) was measured in 93 overweight to obese, prediabetic men (n= 72) and women (n=21) with mean age of 59.0 ± 7.1 years and a mean BMI of 31.8 ± 3.1 kg/m^2, respectively.

Table 1 Characteristics of participants

Characteristic	**Mean ± SD**	**Range**
Male (n) /Female (n)	106/54	
Age (y)	49.6 ± 14.7	20 – 69
Body Weight (kg)	90.8 ± 15.7	52.8 – 125.9
BMI (kg/m^2)	29.8 ± 4.4	19.2 – 41.0
Waist:Hip ratio	0.93 ± 0.13	0.64 – 1.22
HOMA-IR	3.7 ± 1.5	1.0 – 9.0
Fasting plasma insulin (mU/L)	14.9 ± 9.4	2.4 – 82.7
Fasting plasma glucose (mmol/L)	5.6 ± 0.6	3.1 – 7.5
Fasting plasma free fatty acids (µmol/L)	672 ± 243	140.3 – 1620
Fasting plasma triacylglycerol (µmol/L)	1178 ± 613	311.0 – 3944
Fasting free glycerol (µmol/L)	102.1 ± 46	27.1 – 372.1
Plasma acetate (µmol/L)	65.2 ± 64.7	2.8 – 429.4
Plasma propionate (µmol/L)	2.0 ± 1.6	0.06 – 12.0
Plasma butyrate (µmol/L) **Fecal acetate (µmol/g)** **Fecal propionate (µmol/g)** **Fecal butyrate (µmol/g)**	1.7 ± 1.8 44.2 ± 16.1 13.0 ± 7.9 10.9 ± 5.9	0.07 – 6.7 6.80 – 102.8 2.53 – 71.2 0.00 – 33.8
Subgroup hyperinsulinaemic-euglycaemic clamp		
Male (n) /Female (n)	72/21	
BMI (kg/m^2)	31.8 ± 3.1	26.1- 41
Age (y)	59.0 ± 7.1	72 – 69
Fasting glucose (mmol/L)	6.0 ± 0.5	5.0 – 7.5
2h glucose (mmol/L)	7.2 ± 1.8	3.3 – 11.2
M-value (mg*kg^{-1}*min^{-1})	3.6 ± 1.5	1.7 – 8.3

BMI body mass index, HOMA-IR Homeostasis model assessment of insulin resistance, M-value mean glucose infusion rate at steady state during insulinemic- euglycemic clamps

Associations between fecal and circulating SCFA concentrations

Fecal acetate and butyrate were not associated to their respective circulating concentrations, while fecal propionate was positively associated with circulating propionate (standardized (std) std $\beta = 0.262$, $P = 0.002$).

Circulating, but not fecal, SCFA are associated with BMI

Fecal acetate was positively associated with BMI (std $\beta = 0.245$, $P = 0.004$), however, after adjustment for age and sex, the association was not significant anymore (std β adj $= 0.092$, $P = 0.214$). Fecal propionate and butyrate were not significantly associated with BMI with or without adjustment for age and sex (fecal butyrate std $\beta = 0.162$, $P = 0.055$, fecal propionate std $\beta = 0.023$, $P = 0.746$). In contrast, circulating butyrate and propionate were significantly inversely related to BMI, also after adjustment for age and sex (circulating butyrate std $\beta = -0.599$ $P<0.001$, circulating propionate std $\beta = -0.290$, $P<0.001$, Supplementary Figure 1 K, L). Circulating acetate was negatively associated with BMI (std $\beta = -0.285$, $P<0.001$), which was not significant anymore after adjustment for age and sex (std $\beta = -0.115$, $P = 0.108$).

Fecal SCFA were not related to metabolic parameters

None of fecal SCFA were significantly associated with fasting GLP-1, PYY, FFA, TAG, glycerol, glucose, insulin concentrations, HOMA-IR, inflammatory markers or fasting substrate oxidation either with or without adjustment for age, sex and BMI. In the subgroup analysis fecal SCFA were not associated with peripheral insulin sensitivity (Table 2).

Table 2 Simple and multiple linear regression coefficients between fecal SCFA and metabolic parameters in fasting state

	Fecal acetate				**Fecal propionate**				**Fecal butyrate**			
Metabolites	*std ß*	*P*	*std β adj*	*P*	*std ß*	*P*	*std β adj*	*P*	*std ß*	*P*	*std β adj*	*P*
FFA, µmol/L	0.052 ± 0.090	.569	-0.048 ± 0.082	.558	-0.098 ± 0.089	.276	-0.092 ± 0.080	.249	0.001 ± 0.090	.991	-0.030 ± 0.081	.707
TAG, µmol/L	0.058 ± 0.090	.519	-0.057 ± 0.084	.498	0.129 ± 0.089	.151	0.037 ± 0.081	.647	0.094 ± 0.090	.296	-0.007 ± 0.082	.931
Glycerol, µmol/L	-0.047 ± 0.090	.603	-0.152 ± 0.086	.079	-0.019 ± 0.090	.836	-0.022 ± 0.084	.797	-0.185 ± 0.145	.205	-0.160 ± 0.084	.059
Hormones												
Insulin, mU/L	0.275 ± 0.082	.001	0.122 ± 0.071	.088	0.185 ± 0.084	.029	0.114 ± 0.069	.101	0.207 ± 0.084	.015	0.102 ± 0.070	.146
GLP-1, pmol/L	-0.081 ± 0.092	.379	0.114 ± 0.074	.125	0.017 ± 0.092	.852	0.140 ± 0.071	.053	-0.037 ± 0.092	.692	0.116 ± 0.072	.113
PYY, pmol/L	-0.194 ± 0.106	.070	-0.166 ± 0.112	.140	-0.093 ± 0.107	.386	-0.087 ± 0.110	.428	-0.156 ± 0.106	.146	-0.142 ± 0.110	.199
Insulin sensitivity												
M-value, mg/kg/min	-0.071 ± 0.106	.502	-0.035 ± 0.075	.640	-0.029 ± 0.105	.783	-0.050 ± 0.073	.490	-0.100 ± 0.105	.345	-0.111 ± 0.073	.130
HOMA-IR	0.267 ± 0.084	.002	0.080 ± 0.068	.244	0.193 ± 0.085	.025	0.091 ± 0.066	.169	0.214 ± 0.085	.013	0.076 ± 0.067	.254
Glucose, mmol/L	0.174± 0.084	.040	-0.029 ± 0.067	.661	0.112 ± 0.084	.187	0.006 ± 0.065	.925	0.086 ± 0.085	.310	-0.066 ± 0.066	.315
Inflammatory markers												
LBP, pg/ml	0.152 ± 0.091	.095	0.033 ± 0.086	.699	0.024 ± 0.091	.789	-0.010 ± 0.084	.909	0.155 ± 0.090	.088	0.092 ± 0.084	.274
IL-6, pg/ml	0.164 ± 0.085	.055	-0.010 ± 0.071	.892	0.012 ± 0.085	.887	-0.088 ± 0.069	.204	0.039 ± 0.085	.651	-0.094 ± 0.069	.178
IL-8, pg/ml	0.157 ± 0.085	.067	0.106 ± 0.088	.231	0.164 ± 0.084	.053	0.118 ± 0.085	.170	0.115 ± 0.085	.179	0.061 ± 0.086	.479
TNF-α, pg/ml	0.196 ± 0.084	.021	0.070 ± 0.078	.170	0.063 ± 0.085	.459	-0.046 ± 0.076	.548	0.156 ± 0.084	.067	0.039 ± 0.076	.612
Substrate Oxidation												
Fat, E%	0.036 ± 0.092	.691	0.001 ± 0.095	.996	-0.002 ± 0.091	.982	0.004 ± 0.093	.970	0.013 ± 0.092	.885	0.004 ± 0.094	.969
CHO, E %	-0.092 ± 0.094	.329	-0.084 ± 0.098	.397	-0.034 ± 0.094	.716	-0.042 ± 0.096	.666	-0.110 ± 0.094	.243	-0.116 ± 0.096	.233

β, standardized β coefficient + standard error of coefficient of fecal acetate, propionate and butyrate as dependent variable in a simple regression and multiple regression analysis adjusted for age, sex and BMI. M-value (n=93), PYY (n=107). Adj, adjusted, std standardized, E%, percentage of energy expenditure, FFA, free fatty acids, TAG, triglycerides, GLP-1, glucagon-like peptide 1, PYY, peptide YY, HOMA-IR, homeostatic model assessment of insulin resistance, LBP, lipopolysaccharide binding protein, IL-6, interleukin 6, IL-8, interleukin 8, TNFα, tumour necrosis factor alpha.

Table 3 Simple and multiple linear regression coefficients between circulating SCFA and metabolic parameters in fasting state

	Circulating acetate				**Circulating propionate**				**Circulating butyrate**			
Metabolites	*std ß*	*P*	*std β adj*		*std ß*	*P*	*std β adj*		*std ß*	*P*	*std β adj*	*P*
FFA, µmol/L	-0.032 ± 0.084	.701	0.003 ± 0.080	.974	-0.302 ± 0.079	.000	-0.127 ± 0.078	.103	-0.381 ± 0.077	<.001	-0.306 ± 0.090	**.001**
TAG, µmol/L	-0.249 ± 0.081	.003	-0.077 ± 0.081	.340	-0.225 ± 0.081	.006	-0.202 ± 0.078	**.010**	-0.356 ± 0.078	<.001	-0.137 ± 0.094	.150
Glycerol, µmol/L	-0.204 ± 0.082	.014	-0.190 ± 0.083	**.023**	-0.025 ± 0.083	.768	0.155 ± 0.081	.059	-0.286 ± 0.080	<.001	-0.173 ± 0.098	.079
Hormones												
Insulin, mU/L	-0.187 ± 0.079	.019	0.003 ± 0.070	.968	-0.234 ± 0.078	.003	-0.066 ± 0.068	.336	-0.406 ± 0.073	<.001	-0.040 ± 0.082	.625
GLP-1, pmol/L	0.402 ± 0.078	.000	0.187 ± 0.070	**.009**	0.318 ± 0.080	.000	0.218 ± 0.068	**.002**	0.574 ± 0.069	<.001	0.274 ± 0.081	**.001**
PYY, pmol/L	0.125 ± 0.097	.201	0.107 ± 0.107	.317	0.080 ± 0.097	.413	0.018 ± 0.105	.868	0.182 ± 0.096	.060	0.142 ± 0.126	.263
Insulin sensitivity												
M-value, mg/kg/min	-0.224 ± 0.102	.031	-0.294 ± 0.071	**<.001**	0.327 ± 0.099	.001	0.161 ± 0.074	**.033**	0.164 ± 0.103	.113	-0.066 ± 0.091	.469
HOMA-IR	-0.247 ± 0.079	.002	-0.009 ± 0.067	.892	-0.160 ± 0.080	.047	0.007 ± 0.065	.914	-0.461 ± 0.072	<.001	-0.054 ± 0.078	.494
Glucose, mmol/L	-0.228 ± 0.078	.004	0.005 ± 0.066	.939	-0.177 ± 0.078	.025	-0.044 ± 0.064	.499	-0.506 ± 0.068	<.001	-0.200 ± 0.075	**<.001**
Inflammatory markers												
LBP, pg/ml	-0.075 ± 0.088	.399	0.038 ± 0.087	.667	-0.262 ± 0.085	.003	-0.117 ± 0.085	.168	-0.330 ± 0.083	<.001	-0.116 ± 0.102	.254
IL-6, pg/ml	-0.298 ± 0.077	.000	-0.098 ± 0.070	.163	-0.177 ± 0.079	.027	-0.027 ± 0.053	.690	-0.406 ± 0.073	.000	-0.021 ± 0.082	.803
IL-8, pg/ml	-0.079 ± 0.080	.324	0.024 ± 0.086	.778	0.126 ± 0.079	.113	0.164 ± 0.084	.051	-0.023 ± 0.080	.775	0.185 ± 0.100	.067
TNF-α, pg/ml	-0.296 ± 0.076	.000	-0.105 ± 0.076	.168	-0.095 ± 0.079	.232	-0.048 ± 0.075	.523	-0.330 ± 0.075	<.001	-0.047 ± 0.089	.598
Substrate Oxidation												
Fat, E%	-0.156 ± 0.084	.065	-0.021 ± 0.086	.812	-0.117 ± 0.084	.167	-0.052 ± 0.085	.543	-0.317 ± 0.080	<.001	-0.138 ± 0.101	.172
CHO, E %	0.074 ± 0.087	.396	0.159 ± 0.094	.094	-0.092 ± 0.087	.291	-0.167 ± 0.092	.072	0.002 ± 0.087	.986	0.047 ± 0.111	.672

β, standardized β coefficient + standard error of coefficient of fasting circulating acetate, propionate and butyrate as dependent variable in a simple regression and multiple regression analysis adjusted for age, sex and BMI. M-value (n=93), PYY (n=107). Adj, adjusted, std standardized, E%, percentage of energy expenditure, FFA, free fatty acids, TAG, triglycerides, GLP-1, glucagon-like peptide 1, PYY, peptide YY, HOMA-IR, homeostatic model assessment of insulin resistance, LBP, lipopolysaccharide binding protein, IL-6, interleukin 6, IL-8, interleukin 8, TNFα, tumour necrosis factor alpha.

Fasting, circulating SCFA were related to fasting GLP-1, lipid metabolites and insulin sensitivity

All three circulating SCFA were positively associated with fasting GLP-1 concentrations (Table 3). Additionally, circulating acetate, propionate and butyrate were negatively associated with fasting glycerol, TAG and FFA, respectively. Also, circulating butyrate was negatively associated with fasting glucose. These relationships remained significant after adjustment for age, sex and BMI (Table 3, Supplementary Figure 1).
Circulating SCFA were not associated with fasting PYY, LBP, IL-6, IL-8 and TNF-α. Furthermore, circulating SCFA were not related to fat and carbohydrate oxidation, expressed as percentage of energy expenditure. In the subgroup analysis of overweight/obese, prediabetic individuals,

peripheral insulin sensitivity was measured using the M-value derived from the hyperinsulinaemic- euglycaemic clamp technique.

We found that circulating acetate was negatively associated with peripheral insulin sensitivity (M-value) whereas circulating propionate was positively related to peripheral insulin sensitivity (Table 3, Supplementary Figure S1). The relationships between circulating SCFA and insulin sensitivity remained significant after adjustment for age, sex and BMI.

Discussion

We investigated the relationship between fecal and fasting circulating SCFA with fasting plasma metabolites, gut hormones, substrate metabolism and inflammatory markers in a cohort with a wide range of BMI and glucometabolic status. This study shows that only circulating but not fecal SCFA concentrations were related to fasting plasma glucose, FFA, TAG and glycerol, GLP-1 and insulin sensitivity, also after adjustment for age, sex and BMI. Contrary to previous human studies, fecal SCFA were not related to BMI, whereas circulating butyrate and propionate were inversely associated with BMI. Circulating plasma propionate seems to be the most reflective of its respective fecal concentrations, whilst fecal acetate and butyrate were not related to their respective circulating concentrations. In line, previous literature reports that SCFA flux into the circulation and uptake in peripheral tissues rather than microbial SCFA production *per se* is of importance for metabolic health [38-40]. Our data emphasize the need to measure circulating SCFA in human prebiotic/probiotic intervention studies as a biomarker/mediator of effects on host metabolism.

To our knowledge, this is the first study providing evidence that fasting circulating SCFA are positively associated with fasting plasma GLP-1 in humans. High colonic SCFA production is linked to increased GLP-1 and PYY secretion through binding of SCFA to GPR41/43 on the enteroendocrine L-cell [41]. Further, a one year dietary fiber intervention (wheat bran, 24g/d) increased circulating SCFA concentrations accompanied by increased levels of GLP-1 concentrations in hyperinsulinemic participants [42]. Yet, there is little known about the contribution of circulating SCFA to GLP-1 secretion during the fasted state. Circulating SCFA may stimulate GLP-1 secretion from the visceral, basolateral side of enteroendocrine L-cells as observed in isolated rat colons [43]. Besides enteroendocrine L-cells, pancreatic α-cells have been suggested to contribute to systemic GLP-1 concentrations in the fasted state [44, 45], but whether circulating SCFA act as stimuli for GLP-1 secretion warrants further investigation.

In contrast to GLP-1, we did not find an association between circulating and fecal SCFA with fasting PYY. This is in contrast to human and *in vitro* studies reporting a stimulatory effect of SCFA on PYY secretion [12, 46, 47], however to what extent SCFA and/or dietary fibres contribute to fasting PYY secretion remains to be investigated. Although the mechanisms still remain to be elucidated, the present data indicate that despite being the net result of production, uptake and tissue utilization, circulating SCFA are more directly linked to metabolic health as compared to fecal SCFA.

In our study population, only circulating, but not fecal SCFA were associated with fasting plasma metabolites. Circulating acetate was negatively associated with fasting free glycerol, an indicator of whole-body lipolysis. This is consistent with *in vitro* and human *in vivo* studies reporting that acetate has an antilipolytic effect [13, 48-50]. This may be beneficial for metabolic health in the long term, since partial inhibition of adipose tissue lipolysis may reduce systemic lipid spillover thereby attenuating ectopic lipid accumulation [51]. Furthermore, circulating propionate was negatively associated with fasting TAG, which might be explained by the activating effect of propionate on lipoprotein lipase (LPL) in adipose tissue leading to increased TAG extraction as shown *in vitro* [52]. Furthermore, circulating butyrate was negatively associated with fasting FFA concentrations. *In vitro* data about the lipolytic effect of butyrate are contradictive showing pro- and antilipolytic effects of butyrate in white adipose tissue models [50, 53]. Thus, circulating SCFA may be negatively related to systemic glycerol or FFA and/or TAG suggesting that increased circulating SCFA may reduce systemic lipid overflow with a potential beneficial effect on ectopic lipid accumulation and insulin sensitivity.

Nevertheless, with respect to markers of insulin sensitivity, neither fasting circulating nor fecal SCFA were related to fasting insulin or HOMA-IR in the total study population. Yet, fasting circulating butyrate, but not acetate and propionate, was negatively associated with fasting glucose. This is consistent with rodent studies showing that butyrate administration may have glucose lowering effects and may improve insulin sensitivity in the postprandial state [54, 55]. In obesity, insulin resistance and T2DM, the abundance of butyrate-producing bacteria is reduced, which may explain to some extent the inverse association between circulating butyrate and fasting glucose in our study [56-58].

In the subgroup analysis including prediabetic individuals with obesity, circulating acetate was negatively associated with peripheral insulin sensitivity. This is in contrast with previous rodent studies reporting a beneficial role of acetate on insulin sensitivity [47] and with two small-scale human cross-sectional studies including obese women or morbidly obese individuals reported either none or a positive association of circulating acetate and insulin sensitivity measured via hyperinsulinemic-euglycemic clamp, respectively [59, 60]. Additionally, when acetate is administered colonically, overweight participants showed increases in fasting fat oxidation, energy expenditure, and PYY secretion [12, 13], reflective of positive effects on metabolic health. Interestingly, a kinetic study showed that intravenously infused acetate remains longer in the circulation in individuals with T2DM suggesting a disturbed acetate tissue uptake and metabolism in the context of metabolic disorders [47]. Furthermore, exogenous and endogenous acetate production but not colonic acetate absorption differed between hyperinsulinemic and normoinsulinemic individuals after rectal infusion of sodium-acetate [49, 61, 62]. Thus, our findings may reflect an altered endogenous acetate metabolism rather than an altered microbial-derived acetate production in metabolically compromised individuals. In contrast to fasting circulating acetate, fasting circulating propionate was positively associated with clamp-derived insulin sensitivity. Propionate has been reported to stimulate glucose uptake in 3T3-L1 adipocytes and C2C12 skeletal muscle cells *in vitro* and improve insulin sensitivity (HOMA-IR) in mice fed a high fat diet [63, 64]. Possible mechanisms include an increase in peripheral glucose uptake via increased GPR41 stimulation, suppression of hepatic *de novo* lipogenesis and increase formation of beneficial odd chain fatty acids in the liver [65].

The main limitation of our study is the cross-sectional design, which limits causal suppositions. Further, we cannot account for endogenous SCFA production, splanchnic and liver extraction or tissue utilization in this study [66, 67]. Secondly, measures of GLP-1 and SCFA in the postprandial state would have been valuable. However, the study's major strength is the availability of fecal and fasting circulating SCFA in combination with metabolic markers in a relatively large cohort with a broad range of BMI and metabolic health status. This enabled us to investigate the relationship between fecal and fasting circulating SCFA concentrations with markers of lipid and energy metabolism as well as insulin sensitivity measured by the gold standard hyperinsulinemic-euglycemic clamp.

For the first time, we confirmed that fasting circulating but not fecal SCFA were related to whole-body lipolysis, fasting GLP-1 and insulin sensitivity in the fasted state. Furthermore, our study calls for urgently needed mechanistic studies in humans concerning the relationship between SCFA, GLP-1 secretion and lipid metabolism.

In conclusion, our data show that circulating but not fecal SCFA are linked to circulating GLP-1 concentrations, whole-body lipolysis and peripheral insulin sensitivity in humans. Of note, this highlights that circulating SCFA are more directly linked to metabolic heath parameters. Therefore, our data indicate the need to measure circulating SCFA as a biomarker/mediator of effects on host metabolism in future human prebiotic/probiotic intervention studies. This may provide interesting leads for future research, which should aim to modulate the SCFA availability in the systemic circulation and its impact on peripheral tissue function.

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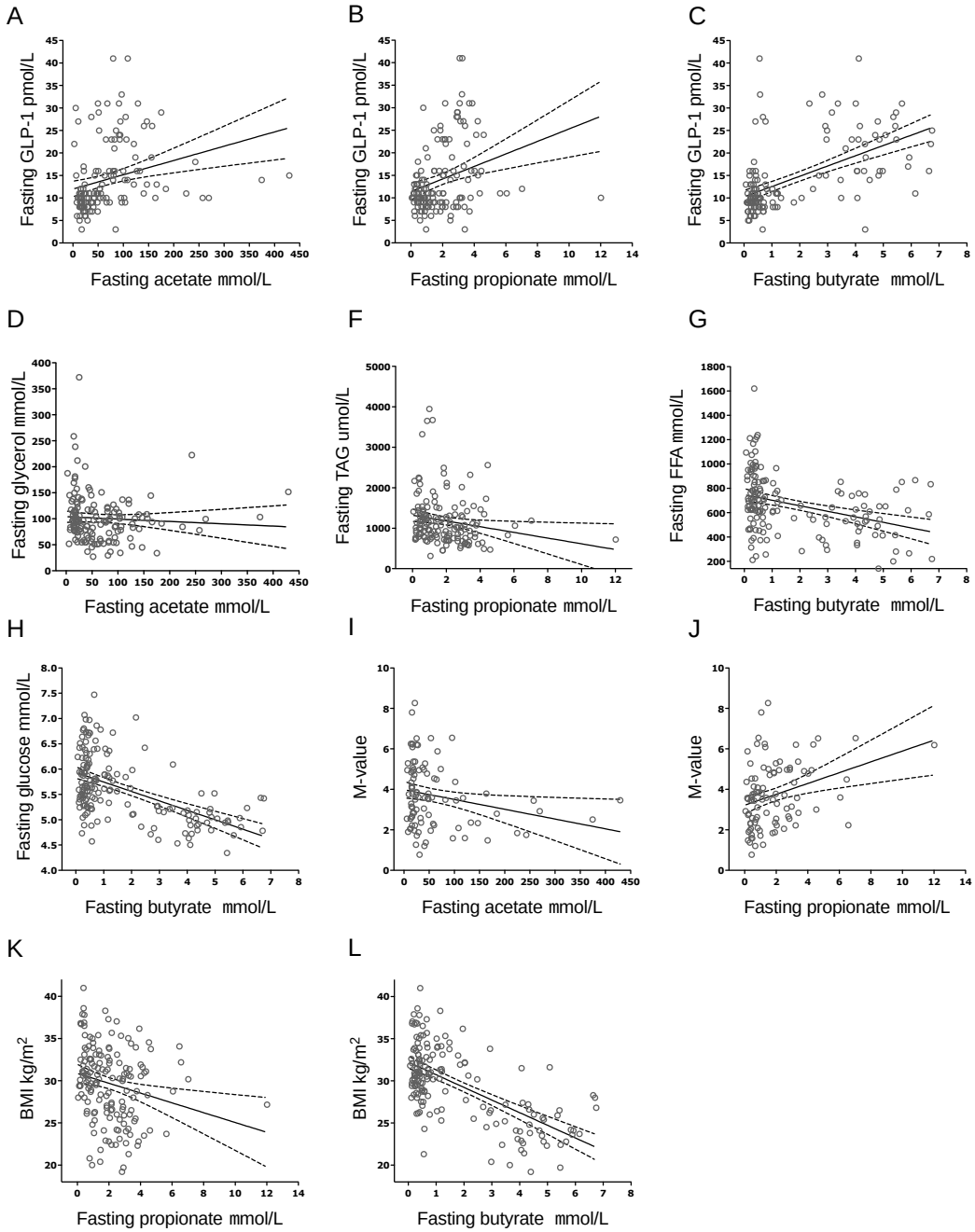
Supplementary Material



Supplementary Figure 1 Associations between metabolic parameters, BMI and insulin sensitivity and fasting circulating SCFA. Linear regression scatter plots of fasting circulating SCFA with fasting GLP-1 (**A-C**), fasting acetate with fasting glycerol (**D**), fasting propionate with fasting TAG (**F**), fasting butyrate with FFA (**G**) and fasting glucose (**H**), fasting acetate with M-value (**I**), fasting propionate with M-value (**J**) and BMI (**K**) and fasting butyrate with BMI (**L**). GLP-1 glucagon like peptide 1, TAG triacylglycerol, FFA free fatty acids, BMI body mass index

CHAPTER 5

Fecal microbial metabolites of proteolytic and saccharolytic fermentation in relation to degree of insulin resistance in overweight and obese individuals

Running title: Microbial metabolites, obesity and insulin resistance

Manuel A. González Hernández, Emanuel E. Canfora and Ellen E. Blaak.

To be submitted

CHAPTER 6

Acetate does not affect palmitate oxidation and AMPK phosphorylation in human primary skeletal muscle cells

Running title: Acetate and fat oxidation in human muscle cells

Manuel A. González Hernández, Ellen E. Blaak, Nicole T. H. Hoebers, Yvonne P. G. Essers, Emanuel E. Canfora† and Johan W. E. Jocken†

† shared last authors

To be submitted

CHAPTER 7

Short-chain fatty acids differentially affect intracellular lipolysis in a human white adipocyte model

Running Title: SCFA and intracellular lipolysis

Johan W.E. Jocken, Manuel A. González Hernández, Nicole T.H. Hoebers, Christina M. van der Beek, Yvonne P.G. Essers, Ellen E. Blaak, Emanuel E. Canfora

Front. Endocrinol., 11 January 2018; 8:372

Abstract

Background & Aims: Gut-derived short-chain fatty acids (SCFA), formed by microbial fermentation of dietary fibres, are believed to be involved in the aetiology of obesity and diabetes. Previous data from our group showed that colonic infusions of physiologically relevant SCFA mixtures attenuated whole-body lipolysis in overweight men. To further study potential mechanisms involved in the antilipolytic properties of SCFA, we aimed to investigate the *in vitro* effects of SCFA incubations on intracellular lipolysis and signalling using a human white adipocyte model, the hMADS cells.
Methods: hMADS adipocytes were incubated with mixtures of acetate, propionate and butyrate or single SCFA (acetate, propionate and butyrate) in concentrations of 1 μmol/L and 1 mmol/L. Glycerol release and lipase activation was investigated during basal conditions and following β-adrenergic stimulation.
Results: SCFA mixtures high in acetate and propionate decreased basal glycerol release, when compared to control (P<0.05), whilst mixtures high in butyrate had no effect. Also, β-adrenergic receptor mediated glycerol release was not significantly altered following incubation with SCFA mixtures. Incubation with only acetate decreased basal (1 μmol/L) and β-adrenergically (1 μmol/L and 1 mmol/L) mediated glycerol release as compared to control (P<0.05). In contrast, butyrate (1 μmol/L) slightly increased basal and β-adrenergically mediated glycerol release compared with control (P<0.05), whilst propionate had no effect on lipolysis. The antilipolytic effect of acetate was accompanied by a reduced phosphorylation of hormone sensitive lipase (HSL) at serine residue 650. In addition, inhibition of Gi G proteins following pertussis toxin treatment prevented the antilipolytic effect of acetate.
Conclusion: The present data demonstrated that acetate was mainly responsible for the antilipolytic effects of SCFA and acts via attenuation of HSL phosphorylation in a Gi coupled manner in hMADS adipocytes. Therefore, the modulation of colonic and circulating acetate may be an important target to modulate human adipose tissue lipid metabolism.

Introduction

Increasing evidence suggests that the human gut microbiota and its products are key players in host metabolism, body weight, and insulin sensitivity, thereby contributing to the aetiology of obesity and related disorders [1]. The gut microbiota can ferment indigestible nutrients into short-chain fatty acids (SCFA), of which acetate, propionate and butyrate are the most abundant [2]. Of note, these SCFA can be taken up by the epithelial lining of the gut and released into the blood stream [3]. They may thereby act as important signalling molecules between gut microbiota and host physiology, by exerting effects on energy and substrate metabolism such as on adipogenesis and lipolysis in the adipose tissue [4].

Disturbances in adipose tissue function, characterised by a reduced capacity to store lipids, seem to play a major role in the development of insulin resistance and type 2 diabetes mellitus in humans [5]. Under normal healthy conditions, the adipose tissue is an important buffering organ for daily postprandial fatty acid (FA) fluxes when endogenous lipolysis is inhibited. The adipose tissue thereby prevents excessive supply of lipids to nonadipose tissues such as liver, skeletal muscle and pancreas. This buffering action may be impaired under obese insulin resistant conditions [6, 7], resulting in increased circulating lipids and ectopic fat storage in nonadipose tissues, thereby provoking disturbances in insulin signalling and substrate metabolism [8, 9].

SCFA may affect adipose tissue lipid buffering capacity by affecting intracellular lipolysis, the process of hydrolysis of stored triacylglycerol into one molecule of glycerol and three FA molecules, and may thereby affect circulating lipid concentrations [4]. Indeed, already decades ago, a decrease in plasma free fatty acids (FFA) after a single oral acetate ingestion was observed, pointing to an antilipolytic role of acetate [10]. The more recent identification of the two pertussis toxin (PTX)-sensitive inhibitory G (Gi) protein-coupled receptors (GPR) for SCFA, Free Fatty Acid Receptor 3 (FFAR3, also known as GPR41) and FFAR2 (also known as GPR43) in human adipose tissue [11], has led to renewed interest in the lipolytic properties of SCFA. In addition, a direct association between the SCFA/FFAR signalling pathway and lipolytic activity in murine adipocytes was recently discovered [12]. Treatment of differentiated murine 3T3-L1 adipocytes with acetate and propionate in a range between 0.1-0.3 mmol/L exhibited FFAR2 activation and a reduction in intracellular lipolytic activity as assessed by a decreased release of glycerol in the culture medium [12]. In contrast, incubation of 3T3-L1 adipocytes with supraphysiological concentrations of propionate (20 mmol/L) or butyrate (5 mmol/L) resulted in enhanced glycerol release [13]. However, in murine 3T3-L1 adipocytes only FFAR2, and not FFAR3, is expressed [14-17]. Therefore, it remains to be determined, whether these findings extend to human adipocytes, in which both FFAR3 and FFAR2 are expressed [11, 18]. Thus, further investigation on the role of SCFA in human adipocyte lipolysis is urgently warranted.

We recently observed that colonic infusions of mixtures of acetate, propionate and butyrate, in ratios and concentrations that can be reached after dietary fibre intake, attenuated whole-body lipolysis in overweight normoglycaemic men [19]. Therefore, the aim of the present study was to elucidate whether an altered intracellular adipocyte lipolytic rate is responsible for the antilipolytic effect of SCFA found *in vivo*, as well as to further investigate underlying mechanisms. Hence, we investigated the *in vitro* effects of incubation with SCFA mixtures and single SCFA on intracellular lipolysis in a human white adipocyte model, the human multipotent adipose tissue-derived stem (hMADS) cells. To study whether these effects are mediated via Gi-coupled receptors, we investigated the effect of SCFA on lipase activation and performed PTX-mediated inhibition of FFARs.

Material and Methods

Cell culture

hMADS cells, a validated human white adipocyte model to study lipid metabolism [20], were obtained from human subcutaneous adipose tissue biopsies and differentiated into the adipogenic lineage. As described previously by Jocken *et al.* [21], cells were seeded at a density of 2000 cells per cm^2 and kept in proliferation medium (Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 Nutrient Mixture (Gibco, Blijswijk, NL, USA), 10% fetal bovine serum (Bodinco BV, Alkmaar, NL, USA) and 50 units per ml penicillin (Gibco), 50 μg/ml of streptomycin (Gibco)). At 70–80% confluence 250 μmol/L IBMX (Sigma, St Louis, MI, USA) and 5 μmol/L rosiglitazone (Enzo Life Sciences, Raamsdonksveer, NL, USA) were added to induce adipogenic differentiation. The lipolytic experiments were carried out between days 12–14 of the differentiation.

Pooled hMADS cells were derived from male human donors with a large range in BMI (20-40 kg/m^2) and glucometabolic status. The male donors were aged between 35-70 years and participated in two different clinical trials performed (ClinicalTrials.gov, NCT02241421 and NCT02598544). The study protocols were approved by the Medical Ethical Committee Jessa hospital, Hasselt and Hasselt University, Belgium, and by the Medical Ethical Committee of Maastricht University Medical Center, Maastricht, The Netherlands. All procedures were according to the declaration of Helsinki (revised version, October 2008).

Lipolysis experiment

Free glycerol release analysis

To study effects of SCFA mixtures on the basal glycerol release, hMADS adipocytes were incubated for 6 hours with 300 μL DMEM 3 % fatty-acid free BSA (Sigma-Aldrich, St. Louis, United States) supplemented with or without a mixture high in acetate containing 80 % acetate, 10 % propionate and 10 % butyrate (80:10:10), a SCFA mixture containing 60 % acetate, 20 % propionate and 20 % butyrate (60:20:20), a mixture high in propionate containing 40 % acetate, 35 % propionate and 25 % butyrate (40:35:25) and a mixture high in butyrate containing 40 % acetate, 25 % propionate and 35 % butyrate (40:25:35) in final concentrations of 1 mmol/L or 1 μmol/L for 6 hours.

To study effects of single SCFA on the basal (non-stimulated) glycerol release, hMADS adipocytes were incubated with 300 μL DMEM 3% fatty-acid free BSA (Sigma-Aldrich) supplemented with or without acetate (Merck, Darmstadt, Germany), propionate (Sigma Aldrich) or butyrate (Sigma Aldrich) at a final concentration of 1 mmol/L and 1 μmol/L concentrations for 6 hours.

To examine effects of single SCFA or SCFA mixtures on the β-adrenergic receptor mediated glycerol release, 30 minutes after the initiation of SCFA incubation the non-selective β-agonist isoprenaline (ISO) was added at a final

concentration of 1 μmol/L.

After 6 hours incubation, the plates were placed on ice to stop the reactions, and subsequently 250 μL supernatant was removed and directly snap-frozen in liquid nitrogen and stored at - 80 °C until analysis. The glycerol concentrations were quantified using a commercial fluorometric assay (EnzyChrome™ Adipolysis assay kit, BioAssay Systems, Hayward, United States).

Gene expression of FFAR3 and FFAR2 in hMADS adipocytes

To determine the FFAR3 and FFAR2 mRNA expression, total RNA was extracted from hMADS adipocytes at day 0, 2, 7, 10 and 12 using TRIzol reagent (Invitrogen) and SYBR-Green based real-time PCRs were performed using an iCylcer (Biolegio, Nijmegen, The Netherlands; primer sequences see Table 1). Results were normalized for 18S ribosomal RNA (calculating delta-delta Ct values). Ct values ranged from 27 – 33 for FFAR3/2 and 6-9 for 18S ribosomal RNA.

Table 1: Primer sequences

Gene	Forward (5′→3′)	Reverse (3′→5′)
18S	AGTTAGCATGCCAGAGTCTCG	TGCATGGCCGTTCTTAGTTG
FFAR3 (GPR41)	TTCACCACCATCTATCTCACCG	GGAACTCCAGGTAGCAGGTC
FFAR2 (GPR43)	CCGTGCAGTACAAGCTCTCC	CTGCTCAGTCGTGTTCAAGTATT

Abbreviations: FFAR, free fatty acid receptor; GPR, G-protein coupled receptor.

Western blotting

To study the effects of acetate on the protein expression and activation (phosphorylation) of the key lipolytic enzymes adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), hMADS adipocytes were incubated with 300 μL DMEM 3 % BSA supplemented with or without acetate at a final concentration of 1 μmol/L for one hour. In addition, 30 min after the start of the acetate incubation, ISO was added to the medium at a final concentration of 1 μmol/L to investigate the effects of acetate on the β-adrenergic receptor mediated HSL phosphorylation. Following one-hour incubation, cells were washed twice with ice-cold phosphate-buffered saline (PBS), and cells were homogenized in radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease and phosphatase inhibitor cocktail (Cell Signaling, Leiden, The Netherlands). 20 μg solubilized proteins were separated on a Criterion TGX precast gel (Bio-Rad), transferred using the Trans Blot Turbo transfer system (Bio-Rad), and incubated with primary antibodies. The HSL antibody was a kind gift from Prof. C. Holm (Lund University, Lund, Sweden). The ATGL (No.: 2138) and the pHSL at serine residue 650 (rat SER660) (No.: 4126) antibodies were both obtained from Cell Signaling Technology, Leiden, The Netherlands. To determine the FFAR3 (antibody No.: 103718, Abcam, Cambridge, UK) and FFAR2 (antibody No.: 131003, Abcam, Cambridge, UK) protein expression, total protein was extracted from

hMADS adipocytes at day 0, 2, 4, 7, 9, 11 and 14. Secondary antibodies (antibody No.: P0161, antibody No.: P0399).

Effect of inhibition of Gi proteins using pertussis toxin

To study the putative involvement of the Gi-type G-protein coupled FFARs in the acetate-induced inhibition of the lipolytic response, we incubated hMADS adipocytes for 6 hours with acetate in a concentration of 1 μmol/L during basal and ISO stimulated conditions (see protocol above), with or without supplementation of PTX for the whole 6 hours to the medium at a final concentration of 100 ng/mL (No.: P7208, Sigma Aldrich). The PTX experiments were conducted according the Guidelines of the European Chemicals Agency (Helsinki, Finland).

Statistical analysis

Values are expressed as mean ± SD. Significance was determined using the nonparametric Mann Whitney U-test when comparing two groups (single SCFA, western blotting and PTX experiment) or the Kruskal-Wallis H-test when comparing more groups (SCFA mixtures). In case of significant Kruskal-Wallis H-test, Dunns posthoc test was performed. Statistics were performed using the GraphPad Prism 5.0a software package (GraphPad Software, San Diego, CA, USA) and $P<0.05$ (two-sided P-value) was considered statistically significant.

Results

SCFA mixtures high in acetate and propionate decrease basal adipocyte glycerol release in vitro

We investigated whether the observed decrease in systemic glycerol found in our *in vivo* study [19] is related to an attenuated adipocyte intracellular lipolysis. Therefore, we incubated hMADS adipocytes for 6 hours with 1 μmol/L and 1 mmol/L of SCFA mixtures. The SCFA mixtures high in acetate and propionate (80:10:10, 60:20:20 and 40:35:25) decreased the basal (non-stimulated) glycerol release, as compared to control (P<0.05, Figure 1A). The SCFA mixture high in butyrate (40:25:35) did not significantly affect basal glycerol release (Figure 1A). In contrast to the decreased basal lipolysis, β-adrenergic receptor mediated glycerol release was not significantly affected following incubation with all SCFA mixtures in concentrations of 1 μmol/L and 1 mmol/L (Figure 1B).

Single SCFA differentially affect adipocyte glycerol release in vitro

Subsequently, we studied whether one particular SCFA was responsible for the observed antilipolytic effect. Therefore, we incubated hMADS adipocytes for 6 hours with acetate, propionate and butyrate in concentrations of 1 μmol/L and 1 mmol/L. Incubation with 1 μmol/L acetate decreased basal glycerol release when compared to control cells (P<0.05, Figure 2A). In addition, acetate blunted the β-adrenergic receptor mediated glycerol release in concentrations of 1 mmol/L and 1 μmol/L, when compared to control (P<0.05, Figure 2B). In contrast, 1 μmol/L butyrate treatment slightly increased basal (P<0.05, Figure 2A) and β- adrenergic receptor mediated glycerol release (P<0.01, Figure 2B), when compared to control treated cells. Neither in the basal state nor during β-adrenergic receptor stimulation, a significant difference between propionate and control treated adipocytes were observed (Figure 2A and B).

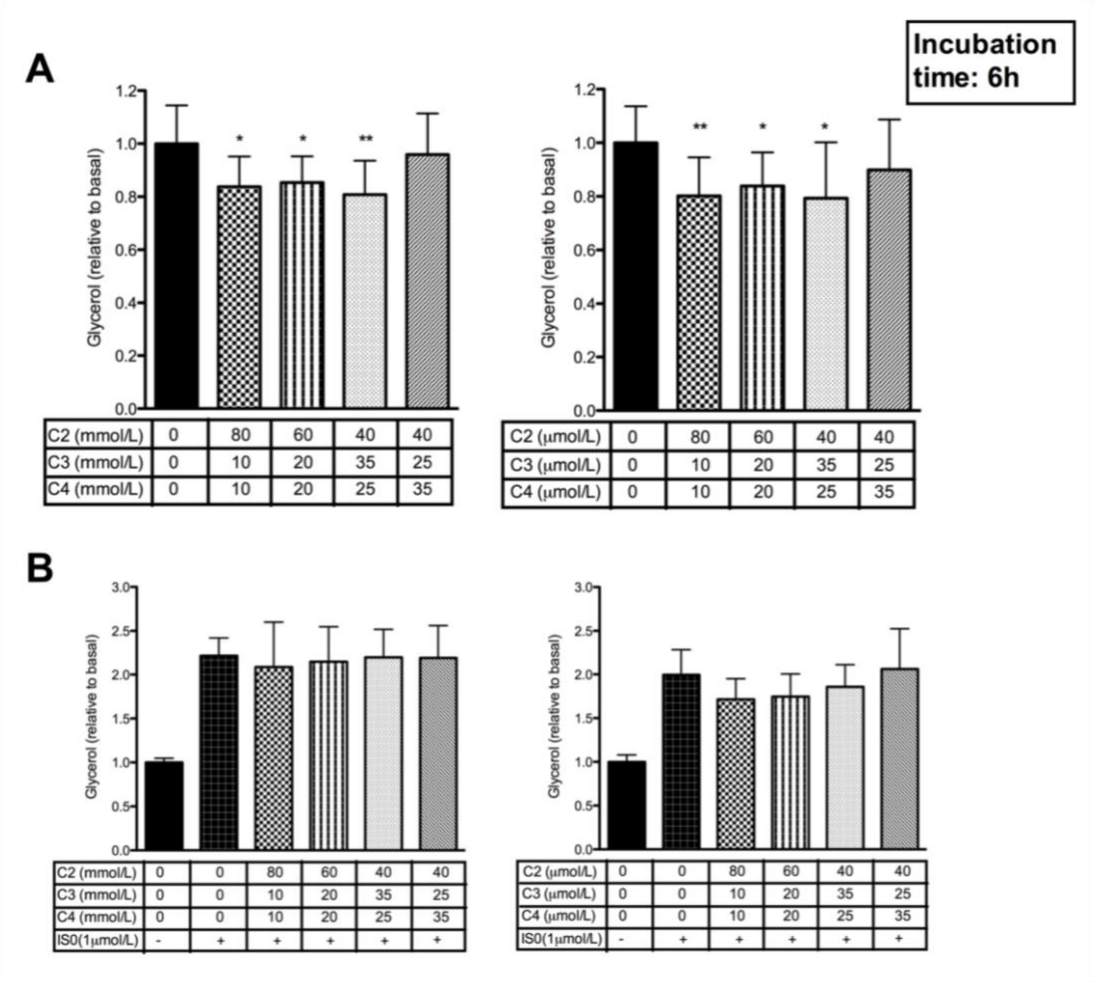


Figure 1. Effect of SCFA mixtures on basal and β-adrenergic receptor **stimulated glycerol release in hMADS adipocytes**

A: Basal (non-stimulated) glycerol concentrations during 6h incubation with 1 mmol/L or 1 µmol/L SCFA mixtures including acetate (C2), propionate (C3) and butyrate (C4)**; B:** Effect of 6h incubation with 1 mmol/L or 1 µmol/L SCFA mixtures including acetate (C2), propionate (C3) and butyrate (C4) on β-adrenergic receptor stimulated (1 µmol/L ISO) glycerol release; Values are given as means ± SD (n = 4-6 independent experiments). Statistical significance compared to basal indicated as asterisk (*) when $P<0.05$ and double asterisk (**) when $P<0.01$.

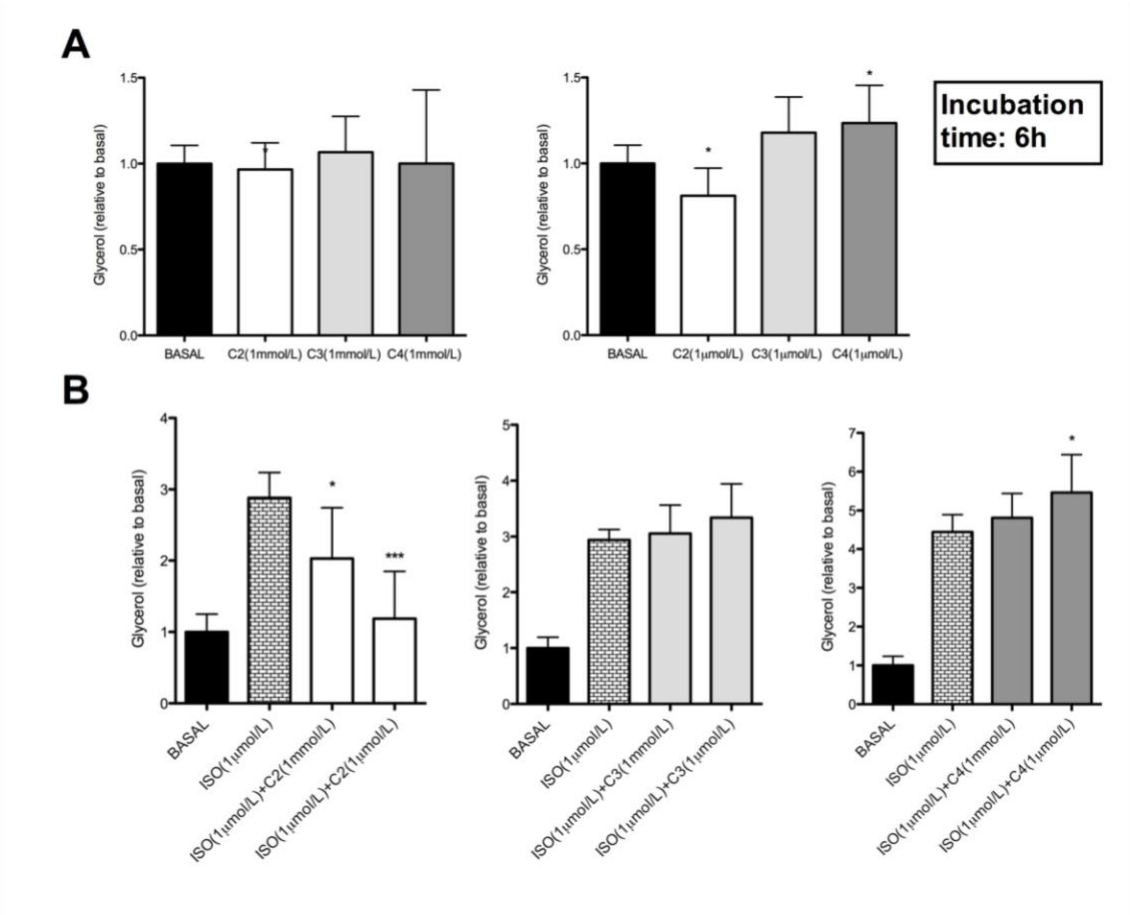


Figure 2. Effect of single SCFA on basal and β-adrenergic receptor **stimulated glycerol release in hMADS adipocytes**

A: Basal (non-stimulated) glycerol concentrations during 6h incubation with 1 mmol/L or 1 µmol/L acetate (C2), propionate (C3) or butyrate (C4); **B:** Effect of 6h incubation with 1 mmol/L or 1 µmol/L acetate (C2), propionate (C3) or butyrate (C4) on β-adrenergic receptor stimulated (1 µmol/L ISO) glycerol release; Values are given as means ± SD (n = 4-7 independent experiments). Statistical significance as compared to basal indicated as asterisk (*) when $P<0.05$ and as triple asterisk (***) when $P<0.001$.

Acetate attenuates HSL phosphorylation in adipocytes

Since the above data indicated that mainly acetate is the driver of the antilipolytic effect of SCFA in human adipocytes, we subsequently investigated the underlying mechanisms in more detail by quantification of key enzymes involved in intracellular lipolysis, including ATGL, HSL and phosphorylated HSL (pHSL). No differences of acetate on total HSL or ATGL protein content were observed (Figure 3A). As indicated in Figure 3B, treatment of hMADS adipocytes with 1 µmol/L acetate resulted in a reduction in the relative amount of phosphorylation of HSL on the serine 650 as compared to control non-treated cells

(P<0.01, Figure 3B). As expected, the phosphorylation of HSL on the serine 650 increased by 5-6 fold in the presence of ISO, as compared to non-stimulated adipocytes (Figure 3A and B). However, pre-treatment of hMADS adipocytes with 1 μmol/L acetate and ISO resulted in reduction in the relative amount of $pHSL_{(SER650)}$ compared to ISO stimulation alone (P<0.05, Figure 3B).

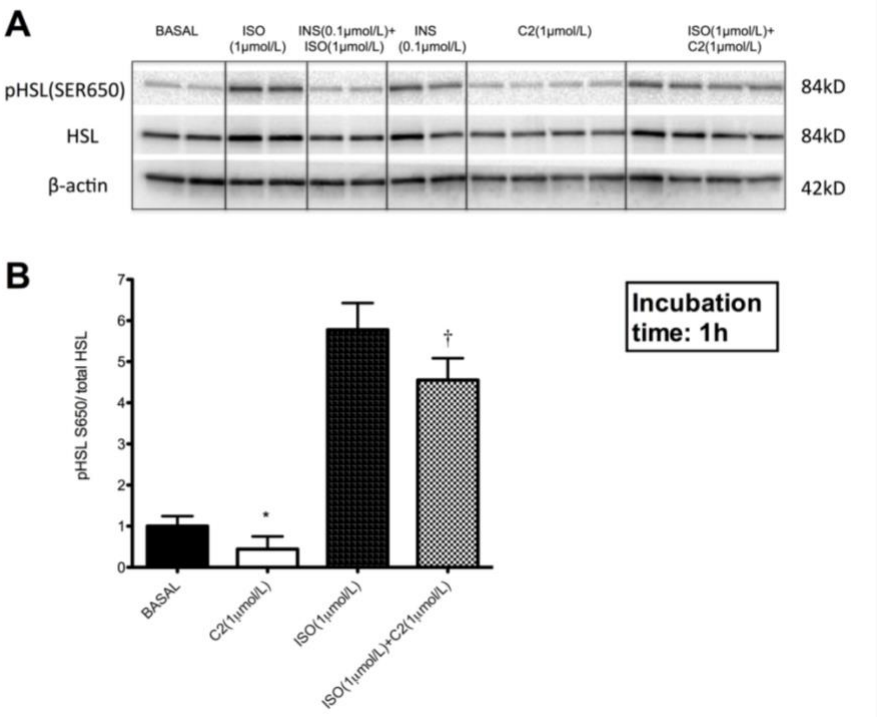


Figure 3. Acetate attenuates HSL (SER 650) phosphorylation in hMADS adipocytes

A: Representative western blot showing that 1 μmol/L acetate (C2) reduced the relative amount of HSL phosphorylated on serine 650 in the presence of isoprenaline (ISO). In this blot, insulin (INS) was used as a control. See for corresponding entire blots supplementary figure 1 **B:** Quantification of western blot using ImageLab 3.0 normalized to total HSL (n = 4). Values are given as means ± SD. Statistical significance as compared to basal indicated as asterisk (*) when P<0.05; and as compared to ISO as dagger (†) when P<0.05.

PTX treatment prevents the antilipolytic effect of acetate in hMADS cells

Finally, we investigated the involvement of inhibitory Gi G protein coupled receptors in this acetate-mediated antilipolytic effect. Both FFAR3 and FFAR2, the major SCFA receptors, were expressed at the RNA (Figure 4A) and protein (Figure 4B) level in our hMADS cells, and expression increased during adipogenic differentiation with a maximal expression at day 12 and 14 (see Figure 4; Figure S3C in Supplementary Material for FFAR2 protein expression in hMADS at day 14).

Next, hMADS adipocytes were incubated with or without PTX, which irreversibly blocks Gi function, thereby inhibiting both FFAR3 and FFAR2 in our hMADS adipocytes. Of interest, PTX prevented the acetate-mediated (1 μmol/L) decrease in basal and β-adrenergic receptor stimulated glycerol release (P<0.01, Figure 5).

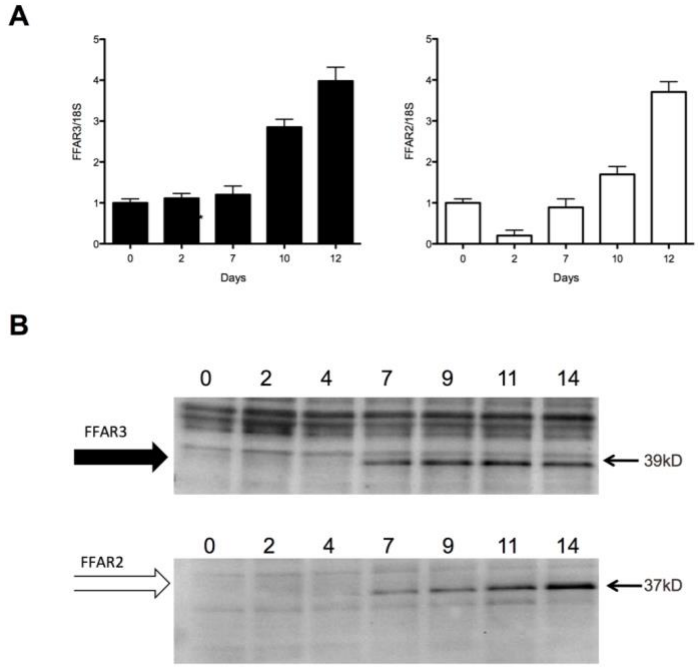


Figure 4: FFAR3 and FFAR2 are expressed at the RNA and protein level in hMADS adipocytes

A: FFAR3/2 mRNA expression during adipocyte differentiation (day 0 - 12). **B:** FFAR3/2 protein expression during adipocyte differentiation (day 0 - 14) (n=1), See for corresponding entire blots supplementary figure 2.

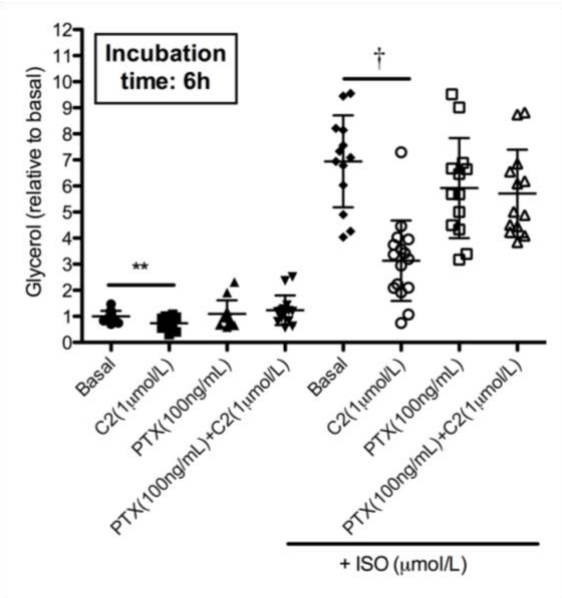


Figure 5: Pertussis toxin (PTX) abrogated acetate-induced inhibition (1 µmol/L) of ISO-mediated glycerol release in hMADS adipocytes

Values are given as individual points and means ± SD (n = 4 independent experiments). Statistical significance when compared to basal indicated as asterisk (**) when $P<0.01$; and as compared to isoprenaline (ISO) as dagger (†) when $P<0.001$.

Discussion

This study provides new insight in the effects of SCFA on human adipocyte lipolysis. We previously showed that acute colonic administration of three physiological-relevant SCFA mixtures, and subsequent elevated circulating acetate concentrations, reduced circulating glycerol concentration in overweight males, indicative of a reduced whole-body lipolysis [19]. However, to further investigate whether the reduction in whole-body lipolysis was related to a putative SCFA effect on white adipocyte intracellular lipolysis, we performed several *in vitro* experiments using our validated hMADS adipocyte model. Our present *in vitro* study in hMADS adipocytes demonstrated that mainly acetate had antilipolytic effects, which was accompanied by a reduced phosphorylation of HSL (at SER650). Incubation with the Gi inhibitor PTX prevented the acetate-mediated antilipolytic effect, suggesting that this antilipolytic effect may be mediated through an acetate-FFAR coupled signalling pathway (for an schematic overview see figure 6).

This study demonstrated that SCFA mixtures at physiological (1 μmol/L) and more supraphysiological (1 mmol/L) concentrations attenuate intracellular lipolysis in human adipocytes. Furthermore, by subsequently incubating human adipocytes with single SCFA, we demonstrated that the intestinally and systemically most abundant SCFA acetate seems to be the main driver of this antilipolytic effect. Acetate is the most abundant circulating SCFA and is found in serum and plasma mean concentrations varying from 5 μmol/L up to 220 μmol/L, depending on the nutritional status [3, 22-24]. Propionate and butyrate are found at much lower maximal mean concentrations of 13 μmol/L and 12 μmol/L, respectively [3, 22, 23]. However, no human data are available reflecting SCFA concentrations that reach the adipose tissue via their capillaries. Based on the scarcely available data on circulating SCFA concentrations, an acetate to propionate to butyrate ratio of 80:10:10 and 60:20:20 could resemble physiologically circulating concentrations and the SCFA concentrations used in this study might be in a physiological (1 μmol/L) to supraphysiological (1 mmol/L) range. Interestingly, the most pronounced effects on lipolysis were found with an acetate concentration of 1 μmol/L, which thus seems to be lower than circulating concentrations. Therefore, it would be of major interest to measure actual acetate concentrations in adipose tissue capillaries or interstitial fluids. To the knowledge of the authors, no data on this are available, which would be very interesting to detect via for example microdialysis techniques.

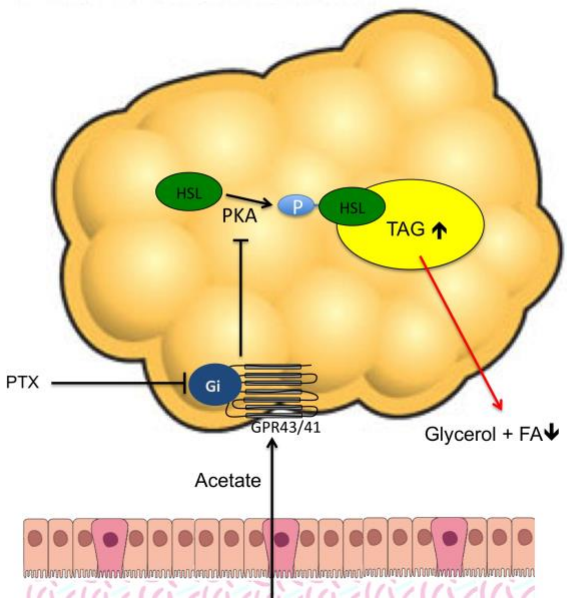


Figure 6: Proposed mechanism of the acetate-mediated antilipolytic effect in human adipocytes.

The blunted FA and glycerol release during acetate incubation is accompanied by a reduced phosphorylation of HSL(SER650), indicating a role for protein kinase A (PKA) in this antilipolytic process. The FFAR inhibitor PTX prevents the acetate–mediated antilipolytic effect, indicating a role for a Gi protein coupled receptor mechanism (i.e. FFAR3 and/or FFAR2) in human adipocyte lipolysis. In addition, we observed that the antilipolytic effect of acetate was accompanied by a reduced phosphorylation of HSL at the serine 650, a major protein kinase A (PKA) regulatory site. In accordance with this observation, Aberdein *et al.* [25] indicated that treatment of murine 3T3-L1 adipocytes with supraphysiological concentrations (4 mmol/L) of sodium acetate reduced the β-adrenergic receptor stimulated non-esterified FA release and decreased HSL phosphorylation at another PKA regulatory site (serine 563) [25]. Ge *et al.* [12] showed that treatment of 3T3-L1 adipocytes with acetate and propionate in a range between 0.1 - 0.3 mmol/L reduced the basal and β-adrenergic receptor stimulated intracellular lipolytic activity as assessed by a decreased release of glycerol in the culture medium [12]. In contrast to the antilipolytic effect of acetate, we observed a slightly increased basal lipolytic response after butyrate treatment in hMADS adipocytes. Comparable results were reported by Rumberger *et al.* [13] showing an increased basal lipolytic response (glycerol release) following incubation of murine 3T3-L1 adipocytes with 5 mmol/L butyrate [13]. However, the underlying mechanism of this lipolytic effect of butyrate needs further investigation in hMADS cells. Together, these results suggest that acetate is the main driver for the antilipolytic effects of SCFA, which is accompanied by an attenuated HSL phosphorylation in both, murine and human adipocytes.

Finally, we observed that the antilipolytic effect of acetate might be FFAR dependent. We first showed in accordance to other reports in human adipocyte models [18] that both, FFAR3 and FFAR2 transcripts and protein are expressed in our hMADS adipocyte model, and that both increased during adipogenic differentiation. Furthermore, we showed that the effects of acetate are abrogated with co-incubation of PTX. PTX is a well-known FFAR inhibitor and irreversible inactivates Gi proteins. Thereby, these data suggest that the acetate effects were mediated via a Gi protein receptor-PKA pathway. In line, a previous study in murine 3T3-L1 adipocytes has shown that the lipolytic effect of acetate was mediated by activation of FFAR2 [12]. However, further investigations are warranted to elucidate whether SCFA effects on human intracellular adipocyte lipolysis are mediated mainly via FFAR2 and/or FFAR3, using specific human knockdown models. In particular the role of FFAR3/2 protein should be further investigated via the use of knockdown and overexpression in human adipocyte models.

Furthermore, evidence is increasing that metabolic phenotype should be considered in future lipolysis studies. Present literature provides evidence that obesity-related metabolic disturbances, such as insulin resistance, are linked to differences in circulating acetate levels and acetate-induced metabolic responses. For example, in our acute studies [19, 24] we included overweight and obese individuals with average fasting acetate concentrations of approximately 20 - 50 µmol/L, whereas in another study of our group with insulin resistant obese individuals markedly higher acetate concentrations of approximately 70 - 90 µmol/L have been found [26]. In addition, a kinetic study showed that the acetate clearance rate is lower and the half-life is longer in type 2 diabetic patients as compared to healthy normoglycaemic controls [27]. This suggests a disturbed uptake and/or metabolism of acetate, which might be relevant to elicit acetate-induced metabolic effects and cell signalling in peripheral tissues. Furthermore, there are indications that overweight insulin resistant compared to normoglycaemic individuals have lower acetate-induced antilipolytic responses on a whole-body level [28]. An acute study demonstrated that intravenously administered acetate resulted in a greater free fatty acid fall and rebound in healthy adults compared with hyperinsulinaemic individuals [28]. Therefore, comparing SCFA-mediated inhibition of the lipolytic response and intracellular signalling mechanism in adipocytes derived from normoglycaemic, insulin sensitive versus metabolically more compromised donors is of major interest. Here, we included cells from human adults with a wide range of BMI and glucometabolic status, therefore we did not distinguish between metabolic phenotypes, which is as limitation.

Nevertheless, this study has clinical implications. If the observed results can be translated into long-term *in vivo* metabolic effects, increased systemic acetate availability might improve human white adipose tissue lipid buffering capacity and reduce adipose tissue lipid spillover. This could ultimately result in

attenuated ectopic fat accumulation and improved insulin action in insulin sensitive tissues such as skeletal muscle, pancreas and liver, preventing insulin resistance. The present study showed that acetate induces a partial inhibition of intracellular lipolysis during basal conditions. Interestingly, combined data derived from rodents and humans demonstrated that a comparable partial inhibition of intracellular lipolysis has beneficial effects on insulin sensitivity without affecting adipose tissue mass in the longer term [29, 30]. In addition to elevated basal lipolysis, β-adrenergic receptor agonist sensitivity is blunted in obese insulin resistant individuals, [31-33] which poses the question whether a further decrease in ß-adrenergically mediated lipolysis by SCFA is positive with respect to metabolic health. Thus, further research including isoprenaline concentration - response curves are needed to ascertain SCFA-induced changes in efficiency or potency of β-adrenergic receptor agonists in different metabolic phenotypes.

With the present *in vitro* study using our human adipocyte model we explored a mechanism that might explain the previously *in vivo* observed antilipolytic effect of physiologically relevant SCFA mixtures [19]. However, other mechanisms, which might also contribute to the SCFA-induced antilipolytic effect on whole-body level, could not be excluded here. For example, in parallel to acetate concentrations, circulating peptide YY (PYY) concentrations were increased after the colonic infusions of SCFA mixtures in our *in vivo* experiment [19]. Indeed, PYY was previously recognized for its antilipolytic property in human adipocytes [34]. In addition, an intriguing study in rodents showed that SCFA can influence energy homeostasis including lipolysis via dorsal sympathetic ganglions and spinal pathways [35].

In conclusion, we demonstrated that in particular the colonic and peripheral most abundant SCFA acetate plays an important role in the regulation of human adipose tissue lipolysis. We showed that the luminal and systemically most abundant SCFA acetate was mainly responsible for the antilipolytic response, via FFAR-mediated attenuation of HSL phosphorylation in human adipocytes. Indicating that the modulation of colonic and systemic acetate might be a target to prevent or improve insulin resistance in human. Therefore, future studies should focus on increasing nutritional strategies to enhance circulating acetate availability, for example via supplementation of specific acetogenic fibres, to improve human lipid metabolism.

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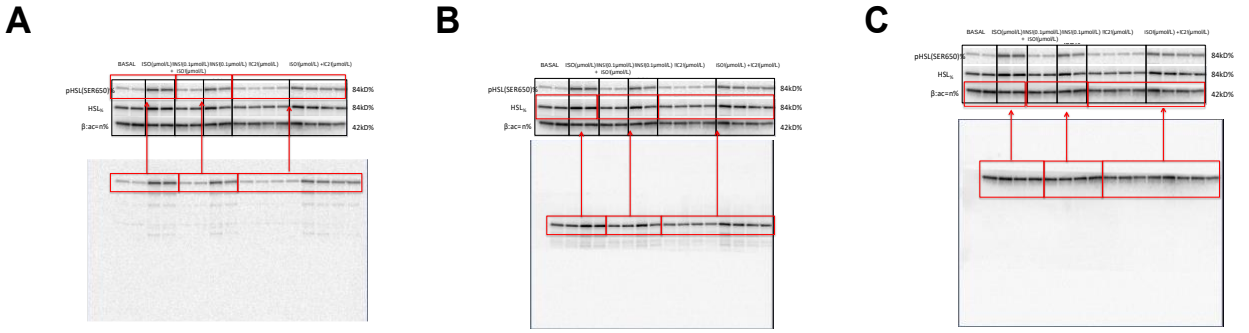
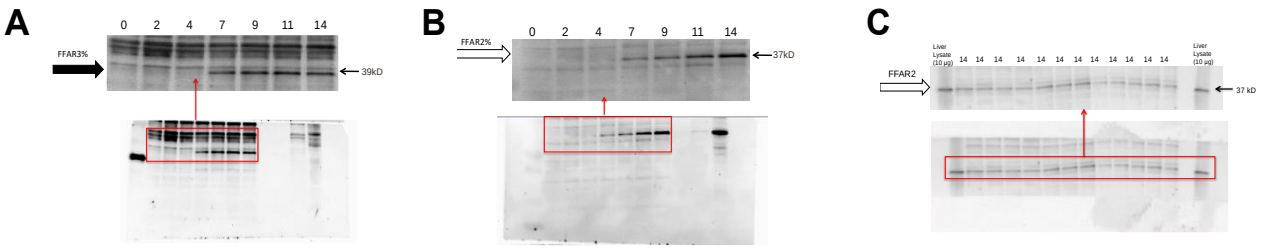
Supplementary Material



Supplementary figure 1: Corresponding blots for **figure 3A** *Acetate attenuates HSL (SER 650) phosphorylation in hMADS adipocytes* of the manuscript.



Supplementary figure 2: A and B: Corresponding blots for **figure 4B** *FFAR3 and FFAR2 are expressed at the protein level in hMADS adipocytes* of the manuscript **C:** Additional blot of FFAR2 protein expression of fully differentiated hMADS adipocytes at day 14 (as positive control 10 µg human fetal liver lysate was used).

CHAPTER 8

General Discussion

Obesity Epidemic

Obesity and comorbidities have dramatically increased worldwide in the last decades [1]. Excessive body weight and the accompanying increased adiposi may result in adipose tissue dysfunction and the development of insul resistance, Type 2 diabetes Mellitus (T2DM) and cardiovascular disease (CVD) [1]. An important factor connecting these pathophysiological conditions is the decreased adipose tissue lipid buffering capacity, which results in lipid overflow and ectopic fat deposition in non-adipose tissues (i.e. skeletal muscle, liver, pancreas, heart) [2]. In addition, an impaired fat oxidation unable to cope with the increased lipid supply as well as increased systemic low-grade inflammation contribute to ectopic lipid accumulation (bioactive lipid metabolites), impairments in insulin signaling (elevated proinflammatory cytokines) and the subsequent development of insulin resistance [3-5].

Several surgical, pharmacological and lifestyle strategies have focused on body weight management to combat obesity and its complications. Currently, the highest prevalence (between 28-36%) of obesity is observed in North America (i.e. Mexico), some parts of Europe and the Middle East. Although the above mentioned strategies improved throughout the last decade, the prevalence of obesity is expected to further increase in the coming decades [6]. Taken together, there is an urgent need to investigate additional strategies for the prevention and treatment of obesity, in particular on long-term maintenance of weight loss- or dietary intervention-induced metabolic improvements. In particular, more targeted or personalized strategies considering individual preferences, genetic, microbial and metabolic phenotypes are required to promote weight loss and improve metabolic health over longer periods of time.

Currently, scientific insights with the focus on the gut microbiota, its metabolites and its inter-organ crosstalk with peripheral tissues highlight the rationale to target the gut and its inhabitants to elicit metabolic benefits [7]. Based on this, interventions (i.e. pre- or probiotics) have focused on microbiota modulation to improve gut and host metabolic health [8]. Predominantly, research has shown that a more pronounced saccharolytic fermentation (prebiotics) in the whole colon may increase the production of postbiotic metabolites such as short chain fatty acids (SCFA). Of note, SCFA administration elicited various beneficial effects on metabolic health in several animal and in a limited number of human studies [9-13]. On the other hand, a less explored field is the modulation of gut microbiota through other saccharolytic metabolites (e.g lactate, succinate) as well as through proteolytic (protein) fermentation and the production of branched chain fatty acids (BCFA) with possible more adverse metabolic effects [12]. Although fecal BCFA are positively related to obesity in humans [14-16], more studies are warranted to elucidate the underlying mechanisms.

Furthermore, it is important to consider that gut microbiota composition and functionality [17-25] and metabolic phenotype [26-28] may determine the effectiveness of a dietary intervention aiming to modulate saccharolytic/proteolytic fermentation ratio.

This doctoral thesis investigated the association of fecal/plasma SCFA (acetate, propionate and butyrate), other fecal metabolites (lactate, caproate, valerate and succinate) as well as fecal BCFA (isobutyrate, isovalerate) with metabolic health parameters in well phenotyped human cohorts. In addition, we investigated, *in vitro,* underlying mechanisms of action of SCFA in peripheral tissue substrate metabolism using human derived primary cells from skeletal muscle and adipose tissue.

Microbial metabolites derived from saccharolytic fermentation and insulin sensitivity in humans

SCFA and metabolic health

In general, various studies have suggested that SCFA may have tissue specific effects and beneficially impact whole body insulin sensitivity and metabolic health [29]. For instance, muscle gastrocnemius extracts from butyrate supplemented mice (C57BL/6J) showed increments in radiolabeled palmitate oxidation by 200% [30]. In line, SCFA may increase oxidative capacity in liver and muscle tissues which may work through an increased phosphorylation of AMPK as shown in *in vitro* and animal studies after acetate treatment [31-33]. In support, a human study showed that oral administration of sodium propionate increased fasting fat oxidation and resting energy expenditure independent of glucose and insulin levels in healthy individuals [34]. Although there is vast literature on SCFA, the majority has focused on acetate, which evidence comes mainly from animal and *in vitro* studies [29]. Therefore, in c**hapter 2,** we extensively reviewed the effects of acetate, the most abundant SCFA in the colon and systemic circulation, in the context of insulin sensitivity and body weight control. Briefly, an increased acetate availability in the systemic circulation resulted in an antilipolytic effects on whole body level as observed in various *in vivo* studies (animal and human) [35-43], increments in fat oxidation and in gut hormone levels (peptide YY and glucagon like-peptide 1 PYY/GLP-1 levels) in humans [44, 45]. In addition, increased plasma acetate concentrations are positively associated with insulin sensitivity markers in humans [46, 47]. Of note, acute colonic infusions (rich in acetate) have shown to beneficially affect whole-body human substrate and energy metabolism as reflected by increments in fasting fat oxidation and energy expenditure as well as in PYY and GLP-1 levels [44, 45, 48].

Interestingly, this effect only occurred after distal and not after proximal colonic acetate infusions [45]. In relation, a study in men conducted during abdominal surgery reported a higher SCFA release (highest for acetate) from distal compared to proximal colon, indicating that colonic absorption may differ between colonic segments [49]. Collectively, these data suggested that an increased acetate availability and absorption in the distal colon (i.e. via supplementation of slowly fermentable fibers), may be a strategy to beneficially affect whole body metabolism through the aforementioned tissue specific effects.

In this regard, a prebiotic intervention in obese/prediabetic men using an acetogenic fiber (galacto-oligosaccharides, 12-week, 15g/d) did not report changes in fecal/plasma levels of SCFA despite an observed bifidogenic effect [50]. This was possibly explained by increased fermentation mainly in the proximal colon and/or an insufficient acetate production, as no increments in fecal and plasma SCFA concentrations were observed after the intervention. Furthermore, research from our group recently investigated whether a mixture of fibers (long-chain inulin and resistant starch), which increased gut microbial acetate production the highest in the distal colon (using an *in vitro* model of the human colon) as compared to other mixtures, could increase acetate production in lean and prediabetic individuals [51]. This intervention indicated an increased energy expenditure, carbohydrate oxidation and postprandial insulin sensitivity in lean individuals, but not in individuals with overweight and prediabetes. These metabolic phenotype-specific effects are in line with a previous human study, in which a 4-week oral butyrate supplementation resulted in increased peripheral and hepatic insulin sensitivity in healthy lean but not in metabolic syndrome individuals [27]. Although these data suggest that effects may depend on metabolic phenotype with a lower or delayed response in metabolically disturbed individuals, there are a few studies that have shown that prebiotic fiber administrations improved insulin sensitivity markers in individuals with obesity and T2DM [52, 53].

Another approach to increase circulating acetate is through oral administrations, such as vinegar supplementations, which have shown improvements in glucose homeostasis, however, individual characteristics (metabolic phenotype and glycemic profile) may again play a role in the metabolic effects induced by acetate. Specially, vinegar supplementations (i.e. containing 6% acetic acid) seem to be more effective in terms of improvements in glycemic control in normo- as compared to hyperinsulinemic individuals [28]. A further interesting aspect to consider is the microbial phenotype and its microbial community stratification in *Bacteroides, Prevotella, Ruminococcus* [17]. For instance, the *Bacteroides* enterotype has been linked to higher consumption of protein and saturated fats, whereas *Prevotella* enterotype has been associated to a lower protein intake [54]. Taken together, these data indicate that further studies aiming to improve metabolic health by increasing the SCFA/acetate availability in tissues such as liver, skeletal muscle and adipose tissue through fiber supplementation or vinegar

supplementations may need to consider metabolic phenotypes and/or microbial phenotypes [55].

Another potential therapeutic intervention to increase SCFA availability in the gut is through probiotic supplementations, which aim to increase SCFA producing bacteria including acetate producers [56, 57]. For instance, *Akkermansia muciniphila* supplementation, an acetate producer and mucin degrader, may exert multiple beneficial effects by influencing host metabolism and immune system [58]. Of note, a recent human intervention study providing *Akkermansia muciniphila* showed safety, tolerability and increased insulin sensitivity markers (+28 %) in overweight-obese insulin resistant individuals [59]. Furthermore, a synbiotic supplementation (fructo-oligosaccharides plus 7 strains of bacteria including *Bifidobacterium longum, Lactobacillus bulgaricus, Lactobacillus casei*) showed improvements in insulin resistance and fasting glucose in individuals with metabolic syndrome [60]. Although no measurements of SCFA were performed here [58, 60], it gives an indication for future studies to investigate the role of acetate or other SCFA using gut microbiota-altering interventions to restore the SCFA producing and metabolizing capacity in metabolically compromised individuals.
In summary, human interventions using pre-, pro- or synbiotics, vinegar supplementations or combinations may have the potential to increase colonic and circulating acetate to exert its whole-body and tissue specific effects (as reviewed **in chapter 2**). Further research should consider colonic production site and the role of the metabolic phenotype in SCFA handling. To further investigate the role of circulating acetate as well as circulating propionate and butyrate, we studied the association of fasting concentrations of SCFA with markers of insulin sensitivity in two large cohorts with well-phenotyped individuals.

Do SCFA have to enter the systemic circulation to exert metabolic effects?

As described above an increased availability of acetate in the distal colon may lead to a high acetate supply to the systemic circulation that may beneficially modulate tissue specific and whole-body metabolism. In view of the fact that previous data showed that fasting plasma acetate levels correlated with fat oxidation and energy expenditure after colonic infusions in humans [48], together with positive associations of circulating acetate concentrations with insulin sensitivity markers in humans [46, 47], we hypothesized **in chapter 3** that fasting systemic acetate concentrations positively associate with insulin sensitivity markers in the DiOGenes study. In contrast, we found a slight positive association between changes in circulating fasting acetate and changes in HOMA-IR (stdβ 0.130, p = 0.033) after the low-calorie diet (LCD) phase in females but not in males even after adjustment for confounders (age, weight and fat free mass). Of importance, this finding should be interpreted with caution considering the

contrasting evidence that has provided valuable insight of acetate tissue specific mechanisms [61] as well as whole-body effects of acetate in humans (increments in fasting fat oxidation, energy expenditure, PYY secretion) [44, 48, 62].

Nevertheless, these findings raise various questions about the association of circulating acetate and insulin sensitivity including the significance of the site of acetate production (gut-derived acetate, endogenously derived from liver), acetate kinetics/clearance a well as the sexual dimorphism of this relationship. First, it should be noted that circulating acetate might be the resultant of colonic acetate production as well as endogenously hepatic acetate production which may strongly depend on metabolic status. Notably, acetate and ketone bodies concentrations were determined after the LCD in the DiOGenes study during energy restricted conditions, and these concentrations partly reflect the increased hepatic ketogenesis and elevate acetate production during energy restriction [63]. Secondly, the sexual dimorphism in this relationship is interesting and warrants further investigation. The latter may possibly relate to sex-specific differences in tissue specific insulin resistance phenotypes, as a previous DiOGenes study reported that despite the fact that hepatic insulin resistance was lower in females compared to males, worsening of the lipid profile was associated with hepatic insulin resistance in females, but not in males [64]. In support to the sexual dimorphism, a previous DiOGenes analysis suggested a sex-specific modulatory role of baseline acetate in energy metabolism, since they reported a negative association with LCD-induced BMI change in morbidly obese females (stdβ -0.23) but not in males [65].

Furthermore, whether sex hormones play a role in this relationship through alterations in SCFA handling and/or hepatic metabolic processes remains unresolved. In this cohort, most women were in the premenopausal state, unfortunately data on hormonal status were unavailable. Thirdly, when interpreting this data it should be considered the complexity of acetate kinetics in the colon, which depends on the microbial composition, its functionality and cross-feeding mechanisms [55]. More studies are warranted to investigate the relationship between acetate concentrations and insulin sensitivity further as well as acetate kinetics where microbially produced acetate and endogenously produced acetate may reflect different metabolic conditions in distinct phenotypes.

Furthermore, we recognize that fecal SCFA are often considered as a biomarker of SCFA production, however, this may have disadvantages as we investigated and discussed in **chapter 4**. Here, we investigated the associations between both fecal and plasma SCFA (acetate, propionate and butyrate) with metabolic health parameters in individuals with a wide range of BMI and glucometabolic status. Our data indicated that in particular circulating fasting SCFA were associated with metabolic health parameters like circulating lipids, GLP-1 and insulin sensitivity whilst there were no associations between fecal concentrations of SCFA and metabolic health parameters [66]. Although various

studies have found increased levels of fecal SCFA in individuals with obesity [25, 67, 68], these findings should be interpreted with caution in presence of the vast amount of data that shows beneficial effects of SCFA on control of body weight and insulin sensitivity [29, 61].

In particular since fecal metabolite concentrations are the net result of various factors including whole gut transit time, gut microbiota composition, microbial cross-feeding mechanisms, complex microbial interactions as well as colonic rates of absorption that may differ along the colon (proximal versus distal) and are not necessarily a good reflection of SCFA production rates [69].

More in detail, in our analysis (**chapter 4**) circulating SCFA (acetate, propionate and butyrate) associated negatively with circulating lipids (glycerol, triacyclglycerols and free fatty acids, respectively). Of note, in this study the antilipolytic effect of acetate was corroborated as previous *in vitro* [35-37, 39], animal [35, 38] and human studies have reported [40-43]. Mechanistically, the antilipolytic effect of acetate may be mediated through a G-protein coupled receptor (GPR)-mediated decrease in hormone sensitive lipase phosphorylation in human adipocytes as observed in **chapter 7**. The negative association between propionate with triacylglycerols may be explained through an increase in lipoprotein lipase in the adipose tissue as observed *in vitro* [70], which may increase lipid extraction from the circulation. With respect to butyrate, data is inconsistent as *in vitro* studies have reported both anti- and pro-lipolytic effects in white adipose tissue [39, 71].

In addition, in our analysis in **chapter 4** butyrate showed a negative correlation with glucose levels, which is in line with rodent studies in which oral butyrate supplementation improved insulin sensitivity [30, 72]. However, butyrate effects in humans may depend on metabolic phenotype, as already explained above, since an oral butyrate supplementation improved glucose metabolism in lean but not in individuals with metabolic syndrome [27]. In addition microbial phenotype may play a regulatory role as well, as it has been consistently reported that insulin resistant/diabetic individuals have low numbers of butyrate-producing bacteria [73-75]. Further studies are warranted to elucidate to what extent the SCFA production and handling is disturbed in metabolically compromised individuals.

In the colon, human enteroendocrine L cells have reported to secrete gut hormones (e. g. GLP-1, PYY) through SCFA stimulation of GPR41/43 receptors [76, 77]. Interestingly, we found that circulating SCFA were positively associated with fasting GLP-1. It is important to interpret this considering that residual (slow-fermenting) dietary fibers may still be fermented in the colon and that whole gut transit time may take longer than an usual overnight fast [78]. To our knowledge this is the first study that reports an association of circulating SCFA with GLP-1 in the fasted state. In rodents, previous studies have reported increments in gut hormones after dietary fiber supplementation in the postprandial state [79].

Circulating SCFA may stimulate GLP-1 secretion from the visceral, basolateral side of enteroendocrine L-cells, as observed in a study using isolated rat colons [80]. Of note, the stimulus was rather weak, thus questioning the physiological relevance. Besides enteroendocrine L-cells, pancreatic α-cells have been suggested to contribute to systemic GLP-1 concentrations in the fasted state, but whether circulating SCFA act as stimuli for GLP-1 secretion warrants further investigation [81].

Circulating acetate and propionate in relation to insulin sensitivity

In a subset of the cohort **in chapter 4**, we investigated the associations of fecal/plasma SCFA with insulin sensitivity as measured via the gold standard hyperinsulinaemic-euglycemic clamp technique. Interestingly, we found that circulating acetate associated negatively (similarly to the above findings), while circulating propionate associated positively with insulin sensitivity. The reason why we did not observe a sexual dimorphism in the analysis in **chapter 4** is not entirely clear and remains to be clarified. A factor which may play a role in explaining the differences between studies is the metabolic phenotype of the individuals since a larger proportion of the participants **in chapter 4** had an impaired glucose metabolism.

Indeed, a recent study from the DiOGenes consortium and the Maastricht Study reported that obese (not diabetic) individuals with hepatic insulin resistance may have decreased ketogenesis [82], and possibly suggesting a differential release of acetate from endogenous sources into the circulation. Thus, the finding of an inverse correlation of circulating acetate with insulin sensitivity is in contrast to vast amount of data in animal studies [31, 32, 83-88], human interventions [28, 44, 45], and association studies [46, 47] that report positive effects on insulin sensitivity. The exact mechanisms for these discrepancies remain to be elucidated, but may relate to metabolic condition (fasting, energy restriction), sex differences as well as metabolic phenotype. In line with our positive relationship of propionate and insulin sensitivity in **chapter 4**, a human study showed that oral administration of sodium propionate increased fasting fat oxidation and resting energy expenditure in healthy subjects [34]. Mechanistically, propionate may attenuate intrahepatocellular lipids, increase glycogen synthesis and decreased inflammatory markers [89, 90]. Overall, circulating SCFA may reflect more accurately metabolic health parameters (fasting GLP-1, circulating lipids and insulin sensitivity) as compared to fecal levels. This suggests that the measurement of circulating SCFA is a requirement when investigating the role of the gut microbiome in metabolic health.

Nevertheless, studies investigating SCFA dynamics are needed to provide a better insight of SCFA colonic production, absorption and how circulating levels may impact the function and metabolism of peripheral tissues.

In the following paragraph, we discuss how SCFA might impact skeletal muscle and adipose tissue substrate metabolism.

Mechanisms of action of SCFA on skeletal muscle and adipose tissue metabolism

According to the literature, SCFA may exert their whole-body effects through effects on various tissues including skeletal muscle and adipose tissue metabolism. Of note, SCFA may be the link between gut and skeletal muscle and adipose tissue glucose homeostasis and lipid metabolism [29, 91]. For instance, in previous *in vivo* human studies distal colonic SCFA infusions (rich in acetate) increased whole-body fasting fat oxidation in men with overweight/obesity [45, 48]. Similarly, oral administration of sodium propionate increased fasting fat oxidation and resting energy expenditure independent of glucose and insulin levels in healthy subjects [34]. With respect to skeletal muscle metabolism, acetate has been reported to have the potential to increase the oxidative capacity through increments in AMP/ATP ratio, AMPK phosphorylation and upregulation of GLUT4 [31, 32]. In line muscle gastrocnemius extracts from butyrate supplemented mice (C57BL/6J) showed increments in radiolabeled palmitate oxidation by 200% [30]. Furthermore, in relation to adipose tissue metabolism, *in vitro*, animal and human studies have reported antilipolytic effects in response to acetate [35-43]. Importantly, *in vitro* mechanistic studies have been performed mainly in murine adipose cells (which may lack GPR41) [92] with incubations of single SCFA using supraphysiological concentrations (4-10 mmol/L). Based on previous findings, we hypothesized that circulating acetate may directly affect fat oxidation in human skeletal muscle contributing to the observed increments in *in vivo* whole-body fat oxidation.

Effect of acetate on fat oxidation in human primary muscle cells

In chapter 6, we investigated *in vitro,* the dose and time effect of sodium acetate on complete and incomplete endogenous and exogenous oxidation of ^{14}C-labelled palmitate. In detail, we investigated the effects of acetate in putatively physiological concentrations (0-0.5 mmol/l) reached after fiber fermentation [93-96] as well as supraphysiological concentrations (1-5 mmol/l). Although, intracellular/interstitial concentration of acetate in skeletal muscle remains unknown, we used a wide spectrum of concentrations, thus likely mimicking *in vivo* cellular and extracellular concentrations after fiber fermentation. As shown in **chapter 6**, our *in vitro* data showed no effect of sodium acetate on neither complete nor incomplete endogenous and exogenous oxidation, irrespective of time and dose in human primary muscle cells (HSkMC).

In addition, we investigated the potential effect of sodium acetate on AMP-activated protein kinase (AMPK) phosphorylation in our HSkMC model. In line with our *in vitro* fat oxidation data, we could not observe an effect of sodium acetate on total and phosphorylated AMPK protein content in our HSkMC model.

In contrast, in rat L6 myotubes, acetate increased AMP/ATP ratio and AMPK (threonine 172) phosphorylation in a dose-dependent (0.05-0.5 mmol/L) and time-dependent (0-30 min) manner [31]. In addition to these *in vitro* observations, oral injection of acetate acutely increased phosphorylation of Thr172-AMPKα, in rat skeletal muscle (*in vivo*) [31, 32].

Furthermore, it needs to be mentioned that AMPK-dependent fat oxidation might be limited in HSkMC and/or an AMPK-dependent effect on glucose uptake/oxidation may be preferentially activated in human skeletal muscle cells [97, 98]. Although acetate increases AMPK phosphorylation in rodents, acetate-mediated effect on AMPK activation in skeletal muscle may be species-specific [99] as well as muscle fiber type dependent, since AMPK subunits may differ between muscle type fibers [100]. Of importance, the higher complexity of the *in vivo* situations and the interorgan crosstalk with other tissues may partly explain the lack of effect in our *in vitro* conditions as compared to our previous human interventions [45]. For instance, sodium acetate may increase oxidative capacity in other tissues, as *in vitro* and *in vivo* rodent studies have shown increments in phosphorylated AMPK and total AMPK in the colon, liver and adipose tissue [32, 33, 86]. Secondly, other SCFA may increase fat oxidation *in vivo*, as discussed above in healthy individuals after oral administration of sodium propionate [34]. Finally, the previously observed increment in fat oxidation after SCFA infusion may be partly explained by the ability of these SCFA to stimulate the secretion of other gut-derived hormones such as PYY and GLP-1, which have been associated with increased whole-body fat oxidation and energy expenditure in humans [101, 102]. Furthermore, fat oxidation might be donor-dependent, as individual metabolic characteristics of donors have been reported to be retained in *in vitro* HSkMC models [103, 104]. Considering that we used muscle cells derived from an insulin sensitive donor and the *in vivo* differences between metabolic phenotypes, it remains a challenge to investigate further donor differences at the *in vitro* level as well as intrinsic donor characteristics such as sex, age, diet. In summary, further studies may focus on other metabolic players such as propionate or butyrate, and/or SCFA mixtures and gut hormones as well as investigate the oxidative capacity of other tissues (i.e. liver, adipose tissue) in response to SCFA.

Nevertheless, this is to our knowledge the first study that investigates the effects of the most abundant SCFA acetate on fat oxidation in a HSkMC model.

Effect of SCFA on lipolysis in human adipocytes

As discussed above with respect to adipose tissue metabolism, several studies have reported antilipolytic effects of acetate *in vivo*. Of importance, this is in line with findings in **chapter 4,** that reported that circulating SCFA (acetate, propionate and butyrate) were negatively associated with fasting circulating lipids (glycerol, triacyclglycerols and free fatty acids, respectively). Therefore, in **chapter 7,** we investigated the *in vitro* antilipolytic properties of single and combinations of SCFA at physiological (1 μmol/l) and supraphysiological (1 mmol/l) concentrations in human multipotent adipose tissue derived stem (hMADS) cells. Interestingly, we showed that SCFA mixtures high in acetate and propionate decreased basal glycerol release, when compared to control, while mixtures high in butyrate had no effect. β-adrenergic receptor mediated glycerol release was not significantly altered following incubation with SCFA mixtures. However, incubation with only acetate decreased both basal (1 μmol/L) and β-adrenergically (1 μmol/L and 1 mmol/L) mediated glycerol release when compared with control treated cells. In contrast, butyrate (1 μmol/L) slightly increased basal and β-adrenergically mediated glycerol release compared with control, while propionate had no effect on lipolysis. Of interest, the antilipolytic effect of acetate was accompanied by a reduced serine phosphorylation of the major cytosolic lipase hormone-sensitive lipase (HSL). In addition, inhibition of inhibitory G proteins following pertussis toxin treatment prevented the antilipolytic effect of acetate.

Taken together, our data shows that acetate, the most abundant SCFA in the colon and in the circulation (19-450 μmol/L), is the main driver of GPR-dependent antilipolytic effect of SCFA in human adipocytes [29, 66]. Even though a study in murine 3T3-L1 adipocytes (4 mmol/l) [36] and human primary adipocytes (10 mmol/l) [37] showed antilipolytic effect using physiological concentrations of acetate (100-300 μmol/l), it is important to consider that animal cells may lack the major acetate GPR, GPR41 [92], thus might not capture the human complexity. In addition, it needs to be mentioned that we used pooled hMADS from different donors with multiple metabolic characteristics. It would be worthwhile to investigate the antilipolytic potential of acetate in adipocytes from insulin resistant compared to insulin sensitive donors and whether this is translated to the *in vivo* situation. Finally, future studies on the microbiome-host metabolism interactions (pre, pro and post-biotics) have to further elucidate the role of SCFA (i.e. acetate) in adipose tissue and skeletal muscle metabolism using stable isotope tracer for studying SCFA dynamics and elucidating pathways in detail in tissue biopsies.

Microbial metabolites derived from proteolytic fermentation and insulin sensitivity

Besides saccharolytic, proteolytic fermentation may occur mainly in the distal colon and produce a wide variety of metabolites including phenolic compounds, indoles, hydrogen sulfide as well as BCFA such as isovalerate and isobutyrate [11-13]. In general, high proteolytic fermentation elicited by high protein diets (HPD) , a relatively high content of plant-based proteins, or a diet low in indigestible carbohydrates may lead to altered production of microbial derived metabolites (i.e. increase fecal BCFA and decrease fecal SCFA), alterations in gut microbiota, colonic epithelium as well as impaired detoxification capacity of gut mucosa and increased inflammation [105]. In addition, as extensively discussed, most research focuses on the major saccharolytic byproducts SCFA acetate, propionate and butyrate, and metabolic roles of other saccharolytic fermentation byproducts (i.e. caproate, lactate) remain largely unexplored. Therefore, **in chapter 5** we investigated the associations of fecal BCFA (isovalerate, isobutyrate) as well as other fecal metabolites (valerate, caproate, succinate and lactate) with parameters of metabolic health in a cohort of 88 participants with a wide range of BMI and glucometabolic status. We did not find associations of fecal metabolites with the metabolic markers as well as no differences with respect to the degree of insulin resistance. These findings are in line with a study that did not find differences in fecal BCFA levels between overweight/obese and healthy individuals [67]. In contrast, elevated fecal BCFA concentrations have been observed in obese [106] and hypercholesterolemic individuals [16] as well as BCFA have been associated to NAFLD progression [15]. Moreover, obese individuals have been reported to have lower circulating isobutyrate and higher isovalerate concentrations as compared to healthy individuals [107]. On the tissue level, an *in vitro* study using rat and human adipocytes showed that both BCFA (isobutyrate and isovalerate) inhibited cytosolic (intracellular) lipolysis and lipogenesis using supraphysiological concentrations (10 mmol/L) [37]. Nevertheless, in our study we could not confirm any differences in fecal BCFA concentrations, which partly reflect BCFA production, between insulin resistant and insulin sensitive individuals.

However, as already indicated above, it is important to mention that fecal metabolites depend on various factors including microbial community [108], intestinal transit time [69], diet [109, 110], age [111], lifestyle [112] among others. Whether gut-derived metabolites need to enter the circulation to affect host metabolic health as previously suggested for SCFA [66] warrants further investigation, in particular considering that BCFA are mainly produced in the distal colon. In conclusion, the present data do not show any correlation in proteolytic fermentation metabolites (fecal BCFA) and the saccharolytic metabolites lactate, succinate, valerate and caproate with degree of insulin resistance.

Future studies with the aim to modulate saccharolytic/proteolytic fermentation should measure the kinetics of these metabolites as well as plasma concentrations taking into account the metabolic [26-28] and microbial phenotypes [17-19].

The role of metabolic phenotype in dietary intervention outcome

It is increasingly evident that microbial and metabolic phenotype should be considered as nutritional interventions may depend on microbial enterotypes (*Bacteroides, Prevotella, Ruminococcus*) [17] or metabolic phenotypes [55]. As discussed above, prebiotic and vinegar interventions have shown beneficial effects on glucose control in lean, but to a lesser extent in individuals with an impaired glucose homeostasis [28, 113]. Additionally, we observed a sexual dimorphism in the relationship between acetate and insulin sensitivity in the analysis of the DiOGenes study (**chapter 3**, as discussed above), which warrants further research. Furthermore, restoring gut microbiota composition using probiotics (i.e. *Akkermansia*), its nutrients (prebiotics), byproducts (postbiotics) and/or combinations (synbiotics) considering colonic site might be of key importance for metabolically compromised individuals in terms of restoring metabolic health. In summary, future nutritional interventions may need to consider tissue specific metabolism as well as microbial phenotype (composition and functionality) to develop targeted intervention strategies in order to optimize metabolic health outcome [114-116].

Conclusion and future perspectives

This doctoral thesis focuses on the role of the gut microbially-derived metabolites SCFA as well as BCFA in human substrate and energy metabolism. To study *in vivo* the metabolic effects and underlying mechanisms, we combined association studies in individuals with a broad range of BMI and glucometabolic status, with *in vitro* mechanistic experiments using stem cells derived from human adipose tissue and skeletal muscle biopsies.

We found that fasting circulating SCFA were associated with metabolic health markers (fasting GLP-1, circulating lipids) and insulin sensitivity in human cohorts. In contrast to previous findings we found that circulating acetate was negatively associated with insulin sensitivity. Additionally, there was a sexual dimorphism in the relationship between acetate and insulin sensitivity in analysis in the DiOGenes study. Further studies on the colonic and endogenous sources responsible for acetate production and acetate clearance are required in different metabolic phenotypes.

Furthermore, while acetate is the major SCFA for the pronounced GPR-dependent antilipolytic effect in human adipocytes, there were no acetate-mediated effects on fat oxidation in our human skeletal muscle cell model.

The main outcomes of this thesis provide important insight in the role of SCFA and BCFA in human substrate and energy metabolism. However, future studies investigating the metabolic effects of microbial metabolites should consider various aspects including:

1. **Metabolic phenotype** as it may possibly affect SCFA and BCFA handling of the host. In this regard, it has been reported that tissue specific metabolism and insulin resistance or microbial phenotype may affect intervention outcome [55, 82]. It would be highly valuable to elucidate to what extent the balance between saccharolytic and proteolytic fermentation (SCFA and BCFA production) and in particular the site of colonic fermentation (proximal vs distal) may affect liver and/or skeletal muscle insulin resistance. Additionally, human studies are lacking investigating SCFA/BCFA dynamics as well as the effect of circulating BCFA, especially derived from proteolytic (animal products and plant based) fermentation on tissue metabolism and whole-body homeostasis.
2. **Sexual dimorphism**. In **chapter 3**, we describe that fasting acetate associated positively with insulin resistance in females, not in males. Of note, various studies have reported that gut microbial diversity is linked to insulin sensitivity [20-25] and in a sex-specific manner [117]. Further studies should investigate our sex-specific findings, possibly by elucidating whether the contribution of gut-derived acetate to fasting circulating acetate may be different in males and females. This may provide a better understanding of how circulating acetate may affect metabolism. Additionally, the difference in associations of acetate and propionate as well as the propionate/acetate ratio with insulin sensitivity should be further investigated.
3. **Fecal *versus* circulating levels.** In **chapter 4**, we show that circulating, but not fecal, SCFA are linked to circulating GLP-1 concentrations, whole-body lipolysis and peripheral insulin sensitivity in humans. It is of great interest to elucidate whether plasma SCFA may induce GLP-1 secretion in other tissues such as the alpha cells in the pancreas.
4. Further studies on the colonic and endogenous sources responsible for **acetate** production and acetate clearance are required in different metabolic phenotypes. In addition, with emphasis on skeletal muscle, liver and pancreas to investigate tissue specific effects of acetate.
5. ***In vitro* mechanistic studies.** In **chapter 6** and **chapter 7**, we provide valuable data on the possible mechanism of action of SCFA in human

derived primary cells models. However, technically challenging and demanding gain and loss-of function experiments in this primary cell model are needed to elucidate the exact intracellular mode of action. Further *in vitro* studies in skeletal muscle cell models should also consider donor characteristics, fiber type dependent AMPK activation as well as SCFA mixtures to mimic an *in vivo* postprandial situation. In addition, future *in vitro* studies may need to consider comparing cell models derived from donors of different metabolic phenotypes. Furthermore, to investigate effects of gut-altering interventions at the tissue level, muscle and adipose tissue biopsies may provide key information on pathways involved in SCFA handling and their putative metabolic effects.

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APPENDIX

Summary

In the last two decades, evidence for a link between the gut microbiome with the host metabolism and metabolic disorders like insulin resistance and type 2 diabetes mellitus has constantly increased. The gut microbiota can ferment indigestible carbohydrates (saccharolytic fermentation) and produce the major short chain fatty acids (SCFA) acetate, propionate and butyrate mainly in the proximal colon. Of relevance, SCFA have been linked to improvements in substrate handling at the tissue level contributing to an amelioration in insulin sensitivity and metabolic health. Additionally, fermentation of peptides and proteins (proteolytic fermentation), mainly occurring in the distal colon, yields products such as branched chain fatty acids (BCFA), which may have adverse effects on gut and metabolic health. However, most literature is based on animal studies and only a limited amount of human studies are available. Hence, in this thesis we focused on the role of gut-derived metabolites (SCFA and BCFA) in *in vivo* metabolic health in human cross-sectional and intervention studies with overweight and obese individuals as well as the role of SCFA in human adipose tissue and skeletal muscle derived in *vitro* models.

In **chapter 2,** we extensively reviewed findings on the most abundant SCFA acetate in the context of body weight control and insulin sensitivity. We discuss that circulating acetate may have tissue-specific metabolic effects (liver, skeletal muscle, adipose tissue and pancreatic beta cells) and potentially impact whole-body energy and substrate metabolism (satiety, energy expenditure, fat oxidation) resulting in an improved metabolic health and insulin sensitivity. In addition, we discuss that the effectiveness of interventions (pre- probiotics, synbiotics) aiming to increase acetate concentrations in the colon as well as in the circulation may depend on colonic production site. In this respect, differences in the site of acetate production (proximal vs distal colon) as well as the differences in colonic acetate release into the circulation have to be taken into account. Furthermore, we discuss that future interventions (using prebiotics, probiotics, synbiotics and/or vinegar administrations) with the aim to increase circulating acetate should be more personalized and thereby consider both metabolic and microbial phenotype of participants.

Interestingly, it has been shown that the relationship between gut microbiota and insulin sensitivity may be sex-specific in humans. Therefore, in **chapter 3**, we investigated whether the associations between circulating acetate with insulin sensitivity/resistance indices (Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), circulating insulin and Matsuda index) were different in male and female participants (BMI >27 kg/m^2, n=478) of the Diet, Obesity and Genes (DiOGenes) study before and after a low-calorie diet (LCD, 800 kcal/d). We found that acetate was positively associated with insulin resistance in females, but not in males, even after adjustment for age, BMI and fat free mass.

Of note, these findings warrant further investigations to understand the sex-specific differences in circulating acetate and their potential role in metabolic health during weight loss. In particular, factors contributing to circulating fasting acetate such as hormonal status, endogenous liver production (ketogenesis) and/or the gut-derived production of acetate should be considered. Furthermore, future studies should investigate acetate dynamics to fully elucidate the role of circulating acetate in metabolic health.

So far, in human studies gut microbial-derived SCFA have been mainly measured in feces. However, fecal SCFA production should be interpreted with caution, since they are the net result of colonic production, absorption rates and release into the circulation, which are intricately influenced by microbial composition, cross-feeding mechanisms and whole gut transit time among other factors. Therefore, in **chapter 4**, we investigated associations between fecal and plasma SCFA (acetate, propionate and butyrate) with metabolic health markers including circulating metabolites, gut hormones, substrate oxidation, inflammatory parameters and markers of insulin sensitivity in a well-phenotyped group of individuals with a large range in BMI and glucometabolic status. Interestingly, we found that plasma, not fecal, SCFA (acetate, propionate and butyrate) associated negatively with circulating markers of lipid metabolism and positively with circulating GLP-1 levels. The negative associations with circulating lipids (glycerol, triglycerides, free fatty acids, respectively) are in line with previous literature, suggesting a relationship between SCFA and adipose tissue metabolism and possible improvements in adipose tissue lipid buffering capacity. An interesting (novel) finding was the positive association of circulating SCFA with fasting GLP-1 levels possibly indicating a link of plasma SCFA with gut-hormone secretion. In a subgroup analysis, we found that insulin sensitivity (as measured by the golden standard hyperinsulinaemic-euglycemic clamp technique) associated with circulating SCFA but that the direction of this association depended on type of SCFA (acetate negatively and propionate positively). Although controversial associations of circulating acetate and insulin sensitivity have been found (in **chapter 3** and **chapter 4**), findings should be interpreted with caution taking into consideration that circulating acetate is affected by various factors including liver endogenous production (ketogenesis), gut-derived production as well as metabolic and microbial phenotype.

Besides saccharolytic fermentation and SCFA production, proteolytic fermentation may yield various metabolites such as BCFA (i.e. isobutyrate, isovalerate), which role in human substrate and energy metabolism remains largely unclear. In addition, research on other fecal metabolites derived from saccharolytic fermentation (i.e. valerate, lactate) is largely lacking. Therefore, in **chapter 5,** we investigated fecal BCFA (isobutyrate and isovalerate) and other fecal microbial metabolites (valerate, lactate, succinate and caproate) in insulin sensitive and insulin resistant individuals. Additionally, we assessed the relationship of fecal microbial metabolites with circulating metabolites, substrate oxidation, and

markers of insulin sensitivity (circulating insulin and HOMA-IR). Here, we found no significant differences in fecal BCFA concentrations between insulin sensitive and insulin resistant individuals. Additionally, there were no associations between BCFA and markers of insulin resistance and metabolic health. Further human studies are warranted to investigate the role of these microbially-derived fermentation products and their kinetics in metabolic health and insulin sensitivity.

In **chapter 6,** we investigated whether our previously observed increments in *in vivo* fat oxidation after colonic acetate administration in humans were mediated through direct effects of circulating acetate on the skeletal muscle fat oxidation. Therefore, we investigated the effects of sodium acetate on fat oxidation in human primary muscle cells (HSkMC) derived from a healthy insulin sensitive donor. We observed no direct sodium acetate-mediated effects neither on endogenous nor on exogenous fat oxidation in our human skeletal muscle cell model using physiologically relevant sodium acetate concentrations. Additionally, we observed no dose and time effect of sodium acetate on total and phosphorylated AMPK levels. However, we cannot exclude that our previously observed *in vivo* human effects on fat oxidation after colonic acetate administrations occur in tissues other than skeletal muscle (i.e. liver fat oxidation) or are mediated by other gut-derived metabolites or hormones (i.e. PYY). Additionally, the lack of effect on *in vitro* muscle fat oxidation may be donor-dependent, muscle fiber type-specific as well as species-specific and warrants further investigation.

In several animal as well as human *in vivo* studies an anti-lipolytic effect of SCFA was demonstrated, which may reduce systemic lipid overflow and ultimately result in improvements in insulin sensitivity. Therefore, in **chapter 7**, we used the human multipotent adipose-derived stem (hMADS) cell model to study the effects of SCFA on (cytosolic) lipolysis and to elucidate the potential underlying mechanisms. We found that SCFA differentially affect adipose tissue lipolysis, and in particular that acetate is the major SCFA responsible for the decreased basal and β-adrenergically stimulated glycerol release in our *in vitro* hMADS cell model. The latter anti-lipolytic effect was mediated through a GPR-dependent reduction in phosphorylation of hormone sensitive lipase (HSL). Thus, these data suggest that circulating acetate affect adipose tissue lipolysis, thereby possibly limiting lipid overflow and improving insulin sensitivity.

In conclusion, this doctoral thesis provides an increased insight in the role of saccharolytic (major SCFA as well as other less studied metabolites) and proteolytic fermentation products (BCFA) in insulin sensitivity and substrate metabolism in human cohorts as well as in *in vitro* studies. The main findings indicate that gut-derived metabolites (SCFA) in the circulation might be a better indicator of their effects on metabolic health as compared to fecal concentrations.

Furthermore, the present data suggest that the sex-specific relationship of circulating acetate with insulin sensitivity exist, which may depend on various

factors such as endogenous liver metabolism, hormonal profile, and microbial and metabolic phenotype.

Lastly, our *in vitro* experiments provided valuable mechanistic insight on the relation between acetate and skeletal muscle fat oxidation and adipose tissue lipolysis, which needs to be investigated in more detail in human *in vitro* and *in vivo* studies. Additionally, future studies need to consider SCFA and BCFA dynamics (production, absorption and release into the circulation), colonic production site as well as the role of these gut-derived metabolites in tissue and whole-body metabolism and metabolic health.

Resumen

En las ultimas décadas, ha aumentado la evidencia de la conexión de la microbiota intestinal con el metabolismo del huésped, así como con enfermedades como la Resistencia a la insulina y la diabetes tipo 2. La microbiota intestinal posee la capacidad de fermentar hidratos de carbono no digeribles (i.e. fibra, prebióticos) y producir ácidos grasos de cadena corta (SCFA, por sus siglas en inglés) principalmente en la parte proximal del intestino grueso. Los SCFA de mayor relevancia son acetato, propionato y butirato. La literatura ha reportado que los SCFA están relacionados con mejoras del metabolismo en distintos tejidos, así como mejoras en la sensibilidad a la insulina. En la parte distal del intestino grueso, el microbiota intestinal fermenta péptidos y proteínas y produce ácidos grasos de cadena ramificada (BCFA por sus siglas en ingles) entre otros metabolitos, los cuales pueden tener efectos adversos en el intestino y en la salud metabólica. La mayor parte de la evidencia al respecto de estos metabolitos proviene de estudios en animales. Por lo tanto, en esta tesis, nos enfocamos en el rol de SCFA y BCFA en estudios *in vivo* en humanos con sobrepeso y obesidad, así como el rol de los SCFA en modelos *in vitro* de tejido adiposo y musculo esquelético derivados de humanos.

En el **capitulo 2**, realizamos una revisión bibliográfica de la literatura del SCFA acetato en contexto de control de peso y mejora de la sensibilidad a la insulina. En el cual, discutimos que las concentraciones de acetato en la sangre pueden tener efectos en distintos órganos importantes (hígado, musculo esquelético, tejido adiposo y las células pancreáticas beta), impactar en el metabolismo sistémico de macronutrimentos (saciedad, gasto energético, oxidación de grasas) y a su vez mejorar la salud metabólica y disminuir la resistencia a la insulina. Además, discutimos que la efectividad de las intervenciones (pre, probióticos y sinbióticos) con el objetivo de incrementar las concentraciones de acetato en el intestino grueso y en la circulación sistémica dependen del sitio de producción en el intestino grueso. Respecto a esto, diferencias de producción de acetato en el intestino grueso (proximal versus distal) así como diferencias en la absorción a la circulación deben ser consideradas. Por ultimo, en la revisión bibliográfica discutimos que las futuras intervenciones (pre, probióticos y sinbióticos y/o vinagre) con el objetivo de incrementar concentraciones de acetato en sangre deben ser personalizadas y considerar la influencia del fenotipo metabólico y de la microbiota intestinal en los individuos.

Curiosamente, se ha reportado una relación entre la microbiota intestinal y la sensibilidad a la insulina con diferencias entre sexos en humanos. Por lo tanto, en el **capitulo 3** investigamos si la relación entre las concentraciones de acetato con la sensibilidad a la insulina (Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), insulina en sangre y Matsuda Index) eran distintas entre hombres y mujeres (IMC>27 kg/m^2, n=478), participantes del estudio DiOGenes (Diet, Obesity and Genes) antes y después de una dieta baja en calorías (800 kcal/d). Encontramos que el acetato estaba positivamente asociado con resistencia a la insulina en mujeres y no en hombres, incluso después de ajustar por edad, IMC y masa magra. Estos hallazgos deben ser investigados a fondo para entender las diferencias en acetato en sangre entre sexos, así como el rol del acetato en la salud metabólica durante la perdida de peso. En particular, se deben investigar los factores que contribuyen a las concentraciones de acetato en ayunas tales como el estatus hormonal, cetogénesis endógena del hígado y/o la producción de acetato en el intestino grueso. Además, estudios futuros deben investigar el flujo de acetato del intestino grueso a la circulación sistémica y su rol en la salud metabólica.

Actualmente, estudios en humanos que estudian los SCFA derivados de la fermentación en el intestino grueso han medido las concentraciones en heces. Sin embargo, las concentraciones de SCFA en heces deben ser interpretadas con cautela, ya que son el resultado de la producción en el intestino grueso, velocidad de absorción y liberación a la circulación sistémica, procesos que están intricadamente controlados por la composición de la microbiota intestinal, mecanismos de alimentación cruzada (cross-feeding), transito intestinal entre otros factores. Por lo tanto, en el **capitulo 4**, investigamos las asociaciones entre concentraciones de SCFA (acetato, propionato y butirato) en heces y sangre con marcadores metabólicos incluyendo metabolitos en sangre, hormonas intestinales, calorimetría indirecta, marcadores de inflamación y marcadores de sensibilidad a la insulina en un grupo de individuos con amplio rango de IMC y de estatus glucometabolico. Curiosamente, encontramos que SCFA (acetato, propionato y butirato) en sangre, no en heces, están asociados negativamente con marcadores de metabolismo de lípidos y positivamente asociados con niveles de péptido similar al glucagón 1 (GLP-1) en sangre. Las asociaciones negativas con lípidos en sangre (glicerol, triglicéridos, ácidos grasos libres, respectivamente) coinciden con la literatura, lo que sugiere una relación entre SCFA con metabolismo del tejido adiposo y posibles beneficios en la capacidad de almacenamiento del tejido adiposo. Un hallazgo interesante es la asociación positiva de los SCFA en sangre con niveles de GLP-1 en sangre, posiblemente indicando una conexión de SCFA en sangre con secreción de GLP-1. En el **capitulo 4**, en un análisis de un subgrupo encontramos que la sensibilidad a la insulina (medida con la técnica hiperinsulinémico-euglucémico clamp) se asocio con SCFA en sangre en direcciones opuestas (acetato negativamente y propionato positivamente).

Aunque las asociaciones de niveles en sangre de acetato con insulina son controversiales (**capitulo 3** y **capitulo 4)**, estas deben ser interpretadas con cautela considerando que los niveles de acetato en sangre son afectados por varios factores incluyendo la producción endógena por medio de la cetogénesis en el hígado, producción del intestino grueso, así como el fenotipo metabólico y de la microbiota intestinal.

Aparte de la fermentación de los hidratos de carbono no digeribles, la fermentación de proteínas y péptidos en el intestino grueso distal puede producir varios metabolitos tales como los BCFA (isobutirato e isovalerato), cuyo rol en la salud metabólica y metabolismo sistémico de macronutrimentos permanece sin investigar. Además, que existe carente investigación respecto a otros metabolitos presentes en heces derivados de la fermentación de hidratos de carbono no digeribles (i.e. valerato, lactato). Por lo tanto, en el **capitulo 5**, investigamos las concentraciones de metabolitos en heces incluyendo BCFA (isobutirato, isovalerato), otros metabolitos (valerato, lactato, succinato y caproato) en individuos con sensibilidad y resistencia a la insulina. Además, evaluamos la relación entre las concentraciones de estos metabolitos en heces con metabolitos en sangre, calorimetría indirecta y marcadores de sensibilidad a la insulina (insulina en sangre y HOMA-IR). No encontramos diferencias significativas en BCFA entre individuos con sensibilidad e individuos con resistencia a la insulina. Además, no encontramos asociaciones entre BCFA y marcadores de resistencia a la insulin y salud metabólica. Estudios futuros deben investigar el rol de estos metabolitos, así como su flujo del intestino grueso hacia la circulación sistémica.

En el **capitulo 6**, investigamos si los resultados previos en humanos ocurren a nivel del musculo esquelético, en los que la administración de acetato en el intestino grueso causaba incrementos en la oxidación de grasas. Por lo tanto, investigamos los efectos del acetato de sodio en la oxidación de grasas en células de musculo esquelético derivadas de una biopsia de un individuo sano (HSkMC). No observamos un efecto directo del acetato de sodio en oxidación de grasas (endogena y exógena) en nuestro modelo HSkMC usando concentraciones fisiológicas de acetato de sodio. Además, no observamos un efecto de dosis o de tiempo en la fosforilación de AMPK. Sin embargo, no podemos excluir que nuestros efectos reportados en humanos en la oxidación de grasas después de una administración en el intestino grueso ocurren en otros tejidos (hígado) o son mediados por otros metabolitos (butirato) u hormonas (PYY). Además, nuestra falta de efecto en la oxidación de grasas en respuesta a acetato de sodio puede ser explicada por diferencias del donante, tipo de fibra muscular as como por diferencias entre especies.

Varios estudios en animales, así como estudios en humanos han reportado un efecto anti-lipolítico de los SCFA, el cual puede reducir los niveles sistémicos de dislipidemia y mejorar la sensibilidad a la insulina. Por lo tanto, en el **capitulo 7**, usamos el modelo celular de adipocito derivado de humanos (human multipotent adipose-derived stem (hMADS) para estudiar los efectos de SCFA en la lipolisis citosolica y elucidar los mecanismos. Encontramos que los SCFA modulan la lipolisis de diferente forma, y en particular el acetato es el mayor responsable de disminuir la liberación basal y estimulada (β-adrenérgica) de glicerol en nuestro modelo hMADS. Este efecto anti-lipolítico fue mediado por la reducción de la fosforilación de la hormona sensitiva a la lipasa (HSL) por medio de los receptores celulares GPR. Estos resultados sugieren que el acetato en sangre disminuye la lipolisis y muestra potencial para mejorar la dislipidemia y resistencia a la insulina.

En conclusión, esta tesis doctoral provee mayor información respecto al rol metabólico de los metabolitos que provienen de la fermentación de hidratos de carbono no digeribles (principales SCFA y otros menos abundantes) y de los metabolitos provenientes de la fermentación de proteínas y péptidos (BCFA) en la sensibilidad a la insulina y metabolismo de macronutrimentos en estudios de cohorte en humanos así como en estudios con modelos celulares derivados de humanos (musculo esquelético y tejido adiposo). Los principales hallazgos indican que las concentraciones de SCFA en sangre pueden ser mejores indicadores de los efectos en la salud metabólica, en comparación con niveles de SCFA medidos en heces. Además, estos resultados sugieren que la relación de los niveles de acetato en sangre con la sensibilidad a la insulina puede ser distinta en hombres y en mujeres y depender de varios factores como metabolismo del hígado, perfil hormonal, y fenotipo metabólico y de microbiota intestinal. Por ultimo, nuestros experimentos *in vitro* proveen más información a nivel de mecanismos respecto a metabolismo de SCFA en oxidación de grasas en musculo esquelético y de efectos en lipolisis en tejido adiposo, que debe ser investigado en mas detalle en estudios futuros. Además, estudios futuros deben considerar el sitio de producción, absorción, y liberación en el intestino grueso de los SCFA y BCFA, su contribución al metabolismo de distintos órganos y efectos sistémicos en la salud metabólica.

Valorization

This thesis describes the potential of gut-derived microbial metabolites as key players in host metabolic health and insulin sensitivity. We investigated the role of short chain fatty acids and branched chain fatty acids (SCFA and BCFA) in *in vivo* metabolic health in human cross-sectional and intervention studies with overweight people and with obesity. In addition, we studied the role of SCFA in human derived adipose and skeletal muscle tissues in *in vitro* models. In this section, we describe the impact of our scientific findings for global society and economy, especially on the implications and possible applications for specific target groups with higher health risks.

Obesity is associated with other comorbidities including cardiovascular disease, type 2 diabetes mellitus, some forms of cancer among other comorbidities [1]. Currently, obesity prevalence and related complications are continuing to increase as reported by the World Health Organization with prevalence rates of obesity of 2 billion according to 2016 report [1]. Most affected areas include parts of Europe, North America (i.e. Mexico) and the Middle East [1]. Of major importance, predictions point towards even higher increments in the near future.

Obesity is a chronic metabolic disease that results from an energy imbalance in the long-term. The major contributing factors to complications include an increased lipid accumulation in adipose and non-adipose organs, adipose tissue dysfunction, lipid spillover and low-grade inflammation that collectively result in the development of insulin resistance. These metabolic alterations may start with a mild insulin resistant state and progress to type 2 diabetes mellitus.

In the last decades, research has shown the intricate connection of the gut and gut microbial-derived metabolites with host metabolic health. In this context, this thesis provides valuable information about major saccharolytic fermentation products (acetate, propionate and butyrate) and less abundant gut-derived metabolites (lactate, caproate, valerate, succinate) as well as proteolytic fermentation products (isovalerate and isobutyrate). This sheds light on the individual as well as collective effects of these metabolites on host metabolic health and insulin sensitivity.

For instance, we discuss the possible higher relevance of circulating SCFA as compared to fecal concentrations as biomarkers of metabolic health effects. Although this particular finding warrants further investigation, this may be of importance in the clinical setting and monitoring of nutritional interventions targeting the gut. Furthermore, we show that the relationship of the major SCFA acetate with insulin sensitivity together with the sexual dimorphism in this

relationship is complex and warrants further investigation. Of note, these findings point towards the need for carefully designed studies to investigate SCFA (i.e. acetate) kinetics (production, absorption and release into the circulation) in relation to metabolic health.

In general, this thesis adds to the knowledge on the potential of gut-derived metabolites to modulate host metabolic health, especially in the human situation. In this regard, our findings may provide useful information for dietary intervention strategies that may ameliorate metabolic health. It is evident that attention should be placed on the balance between saccharolytic and proteolytic fermentation as a determinant of metabolic health. Furthermore, our data may provide clues for a more targeted dietary guidance based on microbial and metabolic phenotype. After further confirmation of these findings in prospective dietary intervention studies, this knowledge may be used by health care professionals when advising patients on their dietary intake as well as relevant leads for food industry to produce functional foods with pronounced metabolic benefits.

Of note, the *in vitro* studies of this doctoral thesis provided valuable scientific input on tissue-specific effects of SCFA. These studies provided important mechanistic insight on previous described metabolic effect on lipolysis and fat oxidation after colonic SCFA administration *in vivo*. However, more research is needed to define effective dietary interventions (pre, pro, synbiotics) that can increase systemic SCFA levels in physiologically relevant concentrations, thereby eliciting similar effects, as reported in vitro, at the tissue level in skeletal muscle and adipose tissue or other tissues.

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All the work in this thesis will be available to the public through scientific publications in internationally peer-reviewed journals. In addition, there has been frequent interaction in research meetings, symposiums (School of Nutrition and Translational Research in Metabolism, NUTRIM) and conferences at the national (Netherlands Association for the Study of Obesity, NASO Utrecht 2018 and 2019) and international level (European Congress of Obesity, ECO Vienna 2018 and Glasgow 2019).

The overall aim of the thesis was to gain more insight into gut microbial metabolites and their impact on metabolic health by utilizing state of the art methodology and extensive human phenotyping. In addition, we used human derived skeletal muscle and adipose tissue *in vitro* models, which provided valuable insight into human physiology that was previously lacking.

Previous work has focused on identifying nutritional interventions (i.e. prebiotics) and their capacity to produce microbial metabolites that can improve metabolic health. Accumulating evidence suggests that the obesity pandemic may increase susceptibility and worsen the current coronavirus disease 2019 (COVID-19) outcomes possibly partially linked to alterations in microbial community [2, 3]. However, future research has to elucidate whether nutritional interventions promoting anti-inflammatory properties, gut and metabolic health may ameliorate COVID-19 infections and the progression of the disease [4].

To conclude, this thesis provides input on the role of saccharolytic (SCFA) and proteolytic fermentation products (BCFA) in metabolic health in human studies and human derived cell models. Of note, this may lead to design more carefully and personalized human interventions and investigate in more detail the role of microbial metabolites in metabolic health in response to nutritional interventions. Finally, this may aid in the development of more personalized nutritional strategies that counteract the impact of obesity and its comorbidities more effectively than or in addition to conventional caloric restriction.

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Curriculum Vitae

Manuel Alejandro Gonzalez Hernandez was born on May 12th, 1989 in Leon Guanajuato, México, where he was raised and completed his bachelor's degree in Human Nutrition at Universidad de Guanajuato in 2013. During undergraduate studies he enrolled in an exchange program for one semester in St. Francis Xavier University (Nova Scotia, Canada) and joined a summer research program at University of Arizona, (Tucson, USA). He continued his education in the Netherlands, where he obtained his master's degree in Biomedical Sciences at Maastricht University in 2016 at the department of Human Biology.

In 2016, Manuel started his PhD at the department of Human Biology at Maastricht University within NUTRIM School of Nutrition and Translational Research in Metabolism under the supervision of Prof. dr. Ellen Blaak, Dr. Emanuel Canfora and Dr. Johan Jocken. His research focused on the role of gut-derived metabolites (SCFA and BCFA) in *in vivo* metabolic health in human cross-sectional studies with overweight and obese individuals as well as the role of major SCFA in human derived adipose and skeletal muscle tissues in *in vitro* models. During his PhD, Manuel has presented his work as oral and poster presentations in national (Netherlands Association for the study on obesity, NASO 2018 and 2019) and international (European Congress on Obesity, ECO 2018 and 2019) conferences. In addition, he was awarded travel grants from NASO to attend ECO in 2018 and 2019. Recently, he was awarded the NASO publication prize in 2020 for the manuscript entitled "Circulating but not fecal short-chain fatty acids are related to insulin sensitivity, lipolysis and GLP-1 concentrations in humans".

List of Publications

Manuel A. González Hernández, Emanuel E. Canfora, Kenneth Pasmans, Arne Astrup, W.H.M. Saris and Ellen E Blaak. The Relationship between Circulating Acetate and Human Insulin Resistance before and after Weight Loss in the DiOGenes Study. *Nutrients. 2020; 12 (2): 339*

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Mattea Müller*, **Manuel A. González Hernández***, Gijs G.H. Goossens, Dorien Reijnders, Jens J. Holst, Johan W.E. Jocken, Emanuel E. Canfora and Ellen E Blaak. Circulating but not fecal short-chain fatty acids are related to insulin sensitivity, lipolysis and GLP-1 concentrations in humans. Scientific Reports 2019; 9(1):1-9. *Shared first authors

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Elisa Cirillo, Martina Kutmon, **Manuel A. González Hernández,** Tom Hooimeijer, Michiel E Adriaens, Lars M T Eijssen, Laurence D Parnell, Susan L Coort, Chris T Evelo. From SNPs to pathways: Biological interpretation of type 2 diabetes (T2DM) genome wide association study (GWAS) results. PLoS One 2018 13(4): e0193515

Manuel A. González Hernández, Ellen E Blaak, Nicole T. H. Hoebers, Yvonne P. G. Essers, Emanuel E. Canfora and Johan W. E. Jocken. 133. Acetate does not affect palmitate oxidation and AMPK phosphorylation in human primary skeletal muscle cells. *In preparation for submission.*

Manuel A. González Hernández, Emanuel E. Canfora and Ellen E Blaak. Fecal microbial metabolites of proteolytic and saccharolytic fermentation in relation to degree of insulin resistance in overweight and obese individuals. *Submitted*

Awards and grants

"Publication prize 2020" for PhD students, granted by Netherlands Association for the Study on Obesity (NASO), Online meeting, The Netherlands.
TITLE: Circulating but not fecal short-chain fatty acids are related to insulin sensitivity, lipolysis and GLP-1 concentrations in humans. Scientific Reports

"NASO Travel grant 2019" for PhD students to attend European Congress of Obesity in Glasgow 2019, granted by Netherlands Association for the Study on Obesity (NASO), Online meeting, The Netherlands

"NASO Travel grant 2018" for PhD students to attend European Congress of Obesity in Vienna 2018, granted by Netherlands Association for the Study on Obesity (NASO), Online meeting, The Netherlands

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Poster and oral presentations

Oral presentation, Organ Cross Talk session at **26th European Congress on Obesity**, 28th April to May 1st 2019, Glasgow, Scotland
TITLE: Circulating but not fecal short-chain fatty acids are related to insulin sensitivity, lipolysis and GLP-1 concentrations in humans.

Oral presentation, annual meeting **Netherlands Association for the Study on Obesity (NASO) 2019**, Utrecht, The Netherlands
TITLE: Circulating but not fecal short-chain fatty acids are related to insulin sensitivity, lipolysis and GLP-1 concentrations in humans.

Oral poster and pitch presentation, at **Annual NUTRIM School of Nutrition and Translational Research**, November 2018, Maastricht, The Netherlands
TITLE: Circulating but not fecal short-chain fatty acids are related to insulin sensitivity, lipolysis and GLP-1 concentrations in humans.

Poster presentation, at **25th European Congress on Obesity**, May 2018, Vienna, Austria
TITLE: The Relationship between Circulating Acetate and Human Insulin Resistance before and after Weight Loss in the DiOGenes Study

Oral presentation, annual meeting **Netherlands Association for the Study on Obesity (NASO) 2018**, Utrecht, The Netherlands.
TITLE: The Relationship between Circulating Acetate and Human Insulin Resistance before and after Weight Loss in the DiOGenes Study