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The intestinal short chain fatty acid production: its complexity and metabolic consequences

Rios Morales, Melany

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General introduction and thesis outline

INTRODUCTION

The incidence of obesity and associated pathologies has nearly tripled since 1975. Until 2016, almost 15% of the world's adult population was obese and 40% were overweight¹, imposing a major health and socio-economic problem². Globally, this condition is driven by the increased availability of high-calorie food and a sedentary lifestyle. This leads to an energy imbalance, in which the calories consumed exceed the calories expended¹. This can result in an overflow of circulating lipids causing ectopic fat storage in non-adipose organs such as the liver and the skeletal muscle. This makes obesity a risk factor for other chronic diseases, such as type 2 diabetes mellitus (T2DM) and metabolic-associated fatty liver disease (MAFLD)³.

Consumption of non-digestible carbohydrates (NDC), complex polysaccharides that resist digestion and absorption in the small intestine, has been associated with improved systemic metabolic health, including improved body weight and glucose control⁴, but the underlying mechanisms are poorly understood. NDC can be used by gut microbiota as a substrate for anaerobic fermentation, resulting in the production of short-chain fatty acids (SCFA), mainly acetate, propionate, and butyrate^{4,5}. SCFA can have local effects in the intestine or can be taken up by the host and affect other organs, notably adipose tissue, liver, and skeletal muscle⁶. Over the past years, the gut microbiome has emerged as an important regulator of host energy metabolism^{4,7-9}. Likewise, SCFA have been suggested to be the link between a high-fiber diet and health improvements^{6,10}. Due to the limited information from human studies, the inner world of luminal NDC fermentation and SCFA production, and via which mechanisms these small molecules affect whole-body metabolism, is still not completely elucidated.

Colonic NDC fermentation by the gut microbiota

NDC as fermentable substrates. Carbohydrates that are not digested nor absorbed in the human intestine, so far called NDC, are classified as fibers if they have at least 3 monomeric units¹¹. Indigestible foods play a major role in shaping the composition of the gut microbiota¹². The types and amounts of NDC that reach the different portions of the large intestine depend on the daily intake and type of food. Current western diets contain low amounts of insoluble and soluble/fermentable fibers, typically around 20 g/day. This does not approach the recommended 25-50 g/day of fiber intake^{13,14}. Only a few fibers could also be categorized as prebiotics, such as GOS and FOS, which are endorsed by International Scientific Association of Pre- and Probiotics (ISAPP). Prebiotics are “substrates that are selectively utilized by host microorganisms conferring a health benefit”^{9,15}. In this thesis, I will focus on the soluble NDC galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS),

also called oligofructose, the two main described prebiotics⁹. GOS and FOS are naturally found in a variety of food. GOS are mostly present in mother milk, beans, and legumes^{16,17}, while FOS can be found in roots, fruits and whole grains^{18,19}. Both oligosaccharides are also industrially produced as dietary supplements because of their low caloric value and beneficial effects as fermentation substrates¹⁵. FOS can be produced by partial enzymatic hydrolysis of inulin, which is extracted mainly from chicory roots²⁰, and GOS via enzymatic hydrolysis and transgalactosylation of lactose²¹. To optimize the guidelines on fiber supplementation with prebiotics, detailed knowledge on their fermentation is needed.

Gut microbiota. The human intestine contains an ecosystem that consists of trillions of bacteria forming a consortium. Although the gut microbial community is composed of mostly five phyla (*Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*), there is considerable diversity at the level of species and their relative abundances^{22,23}. The microbial composition depends on genetic and environmental factors, such as diet composition²⁴. The gut microbiome has important roles in the training of host immunity, digesting food, eliminating toxins, and producing numerous compounds that influence the host²⁵. Throughout this thesis, I will focus on microbial fermentation of NDC and its main product, SCFA. NDC are fermented under the anaerobic conditions that prevail in the proximal colon. There they are degraded into monosaccharides by microbial hydrolysis and subsequently fermented to phosphoenolpyruvate (PEP) via the Embden-Meyerhof pathway. PEP will then be used to produce SCFA, mainly acetate, propionate, and butyrate, via different reactions⁴. These metabolites are a necessary waste product to the microbial community, required to balance the production of redox equivalents in the anaerobic environment of the gut²⁶. For the production of SCFA the gut microbiota must work as a community. Metabolic cross feeding by bacteria in which fermentation products from certain microbes are subsequently used by other microbes, plays a key role in maintaining the microbial ecosystem and is crucial to determine the final SCFA profile in the intestine^{27,28}. Despite detailed knowledge of the biochemistry of SCFA production by specific bacteria, and the extensive culturing and sequencing efforts, the complete microbial repertoire of the human gut and the behavior of the microbiome as a community remain not completely elucidated.

The fermentation products SCFA. SCFA are small organic, monocarboxylic acids with less than six carbon atoms. Acetate, propionate, and butyrate account for more than 95% of the microbially produced SCFA. The type and amount of NDC in the diet, the diversity and absolute amount of the intestinal microbiota, and gut transit time of food play an important role in the production of SCFA²⁹⁻³².

After their production in the lumen of the gut, SCFA are rapidly absorbed by colonocytes. Transport of SCFA may occur via non-ionic diffusion of protonated SCFA, via the monocarboxylate transporters MCT1, MCT2, and MCT4, or via the sodium-coupled monocarboxylate transporter 1 (SMCT1)³³. In the colonocytes, butyrate and possibly acetate are metabolized as the main source of energy. Whereas colonocytes have a higher affinity towards butyrate than towards acetate, this is probably compensated by the high concentration of acetate in the colon^{5,34,35}. The fraction of all three SCFA that escapes metabolism by colonocytes, drains into the portal vein to be further metabolized in the liver. Eventually, only a small fraction of gut SCFA, in the μM range, reaches the peripheral circulation³⁶⁻³⁹, where it can be taken up by other organs and affect their physiology, both in humans and mice^{6,10}.

In a landmark study in humans that had died suddenly, SCFA concentrations were measured within 4 hours after death. The concentration of SCFA in the distal ileum reached 13 mmol/kg, while in the colon concentrations were 10-fold higher, reaching 131 mmol/kg⁴⁰. In initial studies inside the human gut, the concentrations of acetate, propionate, and butyrate were in a molar ratio of 54:20:21 respectively^{41,42}. Assessing SCFA kinetics in humans *in vivo* comes with major challenges because of the inaccessibility of the lumen of the proximal colon where microbial fermentation takes place. For this reason, the field mostly relies on fecal measurements as a proxy of colonic content. Since only 5-10% of the luminal SCFA are excreted, feces concentrations are not considered to be an accurate representation of the colonic situation³². Consequently, fecal measurements should be interpreted with caution. On the one hand, it was found in humans that an increased fecal SCFA concentration correlates with improvement of health. On the other hand, in other studies increased fecal SCFA concentrations correlated with an obese phenotype^{43,44}. Altered SCFA concentrations in feces are the result of altered production, altered colonic absorption, or a combination of both. This highlights the importance of studying the kinetics of SCFA production and absorption to elucidate how fiber fermentation affects human health.

NDC degradation and SCFA production have been widely studied in *in vitro* gut systems. These are dynamic *in vitro* simulators of human digestion that can reproduce physicochemical parameters of the human gut, such as temperature, acidity, electrolyte concentration, and that can even mimic peristalsis^{33,45}. They do, however, not account for the interaction with the host, lacking the *in vivo* process of absorption. Another limitation is that these systems are often inoculated with fecal samples, which are expected to be only a surrogate representative of the microbiota residing in the proximal colon^{46,47}. An alternative option to study this process is by the use of stable isotopes and the quantification of label appearance and dilution

in vivo. In mice fed different amounts of fermentable fiber, SCFA production from the fibers was estimated by measurement of the isotope dilution of ^{13}C -SCFA after continuous infusion of ^{13}C -SCFA directly into the cecum⁴⁸. In this study, only the *in vivo* uptake fluxes of the SCFA, and not their cecal concentrations, correlated linearly with the improvements in metabolic health⁴⁸. This suggests that their beneficial effects on metabolic health are at least partially dependent on uptake by the host and a direct role of SCFA in tissues other than the gut. Boets et al pioneered such stable isotopes studies in humans by estimating the rate of appearance of SCFA in blood derived from inulin ingestion after a continuous intravenous infusion of ^{13}C -SCFA²⁹ and the measurement of isotope dilution of ^{13}C -SCFA in blood³⁶. The same group also delivered ^{13}C -labeled SCFA directly into the proximal colon by degradable capsules with a pH-responsive coating and measured the label appearance in blood⁴⁹. This provided a first estimate of SCFA production and absorption, although it did not account for the unknown degree of first-pass SCFA metabolism in the gut and liver before reaching the peripheral circulation.

Direct sampling in the gut is essential to quantify the rate of intestinal fiber fermentation. Conventional methods of exploring and collecting human lumen samples are invasive and include naso-intestinal catheters, mostly for the small intestine, or colonoscopies, for the more distal colon⁵⁰. Recently non-invasive gastrointestinal capsules have been developed, some of which allow not only delivery but also sampling of the luminal content⁵¹⁻⁵⁵. Despite earlier optimism, however, to my knowledge, these new tools are still under development.

The effect of SCFA on energy homeostasis and improved metabolic health

High dietary fiber intake has been associated with improved weight control, improved insulin sensitivity, glucose homeostasis, and increased energy expenditure in humans^{4,56-60}. These effects are mimicked by SCFA administration in mice^{37,61,62}, and with more limited evidence in humans⁶³⁻⁶⁶. An extensive review of studies after SCFA interventions can be found elsewhere³³. SCFA can influence host health by a combination of their local effects in the gut and their effects on other organs after absorption into the body. In turn, the effect of SCFA on other organs can be through a direct effect on the tissue or an indirect effect due to inter-organ crosstalk.

SCFA regulate host metabolism in the different organs via different mechanisms. All three SCFA can act as signaling molecules, mostly through the G protein-coupled receptors GPR41 and GPR43. These receptors are not only present in human colonic tissue^{67,68}, but also in the liver and peripheral organs, notably white adipose, skeletal muscle, and pancreas⁶⁹⁻⁷¹. Secondly, butyrate, and to a lesser extent propionate⁷²,

regulate gene expression via competitive inhibition of histone deacetylases (HDAC)^{73,74}. As a HDAC inhibitor, butyrate increases the transcription of target genes involved in host metabolism. Lastly, acetate, propionate, and butyrate are used by the host as metabolic substrates. Stable isotope studies have shown that SCFA can be systemically oxidized or assimilated into glucose and lipids^{28,49}. The known effects of SCFA on energy regulation in the gut and other metabolic organs are summarized below.

Gut. SCFA affects energy intake by locally signaling through GPR41 and GPR43, which promotes the release of glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) from enteroendocrine cells⁷⁵⁻⁷⁸. Both hormones help regulate food intake and satiety⁷⁹. Additionally, butyrate and propionate can induce intestinal gluconeogenesis (IGN) via complementary mechanisms. Butyrate increases IGN via a c-AMP-dependent mechanism. In contrast, propionate, itself a substrate of IGN, induces the pathway via a gut-brain neural circuit initiated by activation of GPR41 in the periportal afferent neural system. The resulting glucose is sensed in the walls of the portal vein and induces a nervous signal to the brain that influences food intake and glucose control, among others through decreased hepatic glucose production⁸⁰.

Pancreas. By using a propionate-inulin-ester, it was demonstrated that propionate has beneficial effects on human β -cell function and insulin secretion *in vivo*. This was recapitulated in human islets *in vitro*⁸¹. In obese and insulin-resistant mice, SCFA increased glucose-stimulated insulin secretion via GPR43⁸². In agreement with these findings, GPR43 or GPR41 depletion deteriorated β -cell function and impaired glucose control in mice^{71,83}.

Liver. In the liver, SCFA affect both glucose and lipid metabolism. Oral administration of any of the three individual SCFA in animal models of obesity and T2D resulted in a decrease in hepatic lipid accumulation and improved glucose homeostasis^{61,62,84,85}. In rodents, butyrate supplementation or treatment with butyrate-producing bacteria prevents the progression of obesity-induced fatty liver disease and improves insulin sensitivity⁸⁶⁻⁸⁸. This was ascribed to signaling through AMP-activated protein kinase (AMPK), based on experiments with *ex vivo* liver tissue and HEPG2 cells⁶¹. Bovine hepatocytes also showed increased AMPK activation and expression of genes involved in lipid oxidation upon stimulation with acetic acid⁸⁹. This signaling is likely to be triggered by GPR41 and GPR43.

Adipose tissue. Several *in vivo* studies have shown that acetate inhibits whole-body lipolysis in humans. For instance, rectal delivery of a SCFA mixture decreased the concentration of glycerol, a product of lipolysis³⁸, in blood. Acute intravenous acetate

decreased free-fatty acids in plasma⁹⁰. Reduced lipolysis in adipose tissue prevents excess lipid supply to other tissues^{38,91,92}. *In vitro* studies suggest that the antilipolytic effect of acetate is mediated by a decreased phosphorylation of hormone-sensitive lipase in a GPR-dependent manner^{92,93}. Acetate and propionate also increase adipogenesis via GPR43, at least *in vitro*⁹⁴. Together these results show that SCFA improves the lipid buffering capacity of adipose tissue. This would prevent systemic low-grade inflammation and improve insulin sensitivity⁹¹.

Muscle. Skeletal muscle is a major site of insulin-stimulated glucose uptake and thereby plays a critical role in glucose homeostasis. In one study, dietary acetate supplementation in rats increased the expression of genes that code for proteins involved in glucose metabolism in the abdominal muscle, including the insulin-sensitive glucose transporter GLUT4⁹⁵. In contrast, in another study dietary acetate supplementation decreased glycolysis in the gastrocnemius muscle of rats, likely by suppression of phosphofruktokinase-1 activity (PFK1)⁹⁶. Skeletal muscle insulin sensitivity has been shown to improve after butyrate treatment³⁷. Butyrate enhanced mitochondrial biogenesis and fatty acid oxidation in the skeletal muscle both *in vivo*^{37,97,98} and *in vitro*^{37,99}. Moreover, through HDAC inhibition this SCFA increased transcription of target genes involved in muscle glucose metabolism¹⁰⁰ and insulin signaling^{101,102}.

In conclusion, SCFA undoubtedly have a beneficial effect on whole-body metabolic health. This suggests that a dietary intervention can be a simple, yet effective alternative to treat metabolic syndrome. However, while many studies focus on the regulatory role of SCFA, their quantitative role as a catabolic or anabolic substrate for the host has received relatively little attention. SCFA are also an extra source of energy from otherwise indigestible carbohydrates. To understand what controls the net effect of SCFA supplementation, more precise data on intestinal SCFA kinetics are needed. Moreover, as described above, most of the knowledge about the mechanism by which SCFA affect different tissues directly is derived from only a few studies. Further studies should address the interplay among the different intracellular pathways via which SCFA affect these tissues.

THESIS OUTLINE

Consumption of non-digestible carbohydrates (NDC) has been linked to many health benefits. Nevertheless, detailed knowledge on the kinetics of NDC fermentation and the metabolic fate of the produced short-chain fatty acids (SCFA) in humans is lacking. This thesis has two main goals. The first aim is to assess the fermentation of

non-digestible carbohydrates with production of SCFA in the gut, and the systemic metabolic fate of SCFA (**chapters 2, chapter 3, and chapter 4**). The second aim is to unravel tissue-specific effects of butyrate on fuel handling (**chapter 5, and chapter 6**).

To study the inner world of the human gut, in **chapter 2**, together with my colleagues from the Wageningen University, I used for the first time a custom-made naso-intestinal catheter to monitor the fermentation of a combination of NDC (namely FOS:GOS) fermentation, SCFA production, SCFA interconversion, and absorption inside the human distal ileum lumen, as well as the direct impact of FOS:GOS on the luminal microbiota. In the same study, I also addressed the effect of a preceding NDC supplementation on the acute fermentation. Luminal SCFA kinetics were studied by directly delivering a bolus of ^{13}C -SCFA into the gut and using a stable-isotope dilution approach to estimate the production of SCFA by fermentation of the FOS:GOS mix by the local microbiome. To study the metabolic fate of acetate, propionate and butyrate as metabolic substrates, the label appearance in different important biological metabolites was measured in blood samples. Because of the scarce studies on the intestinal fermentation of FOS and GOS in humans, we give a more extensive description of the luminal degradation of these two NDC in **chapter 3**. To study colonic NDC fermentation in a way less burdensome for volunteers, new gastrointestinal capsules for luminal delivery and sampling are under development. Because of their novelty, there is no methodology associated to ensure representative data from the sampling location, since the capsules may remain up to 48 hours in the gut before they can be collected from the feces. In **chapter 4**, still with the colleagues from Wageningen, I tested a stabilizing reagent to be preloaded in the gastrointestinal capsules and a complete workflow to analyze dietary fibers, microbiota, and SCFA in the collected sample. Despite the original plan to use gastrointestinal capsules in this thesis to assess colonic fermentation, I could not use them because of major delays in their release on the market. Nevertheless, the work done in **chapter 4** helps prepare the road for the use of this novel technology.

After their production in the gut lumen, SCFA are rapidly absorbed by the host. As described above, their uptake flux correlates with improvements in host metabolic health. Thus, besides having local effects in the gut, they derive their beneficial properties at least partially from direct effects on other tissues. In the liver, systemic butyrate supplementation has been reported to decrease hepatic steatosis and to prevent its progression to nonalcoholic steatohepatitis. In **chapter 5**, together with colleagues at the University of Groningen, I optimized an *ex vivo* system, the murine precision-cut liver slices, to mimic early-stage metabolic-associated fatty-liver disease (MAFLD). In this model, I tested whether the most widely used SCFA, butyrate, could directly prevent triglyceride accumulation. In **chapter 6**, together

with a colleague from my own team, I addressed the direct effect of butyrate on the insulin response of a muscle cell line. In this chapter, we systematically unraveled the interplay between transcription regulation, signalling, and the metabolic effects of butyrate in insulin-resistant myotubes. Finally, in **chapter 7** I discuss the main findings of this thesis in the context of future perspectives and challenges to study the complex world of gut fermentation and improve dietary intervention strategies.



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