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

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General discussion

Recommendation of high fiber diets have been suggested as a valuable strategy to reduce the burden of metabolic diseases such as obesity, metabolic-associated fatty liver disease (MAFLD), and type 2 diabetes (T2D). Acetate, propionate, and butyrate, the main short chain fatty acids (SCFA) produced by gut microbial fermentation of fibers, appear to be key mediators of the beneficial effects elicited by high fiber diets[1]. SCFA supplementation has been linked to the prevention of weight gain, improved glucose homeostasis, and insulin sensitivity. Most studies have been performed in rodents, while in humans, evidence is less thorough. Only one study, in which propionate was targeted to the colon, has measured a reduction of body weight[2]. In line with this, colonic, but not oral administration of propionate decreased energy intake[2–5]. Also in humans, proximal and distal colonic administration of only acetate did not have an effect on energy expenditure in the fasted state or after glucose consumption[6], while other studies show that distal colonic infusion of a mix of SCFA or oral propionate supplementation increased energy expenditure in the fasted state[7,8]. The effect of SCFA supplementation on glucose homeostasis has been more widely studied in humans. Oral propionate or butyrate supplementation improved glucose control, insulin sensitivity, and decreased the concentration of free fatty acids in the circulation[9–11]. Rectal infusion of propionate increased the plasma glucose level[12], whereas a rectally infused SCFA mix did not affect circulating glucose or insulin levels, but decreased free fatty acids levels[8,13].

Due to the limited and heterogeneous information from human studies, further investigations are needed that directly study intestinal SCFA kinetics as the link between fibers and host metabolism. Measuring local SCFA production and absorption after fiber supplementation is key to understand the mechanism via which diet modulates health. However, in humans SCFA have only been measured in plasma or fecal samples. The concentrations in these samples are the net result of SCFA production, excretion, absorption, and metabolization, underestimating the complete contribution of these small molecules to host metabolism. For this reason in Part I of the thesis **(chapter 2**, **chapter 3**, and **chapter 4)** I aimed to study NDC fermentation and SCFA production, microbial interconversion, and absorption inside the lumen of the human gut *in vivo*, and the fate of intestinal SCFA as substrates for glucose and lipid metabolism. Moreover, to further understand the mechanism by which SCFA can modulate host health, in Part II of the thesis **(chapter 5** and **chapter 6)**, I assessed the tissue-specific effects of a single SCFA, butyrate, on glucose and lipid metabolism.

Part I. Fermentation of non-digestible carbohydrates and production of short-chain fatty acids in the gut and the systemic metabolic fate of SCFA

Small intestine. Intestinal catheters have been used for decades in human research as delivery and, less often, as sampling devices to get insight into the local gastrointestinal (GI) tract metabolism[14,15]. In **chapter 2** we used for the first time a custom-made naso-intestinal catheter to monitor galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) fermentation, SCFA production, SCFA interconversion, and absorption inside the human distal ileum lumen, as well as the direct impact of the FOS-GOS mixture on the luminal microbiota. It is known from *in vitro* studies that the small intestinal bacteria have the capacity to hydrolyze up to 80% of FOS and GOS *in vitro*[16–18]. In our *in vivo* study, we did not find FOS or GOS breakdown in the distal ileum. NDC, given as an oral bolus, were present for less than an hour at the sampling site. Dynamic changes of the relative microbiota composition, but not increased total bacterial number, were found during the day. Moreover several bacteria known to be stimulated by NDCs, namely *Bifidobacterium*, *Lactobacillus, Bacteroides* and *Streptococcus* [19], did not significantly fluctuate over time. To assess SCFA kinetics we used ^{13}C-labeled SCFA. After delivery of ^{13}C-SCFA through the delivery lumen of the catheter, samples were withdrawn over time through the sampling lumen. In these samples, dilution of ^{13}C-SCFA was measured to estimate the production of SCFA by fermentation of FOS and GOS by the local microbiome. In the distal ileum SCFA production was minimal. By using acetate with a single ^{13}C atom, propionate with three ^{13}C atoms, and butyrate with four ^{13}C atoms, we could in principle see the origin of the label and estimate the interconversion of the SCFA. In our study, however, no interconversion took place. Rapid absorption might have left too little time for microbial SCFA interconversion. The possibility of rapid absorption was supported by the almost immediate appearance of ^{13}C in exhaled CO_2, glucose, and other metabolites. This also pointed to the rapid metabolism of SCFA by the host. The choice of the different SCFA labels also allowed to evaluate the individual contributions of each SCFA to host metabolites. Acetate is most likely metabolized in the cytosol, while propionate and butyrate are metabolized in the mitochondria. As a consequence, ^{13}C atoms from butyrate and propionate contributed the most to label incorporation into glucose. Butyrate was also the major contributor of carbons to citrate. In contrast, the contribution of carbons from acetate to glucose and citrate was minor. It is important to highlight that by using a different number of ^{13}C atoms in the three SCFA, they can be delivered simultaneously in a single dose, avoiding multiple intubations in the same subject or division of the cohort of volunteers for subgroups for the different SCFA to be studied.

In conclusion, opposite to what we expected from *in vitro* studies, our data showed that in the distal ileum SCFA production and interconversion were negligible and SCFA delivered in the lumen were rapidly absorbed and metabolized by the host. Even though the small intestine microbiota may have a certain capacity for fiber fermentation, it is likely that the rapid transit time precludes substantial fermentation at the sampling location. This is not negative news *per se*, since the small intestine is not adapted to handle gas production like the proximal colon.

In **Chapter 3** we described FOS and GOS digestibility in the small intestine in-depth. FOS was not fermented, digested, or absorbed before reaching the distal ileum, and the initial FOS bolus was almost 100% recovered. GOS was partially digested, likely by the small intestine brush border enzymes[20,21,] or absorbed in the small intestine leading to a less than 80% recovery. The abundances of FOS DP ≥ 2 and GOS DP ≥ 3 relative to the initial NDC bolus were not affected, while the relative and absolute abundance of GOS DP2 was lower than in the initial bolus, because it was hydrolyzed in the small intestine in a glycosidic-linkage depend fashion. I think that testing the intestinal digestibility of different NDC with different linkages and degrees of polymerization in humans *in vivo* helps future development of tailored prebiotics to maximize their availability in the proximal colon where they exert most of their metabolic effects[22].

Colon. From the above results and the literature, one can draw the conclusion that the proximal colon has to be sampled since it is the main site of NDC fermentation and SCFA production. According to our experience, naso-intestinal catheters might not be the best option. At first sight, they have many advantages: e.g the number and size of the lumina and other characteristics can be tuned to the requirements of the study, they allow to take multiple samples over time with relatively large sample volumes (1- 2 mL), and they can be reused after proper sterilization[15]. Nevertheless, when they need to reach the colon, they are a major burden for the patients. The positioning of the catheter in the intestine and past multiple valves is not trivial. This can take hours, if not days[15] . In our experimental setup, reaching the more distal part of the GI tract was successful in only two subjects. In these subjects the sampling of colonic content was not always feasible due to the viscous fluid consistency and blockages of the sampling lumen. Other options to study the more distal colon are colonoscopies and rectal/anal catheters[6,13]. However, to facilitate the passage of the catheter, laxatives or enemas to clean the colon are used. These steps utterly perturb the luminal microbiome, the very subject of our study[23–25]. Thus, new tools for assessing colonic NDC fermentation are urgently needed.

Accessing fermentation in the proximal colon now starts to become possible through novel gastrointestinal delivering/sampling capsules that can be swallowed by humans[26–30]. This technology is non-invasive, with a minimal burden to the patients, and avoids crossover contamination from regions of the intestine that are not the region of interest[31]. Some gastrointestinal sampling capsules are equipped with wireless communication to collect real-time temperature and pH data, which allows for an estimation of their location in the gut and to initiate delivery and/or sampling[26] . These capsules have a small size, to enable smooth passage through the GI tract, and can carry a small volume of sample. Retrieval of the sample can only be done after the excretion of the capsules, which can take up to several days. For these reasons, in **chapter 4** we developed a toolbox for the comprehensive analysis of small volume human intestinal samples that can be used with gastrointestinal sampling capsules. The toolbox comprises a set of validated analytical methods to measure fiber fermentation, microbial composition, SCFA concentration and SCFA isotope dilution in the same small sample in the presence of a quenching reagent. Even though the developed toolbox together with gastrointestinal capsules was not used in this thesis, I hope that it will offer a major advantage to researchers and clinicians in this rapidly developing research field and a basis for a more extensive analytical approach to also study other microbial processes in the gut.

Future perspective. What can we learn from our results and the attempt to measure colonic NDC fermentation? Considering our experience, the tools available, and the technology coming, studying the proximal colon fermentation kinetics still has serious obstacles that we need to take into consideration. First, neither the catheters nor the gastrointestinal capsules can be precisely positioned and localized within the colon. This makes standardization of the sampling location among subjects complicated. This holds even more for the capsules, since for different time points different free-floating capsules are needed, which are not in exactly the same place. Moreover, from our experience in **chapter 2**, we expect that for all technologies, sampling the highly viscous and dense content of the proximal colon will be difficult. Even if these issues will be solved by new technologies to come, the proximal colon is intrinsically a difficult structure to study. The gut functions as a flow-through reactor for NDC fermentation, but its content is most likely inhomogeneous and the volume in which the fermentation takes place is not known. Thus, to calculate fluxes from label dilutions, major assumptions will still be needed. Future research should address these issues and, in addition to improve the use of non-digestible/non-fermentable markers to reliably estimate the necessary parameters for proper calculations of the fermentation kinetics *in vivo* and host metabolism of SCFA in humans.

When reviewing the literature on this topic I was struck by the fact that the inclusion of healthy female volunteers in trials is minor compared to that of men. Because of the burden on patients and the high price of the methodologies to investigate luminal fermentation, human trials are relatively small. To ensure that the data have less inter-individual variation, female volunteers are rarely included, since they are more likely to have infrequent bowel movements and to be constipated [32,33]. Women comprise half of the world population, so this gastrointestinal difference far from being a disadvantage, is actually an important reason why women should be included in such trials. Moreover, microbiota and metabolic profile can be modulated by gender[34–36]. Therefore, I strongly advocate including women in future studies aiming to improve prebiotics as a treatment strategy in the general population.

Part II. Tissue-specific effects of butyrate on fuel handling

After SCFA are produced in the gut, they can have local effects, e.g. promoting the release of satiety-inducing hormones, or they can be taken up by the host[1] . In mice, only the uptake flux of SCFA, but not the cecal concentrations, correlated to improved metabolic health[37] . This suggests that SCFA exert their effects at least partially after they have been absorbed by the host and reached other organs. SCFA can modulate host metabolic health through a range of tissue-specific mechanisms related to appetite regulation, energy expenditure, and glucose homeostasis (**chapter 1)**. However, it is important to highlight that SCFA have also been described as a substrate for lipids and glucose metabolism in mice[38] and humans[39]. Label from ^{13}C-SCFA delivered directly in the colon was incorporated in metabolites measured in blood (**chapter 2**). Tissue-specific effects can be brought about by a direct SCFA stimulation or as a consequence of inter-organ crosstalk. Effects of SCFA on different tissues have mainly been studied in animals *in vivo* after SCFA supplementation and less often *in vitro* by direct SCFA stimulation of cells or tissue samples. In this thesis, I focused on the direct effect of butyrate in two metabolically active tissues, namely (i) the liver (**chapter 5**) using murine precision-cut liver slices (PCLS) and (ii) the skeletal muscle (**chapter 6**) using C2C12 murine myotubes. Butyrate was chosen because it is the most widely used SCFA in this context.

Liver. In patients with cirrhosis, decreased circulating levels of SCFA, especially butyrate, are associated with more advanced liver disease[40]. In line with this, *in vivo* supplementation of butyrate or butyrate-producing bacteria in rodents alleviates features of metabolic syndrome, including hepatic steatosis and fibrosis, and thereby prevents the progression of MAFLD[41–45] . To study MAFLD, the PCLS model is a promising tool that recapitulates the multicellular architecture of the liver[46,47]. An early manifestation of MAFLD is an accumulation of triglycerides (TG) resulting in steatosis[48]. In **chapter 5**, we optimized murine PCLS as a model for this first stage

of MAFLD. This allowed us to investigate the direct effect of butyrate on triglyceride storage in liver tissue, in the absence of crosstalk with other organs. The presence of butyrate in the incubation medium did not prevent TG accumulation in the PCLS and contrary to previous findings, butyrate reduced mRNA levels encoding proteins involved in fatty acid oxidation. Moreover, increasing the concentration of butyrate resulted in an almost significant increase in TG levels. In agreement with *in vivo* studies in mice[38] and humans[39], and the results of label incorporation in **chapter 2**, butyrate served as a metabolic substrate. Butyrate increased butyryl-carnitine levels, which suggests that butyrate is oxidized in the mitochondria. This could lead to an increase in acetyl-CoA, which may explain why butyrate did not decrease, but rather increased TG accumulation in the PCLSs. The difference between the *in vivo* and *in vitro* effects of butyrate suggest that the decreased steatosis after butyrate supplementation *in vivo* is mostly a consequence of inter-organ crosstalk. Butyrate has been reported to decrease lipolysis in adipose tissue, which prevents an excess of lipid supply to the liver[1] . Furthermore, butyrate increased GLP-1 and PYY secretion by the gut, which decreases food intake and increases satiety[1,49], which could lead to fewer calories consumed and lower levels of circulating lipids.

Skeletal muscle. In **chapter 6**, we also assessed the direct effect of butyrate in C2C12 murine myotubes. Butyrate has been associated with enhanced insulin sensitivity when administered to mice[41,50] and the presence of butyrate-producing bacteria in the gut correlated with improved insulin sensitivity in humans[51]. Skeletal muscle is a major site of insulin-stimulated glucose uptake and therefore plays a critical role in glucose homeostasis[52]. Yet, less than a handful of studies has addressed the effect of SCFA on this tissue. Gao et al reported that the insulin sensitivity of skeletal muscle improved after butyrate administration to mice[50]. In **chapter 6** we confirmed this effect, also when butyrate was directly added to myotubes. Butyrate acted as an HDAC inhibitor, upregulating the mRNA levels of various target genes (*Mct-1*, *Irs1*, *Slc2a4*), which in turn led to an increased insulin-stimulated glycolytic capacity. Opposite to what was expected, however, long-chain fatty acid oxidation was not enhanced by butyrate stimulation. Instead, in agreement with **chapter 5**, butyrate was actively used as a metabolic substrate. To elucidate the role of butyrate metabolism, we abolished butyrate oxidation by knocking down the medium-chain 3-ketoacyl-CoA thiolase (*Acaa2*, *MCKAT*). Notably, the insulin-sensitizing effect of butyrate was exacerbated. This suggests that butyrate catabolism functions as an escape valve that attenuates HDAC inhibition.

Future perspective. In conclusion, in **chapters 5** and **chapter 6** I described the different effects of butyrate in two important metabolic tissues. Contrary to earlier studies, butyrate did not regulate long-chain fatty acid oxidation in the liver or the

skeletal muscle. Instead, butyrate was actively metabolized in both organs. This agrees with the results discussed in **chapter 2** where I showed that carbon atoms of butyrate enter the carbon skeleton of glucose. Possibly, the enhanced energy expenditure observed in *in vivo* studies in humans and rodents[6,8,41,50,53–55], might be indicative of increased butyrate oxidation. The observation that butyrate acts also as a metabolic substrate needs to be considered when developing intervention strategies for metabolic disease based on butyrate supplementation in any form. This, together with an understanding of how butyrate modulates multiple intracellular pathways could improve the development of simple dietary strategies towards preventing and treating metabolic syndrome.

REFERENCES

1. Canfora EE, Jocken JW, Blaak EE. Short-chain fatty acids in control of body weight and insulin sensitivity. *Nature Reviews Endocrinology*. 2015;11(10):577-591.
2. Chambers ES, Viardot A, Psichas A, et al. Effects of targeted delivery of propionate to the human colon on appetite regulation, body weight maintenance and adiposity in overweight adults. *Gut*. 2015;64(11):1744-1754.
3. Polyviou T, MacDougall K, Chambers ES, et al. Randomised clinical study: inulin short-chain fatty acid esters for targeted delivery of short-chain fatty acids to the human colon. *Alimentary pharmacology & therapeutics*. 2016;44(7):662-672.
4. Byrne CS, Chambers ES, Alhabeeb H, et al. Increased colonic propionate reduces anticipatory reward responses in the human striatum to high-energy foods. *The American journal of clinical nutrition*. 2016;104(1):5-14.
5. Darzi J, Frost GS, Robertson MD. Effects of a novel propionate-rich sourdough bread on appetite and food intake. *European journal of clinical nutrition*. 2012;66(7):789-794.
6. van der Beek CM, Canfora EE, Lenaerts K, et al. Distal, not proximal, colonic acetate infusions promote fat oxidation and improve metabolic markers in overweight/obese men. *Clinical Science*. 2016;130(22):2073-2082.
7. Chambers ES, Preston T, Frost G, Morrison DJ. Role of gut microbiota-generated short-chain fatty acids in metabolic and cardiovascular health. *Current nutrition reports*. 2018;7(4):198-206.
8. Canfora EE, van der Beek CM, Jocken JWE, et al. Colonic infusions of short-chain fatty acid mixtures promote energy metabolism in overweight/obese men: a randomized crossover trial. *Scientific reports*. 2017;7(1):1-12.
9. Venter CS, Vorster HH, Cummings JH. Effects of dietary propionate on carbohydrate and lipid metabolism in healthy volunteers. *American Journal of Gastroenterology (Springer Nature)*. 1990;85(5).
10. Bouter KEC, Bakker GJ, Levin E, et al. Differential metabolic effects of oral butyrate treatment in lean versus metabolic syndrome subjects. *Clinical and translational gastroenterology*. 2018;9(5).
11. Todesco T, Rao AV, Bosello O, Jenkins DJ. Propionate lowers blood glucose and alters lipid metabolism in healthy subjects. *The American journal of clinical nutrition*. 1991;54(5):860-865.
12. Wolever TMS, Spadafora P, Eshuis H. Interaction between colonic acetate and propionate in humans. *The American journal of clinical nutrition*. 1991;53(3):681-687.
13. Wolever T, Brighenti F, Royall D, Jenkins AL, Jenkins DJA. Effect of rectal infusion of short chain fatty acids in human subjects. *American Journal of Gastroenterology (Springer Nature)*. 1989;84(9).

14. Kastl Jr AJ, Terry NA, Wu GD, Albenberg LG. The structure and function of the human small intestinal microbiota: current understanding and future directions. *Cellular and molecular gastroenterology and hepatology*. 2020;9(1):33-45.
15. Ph van Trijp M, Wilms E, Ríos-Morales M, et al. Using naso-and oro-intestinal catheters in physiological research for intestinal delivery and sampling in vivo: practical and technical aspects to be considered. *The American Journal of Clinical Nutrition*. Published online 2021.
16. Takeshita T, Kageyama S, Furuta M, et al. Bacterial diversity in saliva and oral health-related conditions: the Hisayama Study. *Scientific reports*. 2016;6(1):1-11.
17. Knudsen BKE, Hessov I. Recovery of inulin from Jerusalem artichoke (Helianthus tuberosus L.) in the small intestine of man. *British Journal of Nutrition*. 1995;74(1):101-113.
18. Nardone G, Compare D. The human gastric microbiota: Is it time to rethink the pathogenesis of stomach diseases? *United European gastroenterology journal*. 2015;3(3):255-260.
19. Swanson KS, de Vos WM, Martens EC, et al. Effect of fructans, prebiotics and fibres on the human gut microbiome assessed by 16S rRNA-based approaches: a review. *Beneficial microbes*. 2020;11(2):101-129.
20. Ferreira-Lazarte A, Gallego-Lobillo P, Moreno FJ, Villamiel M, Hernandez-Hernandez O. In vitro digestibility of galactooligosaccharides: effect of the structural features on their intestinal degradation. *Journal of agricultural and food chemistry*. 2019;67(16):4662-4670.
21. Ferreira-Lazarte A, Montilla A, Mulet-Cabero AI, et al. Study on the digestion of milk with prebiotic carbohydrates in a simulated gastrointestinal model. *Journal of Functional Foods*. 2017;33:149-154.
22. Akbari P, Fink-Gremmels J, Willems RHAM, et al. Characterizing microbiota-independent effects of oligosaccharides on intestinal epithelial cells: Insight into the role of structure and size. *European journal of nutrition*. 2017;56(5):1919-1930.
23. Shobar RM, Velineni S, Keshavarzian A, et al. The effects of bowel preparation on microbiota-related metrics differ in health and in inflammatory bowel disease and for the mucosal and luminal microbiota compartments. *Clinical and translational gastroenterology*. 2016;7(2):e143.
24. Jalanka J, Salonen A, Salojärvi J, et al. Effects of bowel cleansing on the intestinal microbiota. *Gut*. 2015;64(10):1562-1568.
25. Bucher P, Gervaz P, Egger JF, Soravia C, Morel P. Morphologic alterations associated with mechanical bowel preparation before elective colorectal surgery: a randomized trial. *Diseases of the colon & rectum*. 2006;49(1):109-112.
26. Koziolek M, Grimm M, Becker D, et al. Investigation of pH and temperature profiles in the GI tract of fasted human subjects using the Intellicap® system. *Journal of pharmaceutical sciences*. 2015;104(9):2855-2863.

27. Jin G, Wang G, Liu X, Cao H, Wang B. 977–Intestine microbiome aspiration (IMBA) capsule: a new autonomous and minimally-invasive device for whole gut microbiome sampling and mapping. *Gastroenterology*. 2019;156(6):S-205.
28. Amoako-Tuffour Y, Jones ML, Shalabi N, Labbé A, Vengallatore S, Prakash S. Ingestible gastrointestinal sampling devices: state-of-the-art and future directions. *Critical Reviews™ in Biomedical Engineering*. 2014;42(1).
29. Rezaei Nejad H, Oliveira BCM, Sadeqi A, et al. Ingestible osmotic pill for in vivo sampling of gut microbiomes. *Advanced Intelligent Systems*. 2019;1(5):1900053.
30. van der Schaar PJ, Dijksman JF, Broekhuizen-de Gast H, et al. A novel ingestible electronic drug delivery and monitoring device. *Gastrointestinal endoscopy*. 2013;78(3):520-528.
31. Tang Q, Jin G, Wang G, et al. Current sampling methods for gut microbiota: a call for more precise devices. *Frontiers in cellular and infection microbiology*. 2020;10:151.
32. McCrea GL, Miaskowski C, Stotts NA, Macera L, Paul SM, Varma MG. Gender differences in self-reported constipation characteristics, symptoms, and bowel and dietary habits among patients attending a specialty clinic for constipation. *Gender medicine*. 2009;6(1):259-271.
33. Mitsuhashi S, Ballou S, Jiang ZG, et al. Characterizing normal bowel frequency and consistency in a representative sample of adults in the United States (NHANES). *Official journal of the American College of Gastroenterology| ACG*. 2018;113(1):115-123.
34. Mauvais-Jarvis F. Gender differences in glucose homeostasis and diabetes. *Physiology & behavior*. 2018;187:20-23.
35. Haro C, Rangel-Zúñiga OA, Alcala-Diaz JF, et al. Intestinal microbiota is influenced by gender and body mass index. *PloS one*. 2016;11(5):e0154090.
36. Varlamov O, Bethea CL, Roberts Jr CT. Sex-specific differences in lipid and glucose metabolism. *Frontiers in endocrinology*. 2015;5:241.
37. den Besten G, Havinga R, Bleeker A, et al. The short-chain fatty acid uptake fluxes by mice on a guar gum supplemented diet associate with amelioration of major biomarkers of the metabolic syndrome. *PloS one*. 2014;9(9):e107392.
38. den Besten G, Lange K, Havinga R, et al. Gut-derived short-chain fatty acids are vividly assimilated into host carbohydrates and lipids. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. Published online 2013.
39. Boets E, Gomand S v, Deroover L, et al. Systemic availability and metabolism of colonic-derived short-chain fatty acids in healthy subjects: a stable isotope study. *The Journal of physiology*. 2017;595(2):541-555.
40. Juanola O, Ferrusquía-Acosta J, García-Villalba R, et al. Circulating levels of butyrate are inversely related to portal hypertension, endotoxemia, and systemic inflammation in patients with cirrhosis. *FASEB Journal*. 2019;33(10):11595-11605. doi:10.1096/fj.201901327R

41. den Besten G, Bleeker A, Gerding A, et al. Short-chain fatty acids protect against high-fat diet–induced obesity via a PPARγ-dependent switch from lipogenesis to fat oxidation. *Diabetes*. 2015;64(7):2398-2408.
42. Jakobsdottir G, Xu J, Molin G, Ahrne S, Nyman M. High-fat diet reduces the formation of butyrate, but increases succinate, inflammation, liver fat and cholesterol in rats, while dietary fibre counteracts these effects. *PloS one*. 2013;8(11):e80476.
43. Mattace Raso G, Simeoli R, Russo R, et al. Effects of sodium butyrate and its synthetic amide derivative on liver inflammation and glucose tolerance in an animal model of steatosis induced by high fat diet. *PloS one*. 2013;8(7):e68626.
44. Endo H, Niioka M, Kobayashi N, Tanaka M, Watanabe T. Butyrate-producing probiotics reduce nonalcoholic fatty liver disease progression in rats: new insight into the probiotics for the gut-liver axis. *PloS one*. 2013;8(5):e63388.
45. Ye J, Lv L, Wu W, et al. Butyrate protects mice against methionine–choline-deficient diet-induced non-alcoholic steatohepatitis by improving gut barrier function, attenuating inflammation and reducing endotoxin levels. *Frontiers in microbiology*. 2018;9:1967.
46. Fontana F, Figueiredo P, Martins JP, Santos HA. Requirements for Animal Experiments: Problems and Challenges. *Small*. 2021;17(15):2004182.
47. Dewyse L, Reynaert H, van Grunsven LA. Best Practices and Progress in Precision-Cut Liver Slice Cultures. *International journal of molecular sciences*. 2021;22(13):7137.
48. Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. *Hepatology*. 2010;51(2):679-689.
49. Kaji I, Karaki S ichiro, Kuwahara A. Short-chain fatty acid receptor and its contribution to glucagon-like peptide-1 release. *Digestion*. 2014;89(1):31-36.
50. Gao Z, Yin J, Zhang J, et al. Butyrate improves insulin sensitivity and increases energy expenditure in mice. *Diabetes*. 2009;58(7):1509-1517.
51. Vrieze A, van Nood E, Holleman F, et al. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology*. 2012;143(4):913-916.
52. DeFronzo RA, Tripathy D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes care*. 2009;32(suppl 2):S157-S163.
53. Li Z, Yi CX, Katiraei S, et al. Butyrate reduces appetite and activates brown adipose tissue via the gut-brain neural circuit. *Gut*. 2018;67(7):1269-1279.
54. Mollica MP, Raso GM, Cavaliere G, et al. Butyrate regulates liver mitochondrial function, efficiency, and dynamics in insulin-resistant obese mice. *Diabetes*. 2017;66(5):1405-1418.
55. Chambers ES, Byrne CS, Aspey K, et al. Acute oral sodium propionate supplementation raises resting energy expenditure and lipid oxidation in fasted humans. *Diabetes, Obesity and Metabolism*. 2018;20(4):1034-1039.


7