Charles University in Prague

First Faculty of Medicine

Doctoral program: Doctoral Program in Biomedicine

Study field: Biochemistry and Pathobiochemistry

UNIVERZITA KARLOVA
1. lékařská fakulta

Ing. Nikola Ďásková

Doctoral Thesis

The modification of gut microbiota composition by dietary intervention: the effect of plant-based and western-type diet

Modifikace složení střevní mikroflóry dietní intervencí: vliv rostlinné a western-type diet

Supervisor: doc. RNDr. Monika Cahová, Ph.D.

Prague, 2023

Declaration:

I declare that I have processed this dissertation research thesis independently and that I have cited all the important sources of information. I agree that this dissertation research thesis may be stored in the Medical Library of Charles University at the 1st Faculty of Medicine and uploaded at the Charles University Digital Repository and may be used for any study purposes, provided that anyone who may use it for their publishing or lecturing activities should quote it as their source of information appropriately.

In Prague, 12. 6. 2023 Nikola Ďásková

Identification Record:

ĎÁSKOVÁ, Nikola. The modification of gut microbiota composition by dietary intervention: the effect of plant-based and western-type diet [Modifikace složení střevní mikroflóry dietní intervencí: vliv rostlinné a western-type diet]. Prague, 2023. 127 pages, 1 appendix. Dissertation research thesis (PhD.). Charles University in Prague, First Faculty of Medicine, Laboratory of Pathophysiology of Metabolism, Institute for Clinical and Experimental Medicine. Supervisor: doc. RNDr. Monika Cahová, Ph.D.

Acknowledgments

I am indebted to numerous individuals whose support and guidance played an instrumental role in the completion of my thesis. I would like to express my deepest appreciation to my supervisor, doc. RNDr. Monika Cahová, Ph.D., whose unwavering encouragement, trust, and invaluable insights propelled me forward. I am incredibly grateful for her generosity in providing me with the necessary space to learn and enhance my bioinformatical skills, which were crucial for our research. Several years ago, she welcomed me as her student, and since then, she has introduced me to the captivating world of the microbiome, for which I am immensely thankful.

In addition, I extend my sincere thanks to all my colleagues at the Institute for Clinical and Experimental Medicine for their support throughout my academic journey. Also, I would like to express my gratitude to Ing. István Módos, Ph.D., and doc. Mgr. Maria Králová, Ph.D., for their guidance and assistance during the challenging statistical and bioinformatical aspects of my work. Their expertise and encouragement were invaluable in overcoming hurdles and achieving significant progress.

I would like to acknowledge and express my deepest gratitude to my parents and my entire family for their support and encouragement throughout my studies. Their constant belief in me and their unwavering faith were invaluable sources of strength. I am grateful for their love, understanding, and sacrifices that allowed me to pursue my educational goals. Furthermore, I would like to extend my heartfelt appreciation to my partner, Petr, who has been a constant source of support and encouragement throughout my academic journey. His unwavering belief in my abilities, patience, and understanding have been crucial in keeping me motivated during challenging times.

Additionally, I would like to express my gratitude to Hlavkova Nadace and Cesky Literarni Fond for providing me with the necessary financial support. Their funding enabled me to attend the Strategies and Techniques for Analyzing Microbial Population Structures course in the USA. The knowledge and skills gained from this course have significantly contributed to my scientific development and greatly influenced the trajectory of my research.

In conclusion, I am deeply grateful to each person and organization mentioned above for their unwavering support, guidance, and encouragement throughout my thesis journey. Without their assistance and belief in my abilities, I would not have been able to successfully complete my studies.

CONTENTS

ABSTRACT

This thesis explores the relationship between diet, gut microbiota, and metabolic health, with a particular focus on their association with non-communicable metabolic diseases (NCDs) such as obesity and type 2 diabetes (T2D).

The aim of the first study is to assess compositional and metabolic differences in gut microbiota between healthy lean long-term vegans and omnivores. The study reveals that while the gut microbiota composition is not significantly different between the two groups, there are significant differences in the fecal, serum and urinary metabolome. These differences may be attributed to the different availability of substrates in the diet, as the vegan diet is associated with a shift from a proteolytic to a saccharolytic fermentation program. Our results support the hypothesis of both resilience and metabolic flexibility of the adult gut microbiota.

In addition to taxonomic analyses, this dissertation also includes metabolomics to evaluate the functional manifestations of the gut microbiota. We introduce a novel method to assess the ability of the gut microbiota to produce beneficial metabolites with a specific focus on butyrate synthesis using qPCR quantification of bacterial butyryl-CoA:acetate CoA-transferase. *In silico*, we identified bacteria among the human gut microbiota that possess the *but* gene, designed and validated six sets of degenerate primers covering all selected bacteria and developed a method to normalize gene abundance in human fecal DNA. We validated this method in subjects with opposite dietary habits and metabolic phenotypes - lean vegans (VG) and healthy obese omnivores (OB) - with known fecal microbiota and metabolome composition.

Furthermore, the effects of inulin treatment on glucose homeostasis in pre/diabetic patients were investigated. A three-month intervention with inulin under clinical trial conditions was associated with an overall improvement in glycemic indices, although the response was highly variable, with a shift in microbial composition towards a more favorable profile and an increase in serum butyric and propionic acid concentrations. Using multi-omics analysis, we identified biomarkers that predict treatment success. If further validated, these predictors could improve the estimation of outcomes of inulin interventions and contribute to personalized dietary management in early-stage diabetes.

Finally, the fourth study investigates the therapeutic potential of fecal microbial transfer (FMT) using vegan microbiota to treat non-infectious diseases. It uses a humanized mouse model to examine the effect of a Western-type diet (WD) and inulin supplementation on obesity, hepatic steatosis, and glucose metabolism. We found that vegan microbiota alone did not protect against the adverse effects of WD and inulin supplementation reversed steatosis and normalized glucose metabolism. This phenomenon was related to a change in microbiota composition and an increase in saccharolytic fermentation at the expense of proteolytic fermentation. Our results highlighted that the success of fecal microbiota transfer in the treatment of metabolic noninfectious diseases depends not only on the microbiota transfer itself but also on subsequent dietary interventions involving inulin or other fiber and/or dietary changes.

This dissertation provides some new insights into the relationship between diet and the gut microbiome, particularly in relation to the therapeutic potential of targeted manipulation of the gut microbiota in the treatment of obesity and T2D. The study highlights the importance of dietary

interventions, such as inulin or fiber supplementation, and emphasizes personalized dietary approaches to modify gut microbiota and improve metabolic health.

Key words:

Gut microbiome, metabolome, metabolic diseases, vegan diet, western type diet, animal models, type 2 diabetes, OMICS data

ABSTRAKT V ČEŠTINĚ

Tato dizertační práce zkoumá vztah mezi stravou, střevní mikrobiotou a metabolickým zdravím. Konkrétně se zaměřuje na vztah mezi složením střevní mikrobioty a nepřenosnými metabolickými chorobami, jako je obezita a diabetes 2. typu (T2D).

Cílem první studie je posoudit rozdíly ve složení a metabolismu střevní mikrobioty mezi zdravými štíhlými dlouhodobými vegany a omnivory. Studie ukazuje, že zatímco složení střevní mikrobioty se mezi oběma skupinami významně neliší, jsou zde významné rozdíly ve fekálním, sérovém a močovém metabolomu. Tyto rozdíly lze přičíst odlišné dostupnosti substrátů ve stravě, protože veganská strava je spojena s přechodem od proteolytického k sacharolytickému fermentačnímu programu. Naše výsledky podporují hypotézu o odolnosti i metabolické flexibilitě střevní mikrobioty u dospělých jedinců.

Kromě taxonomických analýz zahrnuje tato disertační práce také metabolomiku pro vyhodnocení funkčních projevů střevní mikrobioty. Zavádíme novou metodu hodnocení schopnosti střevní mikrobioty produkovat prospěšné metabolity se specifickým zaměřením na syntézu butyrátu pomocí qPCR kvantifikace bakteriální butyryl-CoA:acetát CoA-transferázy. *In silico* jsme identifikovali lidské střevní bakterie, které jsou vybaveny *but* genem, navrhli jsme a ověřili šest sad degenerovaných primerů pokrývajících všechny vybrané bakterie a vyvinuli metodu normalizace množství tohoto genu v lidské fekální DNA. Tuto metodu jsme ověřili u osob s opačnými stravovacími návyky a metabolickými fenotypy – u štíhlých veganů (VG) a zdravých obézních omnivorů (OB) – se známým složením fekální mikrobioty a metabolomu.

Dále jsme zkoumali účinky léčby inulinem na homeostázu glukózy u pre/diabetiků. Byla provedena klinická studie zahrnující tříměsíční inulinovou intervenci, která byla asociována s celkovým zlepšením glykemických parametrů, ačkoli individuální odpověď byla velmi variabilní, s posunem mikrobiálního složení směrem k příznivějšímu profilu a se zvýšením sérových koncentrací kyseliny máselné a propionové. Pomocí multi-omické analýzy jsme identifikovali biomarkery, které predikují úspěch léčby. Pokud budou tyto prediktory dále validovány, mohly by zlepšit odhad výsledků inulinových intervencí a přispět k personalizovanému dietnímu managementu v časném stadiu diabetu.

A konečně čtvrtá studie zkoumá terapeutický potenciál fekálního mikrobiálního transferu (FMT) s využitím veganské mikrobioty k léčbě nepřenosných metabolických onemocnění. Pomocí humanizovaného myšího modelu jsme sledovali vliv diety západního typu (WD) a podávání inulinu na obezitu, jaterní steatózu a metabolismus glukózy. Zjistili jsme, že samotná veganská mikrobiota nechrání před nepříznivými účinky WD, ale naopak přídavek inulinu zvrátil steatózu a normalizoval metabolismus glukózy. Tento jev souvisel se změnou složení mikrobioty a zvýšením sacharolytické fermentace na úkor proteolytické fermentace. Naše výsledky zdůraznily, že úspěšnost přenosu fekální mikrobioty při léčbě metabolických onemocnění závisí nejen na samotném přenosu mikrobioty, ale také na následných dietních intervencích zahrnujících inulin nebo jinou vlákninu a/nebo změny stravy.

Tato disertační práce přináší některé nové poznatky o souvislostech mezi stravou a střevním mikrobiomem, zejména ve vztahu k terapeutickému potenciálu cílené manipulace se střevní mikrobiotou při léčbě obezity a T2D. Studie poukazuje na význam dietních intervencí, jako je suplementace vlákninou, a zdůrazňuje personalizované dietní přístupy k úpravě střevní mikrobioty a zlepšení metabolického zdraví.

Klíčová slova:

Střevní mikrobiom, střevní a sérový metabolom, metabolické poruchy, veganská dieta, dieta západního typu, zvířecí modely, diabetes druhého typu, OMICS data

1 INTRODUCTION

1.1 General preface

„Let food be thy medicine and medicine be thy food."

„All disease begins in the gut."

Hippocrates, 460–370 BC

I want to introduce my dissertation with two statements attributed to the founder of modern medicine, the Greek physician Hippocrates. Nutrition has always been one of the most important components of human life. Over the years, its perception has changed and modern medicine now regards it as a means of influencing the course and development of certain civilization diseases. Nutrition is also one of the basic factors that influence the composition and functional manifestations of the gut microbiome, which has been extensively studied in recent years.

During my postgraduate studies, I focused on the relationship between microbial composition and some non-communicable metabolic diseases, particularly obesity and T2D. In our research, we did not limit ourselves to describing the taxonomic composition of the microbiota, but also analyzed its functional manifestations through fecal metabolome analysis. We attempted to place the obtained results in the broader context of the interaction between microbiome and the host organism by analyzing serum and urine metabolomes as well as health status indices and nutritional parameters. Furthermore, we described the microbiome and metabolome of vegan populations, which, according to numerous epidemiological studies, are metabolically healthier than the general omnivorous population. We were interested in whether the vegan diet affects the composition of the microbiome and metabolome and whether any favorable effects of this diet could be explained by their potential changes.

Targeted modulation of the gut microbiota is discussed as a potentially promising therapeutic strategy for diseases in which the pathophysiology of gut dysbiosis plays a role. However, the wider use of this approach is confronted with a lack of knowledge about the functioning of a system as complex as the gut microbiome and its interaction with the environment and the host. My work has addressed this issue both in humans (clinical intervention trial) and in an experimental study conducted in ex-GF mice. In the intervention study, we tested the hypothesis that the success of inulin (dietary fiber) intervention to improve insulin sensitivity in T2D patients is variable and depends, at least in part, on the composition of the microbiome. In line with the hypothesis, we observed significant inter-individual differences in response to administered fiber and attempted to identify specific markers that would predict the success of this therapy. In the experimental study, we explored the potential use of vegan microbiota transfer in treating diet-induced obesity and insulin resistance.

1.2 Microbiome and microbiota in general

A microbiota is a community of microorganisms living in a particular environment and refers to their taxonomy (each microorganism belongs to a set of taxonomic classification units from kingdom to species or even strains), which serves as an organizational tool (Parks et al., 2018). The broader and more recent term microbiome encompasses the set of all microorganisms (bacteria, archaea, lower and higher eukaryotes and viruses) inhabiting a particular environment and their genomes and surrounding environmental conditions (Marchesi & Ravel, 2015) and was first defined and highly

emphasized by professor and Nobel laureate Joshua Lederberg (Lederberg & McCray, 2001). Nowadays, the two terms are often used interchangeably, although the original definitions should be kept in mind (Ursell et al., 2012).

1.2.1 Humans as holobionts

In the past few decades, research on the microbiome has been developing at a tremendous pace. The concept of the so-called "holobiont" was introduced in 1991 by evolutionary theorist and biologist Lynn Margulis and her graduate student René Fester (Margulis & Fester, 1991), although the concept itself has existed in nature since the first symbiosis, which is considered an essential part of evolution and the basis of the first eukaryotic cell, based on a theory proposed by Lynn Margulis in 1967 (Margulis, 1967) and widely accepted by scientists today. Currently, a holobiont is described as an organism consisting of a host and many microorganisms living in close association with the host (Bordenstein & Theis, 2015) (Simon et al., 2019). It is noteworthy that, based on currently available data, the number of bacterial cells present in the average human being is approximately the same order as the number of human cells, although it has been assumed that microbial cells outnumbered human cells (Sender et al., 2016b). Based on the estimates by Sender *et al*., the number of human cells in the 70 kg "reference" adult human is $3 \cdot 10^{13}$ with the major contributors being red blood cells, platelets, bone marrow cells, lymphocytes and endothelial cells (Sender et al., 2016a) (Sender et al., 2016b). The estimated number of bacterial cells in the same "reference" human is $3.9 \cdot 10^{13}$ (Sender et al., 2016a), but this number do not consider other types of microbes such as viruses and phages (Gilbert et al., 2018).

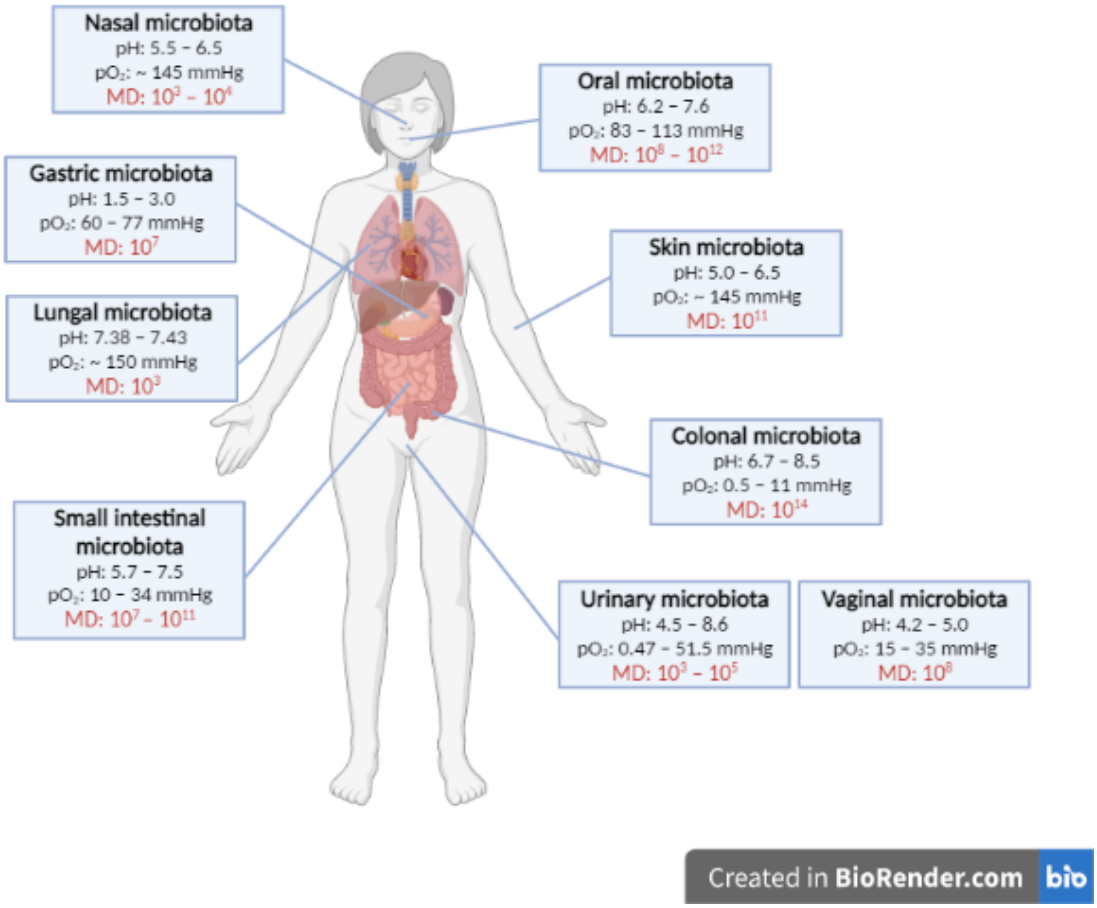
The concept of the holobiont has been greatly expanded by the introduction of new techniques that have enabled much faster and cheaper analysis compared to traditional techniques, one of the most important of which is a high-throughput next-generation sequencing (NGS). NGS was the foundation of the Human Genome Project (HGP) which has undoubtedly changed biological research as we know it (Lander et al., 2001) (Craig Venter et al., 2001). The success of the HGP has been followed by another groundbreaking project, the Human Microbiome Project (HMP), which aims to characterize the human microbiome and answer complex questions about microbial diversity, stability and evolution, i.e. factors that may influence an individual's microbiota, microbial relationship to pathological conditions, and much more. (Turnbaugh et al., 2007). With the introduction of revolutionary sequencing methods, scientists are now able to sequence the genome of both the host and its microorganisms to study their complex relationships. The resulting collective genome can be referred to as a hologenome, which consists not only of the nuclear genome but also includes organelles and the microbiome, resulting in a complex gene system (Bordenstein & Theis, 2015). The size of the male diploid human genome has been estimated at $6.27 \cdot 10^9$ base pairs (Piovesan et al., 2019). If we consider that the "typical" bacterial genome is $5 \cdot 10^6$ base pairs in size (Land et al., 2015), and then multiply this by the estimated number of bacteria inhabiting the human gut, we get an estimate of the genome size of human microbes of approximately $1.95 \cdot 10^{20}$, a huge number compared to the human genome. Interestingly, the set of gut microbial genes was also found to be 150 times larger than the entire set of human genes, with 3.3 million unique microbial genes identified compared to approximately 20,000 human protein-coding genes. (Qin et al., 2010) (Gilbert et al., 2018).

1.2.2 Human niches colonized by microbes

For a long time, the inner body was considered nearly sterile and any presence of microorganisms was considered as the consequence of "breaking the defense systems" and "wrong". Now, increasing

evidence shows that almost every part of our body is populated by microbes. The abundance of microbes across human niches varies according to the chemical and physical aspects of each site or organ such as pH, concentration of oxygen, availability of nutrients, temperature, and presence of antimicrobial compounds or mucus (De Vos et al., 2022) (Milani et al., 2017) (Figure 1).

Figure 1. Main microbial body sites and their environmental characteristics across the human body (De Vos et al., 2022) (Mathieu et al., 2018) (Neugent et al., 2020) (Yagi et al., 2021). Created by using https://www.biorender.com/.



The gastrointestinal tract (GIT) is the largest reservoir of diversified microbes at any site in the human body, and gut microbiota is currently the focus of many researchers due to its extensive functions, which will be discussed in more detail in the following chapters. The second most diverse place of microbial colonization in humans is the mouth including oral mucosa, saliva, all oral tissues, tongue and teeth surfaces (Reynoso-García et al., 2022). Each oral niche is unique in its bacterial composition due to differences in the environment, but all microbes communicate with each other, forming complex multi-species biofilms, communicating through chemical signals, and are also in symbiosis and close association with some fungal microbes (X. Li et al., 2022). Another huge microbial ecosystem, known to be dominated by lactic acid-producing bacteria belonging to the genus *Lactobacillus*, is found in the vagina (X. Chen et al., 2021). Under normal conditions, the *Lactobacillus*-dominated vaginal microbiota protects this niche against invasion of potentially pathogenic species by producing many antimicrobial substances including lactic acid creating low pH, bacteriocins and hydrogen peroxide, which make the environment uninhabitable for other microbes (Hanlon et al., 2013) (S. Wu et al.,

2022). This phenomenon is called colonization resistance and occurs in various modifications in many microbial ecosystems associated with humans.

Compared to the microbially rich environment of the gut, mouth or vagina, many environments are still home to microbes, albeit in smaller densities. Human skin can provide almost 30 m^2 of microbial environment and is the largest epithelial surface for microbial interactions (Gallo, 2017). However, compared to other habitats, this niche contains fewer bacterial taxa and generally lower microbial biomass (Chaudhari et al., 2020). Interestingly, complex interactions between fungi and bacteria occur on the skin surface, and these microbes can form dense biofilms together, which are thought to stabilize the microbial community (Swaney, 2021). Microbes that naturally occur in a healthy human state can be found even in the strangest of places, such as the eye, ear or the bladder (Reynoso-García et al., 2022) (Wolfe et al., 2012). In recent years, microbes have also been found in many cancerous tissues, although studying them is extremely challenging (Y. Chen et al., 2022). Microbes are present in the blood even in a resting state, when they cannot multiply under normal conditions. During pathogenic situations, the gut is the origin of these bloodstream microbes, or it's possible for them to migrate from the oral cavity through translocation (Potgieter et al., 2015). Bacterial communities also inhabit the healthy lungs and respiratory tract, which have long been considered aseptic (Anand & Mande, 2018) (Whiteside et al., 2021). Somewhat problematic and controversial is the presence of microbiota in the placenta or uterus. Chen et. al brought evidence showing the possible presence of living bacteria in the cervical canal, uterus, fallopian tubes and peritoneal fluid (C. Chen et al., 2017), the latest study on the other hand suggests that no placental microbiota exists and the previously observed bacteria were merely the result of contamination (Goffau et al., 2019). Currently, the only human niche we consider truly sterile is the brain. Some studies suggest that a few bacteria live harmlessly in it, but this is still not confirmed given that most human brain studies can only be done post-mortem (Link, 2021). Therefore, more research and new methods are needed to study possible brain microbial communities.

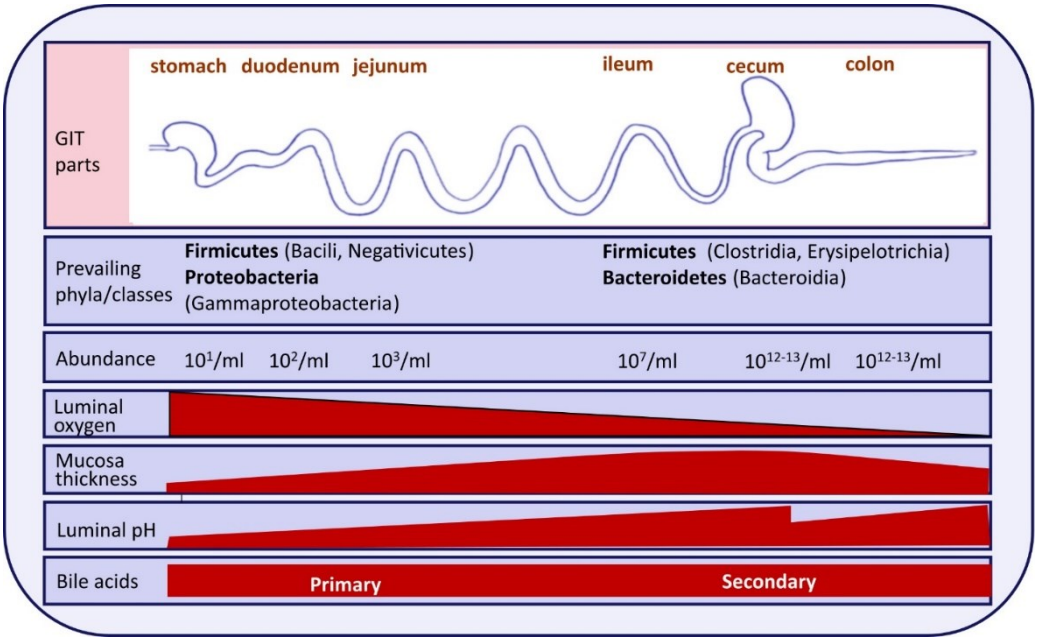
All these microbes from different niches interacts among themselves and form a complex network of relationships. Furthermore, they interact with and influence human cells, affecting human metabolism and health in general. As most studies on the human microbiome have shown, it is inevitable to understand the mechanism of these interactions between microbes living in the human environment itself, but also between microbes and the metabolic and biological processes of the host, in which they undoubtedly play an important role.

1.3 Gut microbiome

The human gut microbiome is currently attracting a great deal of scientific attention. The GIT is home to complex and diversified microbial communities that influence many processes in our body. These communities vary greatly throughout the digestive tract due to the different physical and biochemical conditions in each part of the tract (Figure 2). The stomach has a highly acidic environment with a pH around two because of hydrochloric acid that is released by the parietal cells in the gastric wall (Boland, 2016). Due to its extreme conditions, it serves as the first defense against pathogenic microbes from food (Hunt et al., 2015). Contrary to earlier beliefs, a specific bacterial community is found in the stomach, often dominated by *Helicobacter pylori*, which is known to be associated with gastric diseases (Amieva et al., 2016) (Cover & Blaser, 2009). Nevertheless, the gastric bacterial community surprisingly varies depending on the presence or absence of *H. pylori* (Maldonado-Contreras et al., 2011). When this microbe was present in the stomach, subjects had higher relative abundances of Proteobacteria and Acidobacteria phyla, whereas the negative status of *H. pylori* was associated

with the higher relative abundance of Actinobacteria and Firmicutes. In addition, the richness of bacterial communities did not differ based on *H. pylori* status (Maldonado-Contreras et al., 2011).

Figure 2. Different physical and chemical properties of each part of the digestive tract determine variations in the microbiota. Figure adapted from (Najmanová et al., 2022).

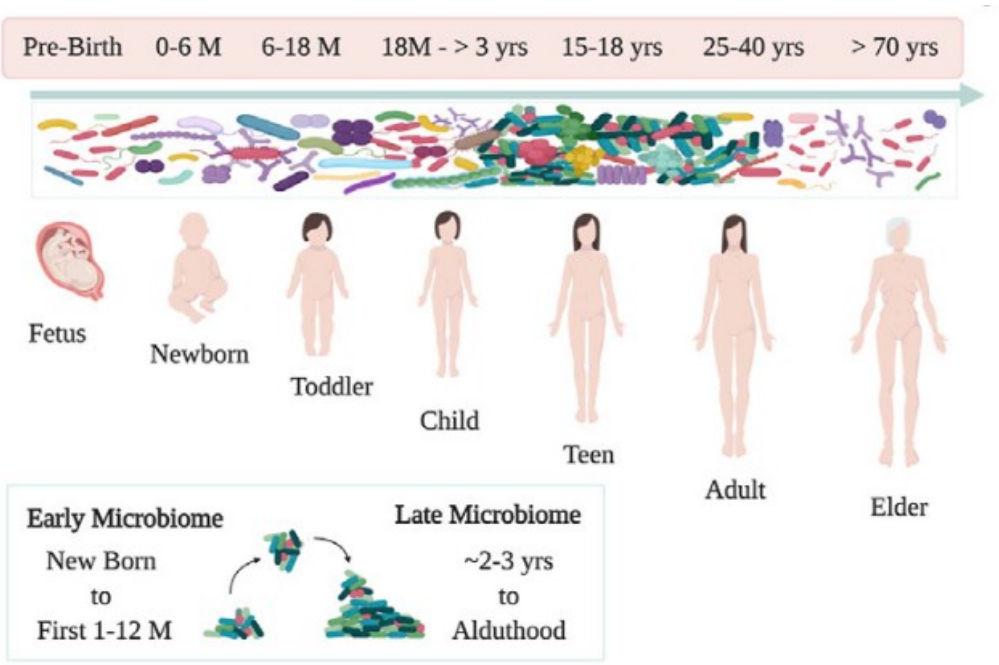


The small intestine is anatomically divided into three parts - the duodenum, jejunum and ileum. The duodenum, the first 30 centimeters of the small intestine, is an important part where the environment changes from acidic to neutral. The jejunum and ileum are areas where digestive enzymes with the help of bile acids break down food components and the absorption of the resulting products occurs over a large absorptive surface area due to villi and microvilli as part of the intestinal surface (Boland, 2016). It is important to note that low concentrations of oxygen are always present in the small intestine, allowing the life of facultative anaerobes. In contrast, many bacteria could not survive in these conditions, either because of the presence of oxygen, antimicrobial peptides (AMPs) or bile acids. *Lactobacillus*, *Enterococcus*, *Prevotella* and *Streptococcus* are the most abundant genera of the duodenum and jejunum, probably due to their higher tolerance to this specific environment (Adak & Khan, 2019) (Reynoso-García et al., 2022). The ileum is dominated by *Streptococcus*, *Escherichia/Shigella* and *Clostridium*, in contrast to its distal part, which is more similar to the microbiota of the colon (Reynoso-García et al., 2022) (Zoetendal et al., 2012). Colon is the site of the most diverse microbial community with its anaerobic environment allowing the thriving of the major gut genera such as *Bacteroides*, *Parabacteroides*, *Clostridium, Lachnospiraceae, Faecalibacterium, Escherichia/Shigella* and *Bilophila* (James et al., 2020). The composition of the cecal microbiota found in the initial part of the colon poses a problem due to sampling difficulties, however, it has been shown to be different from that of samples obtained from feces resembling the distal part of the colon (Be et al., 2001). Surprisingly, the human appendix, previously thought to have lost its function during evolution, turned out to contain diversified microbes with a dominant phylum Firmicutes (Guinane et al., 2013). Scientists are now reassessing the function of the appendix, suggesting that it serves as a "safe place" for many bacteria that can enter the gut when needed (Guinane et al., 2013).

1.3.1 Gut microbiome development during ontogeny

The human gut microbiota is not stable throughout the whole life but changes during human ontogeny (Figure 3). Microbial colonization of the GIT begins during labor when the composition of the microbiota depends on the mode of delivery. It has been shown that vaginally born infants appeared to have microbial communities very similar to their own mother's vaginal microbiota with a predominance of *Lactobacillus* and *Prevotella*, whereas infants born by cesarean section acquired a community that resembled microbes found on the skin surface of their mothers with predominant taxa such as *Staphylococcus* and *Corynebacterium* (Dominguez-Bello et al., 2010). There are other factors that can affect the microbial communities in the gut of infants, such as the type of feeding, the term of the delivery, hospitalization, and the possible use of antibiotics (Ottman et al., 2012) (Vandenplas et al., 2020). Environmental factors such as geographical location, and the presence of siblings or pets in the households also play a key role in the development of the microbiome at an early age (Stewart et al., 2018).

Figure 3. Changes in microbial diversity and abundance during human development from the prenatal stage to adulthood. The late relatively stable microbiome is thought to be established around 3 years of age. In the elderly, microbial diversity is reduced compared to adults. Figure adapted from (Reynoso-García et al., 2022).



Human milk is undoubtedly a key source of nutrition for newborns and, due to its probiotic and prebiotic function, it also plays a key role in determining the microbial composition of the infant's gut with increased diversity and functionality of its microbiome (Kundu et al., 2017) (Vatanen et al., 2018). Significant differences in the composition of the gut microbiota were found in infants who were breastfed, in contrast to infants fed an artificial diet, where *Bifidobacterium* predominated in the gut of breastfed children, while the proportion of *Bifidobacterium* and *Bacteroides* was the same in children that were formula fed (Harmsen et al., 2000). This is mainly due to the fact that human milk is composed of a perfect mixture of nutrients that supports the development of the infant. It contains lactose, many lipids, and also human milk oligosaccharides (HMOs), which are considered to be milk prebiotics that shape the microbial composition of the infant's gut, specifically in favor

of *Bifidobacterium* taxa (Milani et al., 2017). The next stage of gut microbiome development occurs with the introduction of solid food instead of milk or formula when a more complex and mature community replaces the infant one. This transition is also related to the further maturation of the immune system and gut-brain axis (Kundu et al., 2017).

After the period of relative stability during adulthood, changes in the composition and activity of the intestinal microbiota also occur in old age, with a strong dependence on lifestyle, diet or inflammatory diseases (Ottman et al., 2012). As in the neonatal period, the intestinal microbiota of the elderly is again unstable and its changes are often related to dysbiosis partly caused by the natural senescence of the gastrointestinal tract, which is characterized by increased oxidative stress and inflammation. Although the observed changes have high interindividual characteristics, some common features and trends were found throughout the elderly population, including reduced microbial diversity, a decrease in total short-chain fatty acids (SCFAs), a decrease in bacterial genera considered beneficial such as *Bifidobacteria* or *Faecalibacterium*, or an increase in facultative anaerobes (Salazar et al., 2019). Interestingly, reduced microbial diversity appears to be increasing again in populations with extreme longevity, where some new microbes have also been observed (Biagi et al., 2016).

1.3.2 The variability of human gut microbiota

Typical aspect of the human gut microbiota that has not been mentioned so far is its interindividual variability, which is strongly related to environmental factors. Each human individual has distinct gut microbiota, even identical monozygotic twins do not have the same composition of gut microbes. Although some studies suggest that heritable taxa exist and are thought to play a role in shaping microbial diversity (Goodrich et al., 2016), there are other opinions suggesting that the environment is still a major driving factor with little genetic influence (Turnbaugh, Hamady, et al., 2009). It is important to mention that the Turnbaugh group's research had a smaller number of cases available for the proposed study.

Although much of the individual variability remains unexplained, the main sources are thought to be mostly environmental, including diet, geography, lifestyle or antibiotic use, but also include host genetics, age, and early microbial exposure (Gilbert et al., 2018) (Pasolli et al., 2019) (Rothschild et al., 2018) (The Human Microbiome Project Consortium, 2012). Diet has been extensively studied in relation to the composition of the gut microbiota, and indeed there is evidence that it is essential in modulating the gut microbial community (De Filippo et al., 2010) (Ley et al., 2008) (Muegge et al., 2011). Exercise has been shown to be associated with higher microbial diversity (Clarke et al., 2014), but the relationship between exercise and gut microbiota is quite complex and may very well reflect the fact that people with higher physical activity generally have healthier lifestyle habits, which influence gut microbes as well. Another lifestyle habit that has an impact on microbial communities is smoking. Compositional differences between smokers and non-smokers were observed in the oral cavity and also in the gut (Gomaa, 2020). Antibiotics are known to be beneficial when needed to defeat a bacterial infection, but also harmful to the rest of the microbial community. It is therefore not surprising that their use can affect the gut microbiota, specifically negatively by disrupting the balance of the community, reducing its diversity, and also creating an environment in which antibiotic-resistant strains can over-grow (Patangia et al., 2022).

Studies are being conducted to explore and understand the variability in microbial communities in relation to geography. For example, differences in microbiota characteristics and functional genes have been observed in infants, children, and adults in the United States of America, Venezuela, and Malawi (Yatsunenko et al., 2012). Another study performed in Russia in cohorts living in different

environmental conditions confirmed that in comparison with the global large-scale studies, dominant gut bacteria were similar, but some unique taxonomic and functional properties were found in the Russian population (Tyakht et al., 2013). Any observed differences in microbiota may not just reflect geographical differences, but may also include differences in the diet, lifestyle and environment of these locations. Nevertheless, given these results, emphasis should be placed on sampling a wider range of people in terms of dietary habits, geography, ethnicity and age to identify microbiota associated with disease or health.

As far as the microbiota of a healthy adult is concerned, there seems to be a so-called core microbiota that is similar between individuals. The core gut microbiota refers to a set of similar microbial characteristics that can be observed in all adults, but the task of identifying the core microbiome is quite complex, and in recent years attention has shifted from examining the mere taxonomy of the common microbiota to the functional potential of the core microbiome (Sharon et al., 2022). The logic of this shift is that several different taxa can perform the same function (Lozupone et al., 2012). For example, Turnbaugh's group indeed suggested that idea of the core microbiota at the level of commonly shared abundant taxa is incorrect and we should focus more on the core microbiome at the level of metabolic function (Turnbaugh, Hamady, et al., 2009). The problem with the core microbiome is also encoded in the limitations of the studies, with most microbiome studies being conducted on Western populations, even though microbial composition can vary depending on geographic location, environment and lifestyle, and few of these studies access both microbial composition (what type of microbes are present) and their functional potential (what these microbes do). The next layer of uncertainty is the bioinformatic analysis, as there is no universal bioinformatical pipeline for data processing despite the known fact that different ways of data processing result in different outcomes (Sharon et al., 2022).

Clearly, given the large variability of gut microbiota in individuals in any population, studying the microbiome and its potential causal links to disease and overall health is a complex task. Exact matching of possible confounding factors between patients and controls and sufficient power is much needed in these types of studies (Vujkovic-Cvijin et al., 2020). However, microbial variability observed between individuals also suggests that we should focus more on personalized medicine in relation to the microbiome, which has enormous metabolic potential compared to our own human cells.

1.3.3 Functions of gut microbiota

As mentioned in previous chapters, gut microbes perform many important functions in our bodies and are essential for human metabolism and overall health. Scientists are currently using two main models to understand the complex relationship between gut microbiota and human health and to further investigate the impact of these microbes on our physiology and the possible link between microbes and certain diseases. The first method is to use mice after treatment with broad-spectrum antibiotics, which results in mice with depleted gut microbes. However, the current gold standard in this field is the use of germ-free (GF) mice that are kept in special conditions without exposure to any microbes (Kennedy et al., 2018). This GF mouse can be colonized with a defined community of microbes to create a gnotobiotic mouse (Rosenbaum et al., 2015), which allows the microbiota to be studied in direct relation to specific conditions such as diet, drugs or a particular disease state. Albeit mice are by far the most popular animal GF/gnotobiotic model, GF rats and pigs exist as well.

1.3.3.1 Energy homeostasis

Based on the studies performed on GF mice, it was shown that gut microbes play an important role in energy homeostasis. After the colonization of GF mice with microbes from conventional mice, these colonized mice gained weight even though their daily energy intake was 29% less in comparison to GF mice (Bäckhed et al., 2004). This suggests that the microbiota plays a vital role in regulating appetite and processing energy from food, which contradicts the widely accepted "calories in versus calories out" theory that claims that weight gain is only a result of eating more calories than we burn, but obviously, other factors can also influence energy balance.

Bäckhead's findings were supported and further investigated by Turnbaugh and his group, who showed that different composition of the microbiota is associated with the obese and lean phenotype. Microbiota inhabiting the gut of obese animals was associated with a greater ability to obtain energy from the diet (Turnbaugh et al., 2006). The authors also confirmed that this trait is transferable when colonization of GF mice with microbiota derived from genetically obese mice resulted in a significant increase in total body fat compared to GF mice colonized with microbes from lean conventional mice fed the same diet. They subsequently conducted a similar study with humanized GF mice colonized with microbes derived from the fecal contents of a healthy adult, where the mice were then fed either a high-fat Western diet or a standard low-fat, high-polysaccharide diet (Turnbaugh, et al., 2009). As expected, mice fed a Western diet gained more weight and gut microbial community composition and microbial gene expression in both groups was significantly different. Furthermore, the obese phenotype was transferable by gut microbiota from obese humanized mice to GF lean recipients (Turnbaugh, et al., 2009).

Quite recently, it has been shown that not only colonic but also upper intestinal microbiota is involved in energy extraction and regulation of energy homeostasis. Martinez-Guryn and her colleagues showed that GF mice populated with a jejunal microbiota of mice fed a high-fat diet had increased lipid absorption even when fed a low-fat diet (Martinez-Guryn et al., 2018). Indeed, further research in humans is needed to explore how the gut microbiota is involved in weight control and obesity in order to find a sensible solution to the global obesity epidemic.

1.3.3.2 Biotransformation and formation of fermentation products

The human intestinal microbiota have a high capacity to produce a number of bioactive substances that can directly or indirectly influence host metabolism. One important group of these compounds of microbial origin are bile acids, the balance of which is important for the proper functioning of our metabolism, and gut microbes are the main mediators of their biotransformation and homeostasis. Primary bile acids are synthesized in the liver from cholesterol and are further metabolized by gut microbes into secondary bile acids and various other products that greatly increase the diversity of bile acids and their biological potential (Guzior & Quinn, 2021).

In humans, chenodeoxycholic acid (CDCA) and cholic acid (CA) are primary bile acids formed in the liver hepatocytes (Ridlon et al., 2016). Before secretion from the liver, these primary bile acids are conjugated to amino acids glycine or, to a lesser extent, taurine to increase their solubility (Wahlström et al., 2016). After secretion, these conjugated bile acids are deposited in the gallbladder along with phosphatidylcholine and cholesterol (Ridlon et al., 2016). The meal intake stimulates the gallbladder and its contents are released into the small intestine, where bile salts activate pancreatic lipase and, due to their amphipathic properties, help to form micelles composed of cholesterol, monoglycerides, fatty acids and fat-soluble vitamins, which can then be absorbed by enterocytes (Ridlon et al., 2016). Most of these bile acids are then reabsorbed from the distal ileum via a specific transporter

in the enterocyte membrane and then returned to the liver via the bloodstream, where their excretion into bile can begin again (Adak & Khan, 2019).

A small portion of bile acids that escape reabsorption is converted to secondary bile acids by gut microbes through 7-α-dehydroxylation or other transformations (De Aguiar Vallim et al., 2013). In humans, this group of secondary bile acids includes lithocholic acid (LCA), deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA). The essential role of the microbiota in bile acid metabolism has been demonstrated in a GF mouse model. In contrast to normal mice, GF mice have a disrupted primary bile acid profile with the balance shifted exclusively towards taurine conjugates (Swann et al., 2011) and completely lacking secondary bile acids (Sayin et al., 2013).

In addition to their function in dietary lipid absorption, bile acids also act as signaling molecules and regulators of metabolism, primarily through binding and activation of the nuclear farnesoid X receptor (FXR) and the plasma membrane G-protein coupled receptor TGR5 (De Aguiar Vallim et al., 2013) (Yu et al., 2019). By activating these receptors, bile acids are able to control many biological processes including their own synthesis, conjugation and transport, as well as lipid, glucose and energy metabolism (T. Li & Chiang, 2015). It is also a known fact that bile acids have antimicrobial properties and can therefore influence microbial growth rate due to their detergent properties as well as through inducing the genes encoding anti-microbial peptides via FXR-dependent signaling (Li & Chiang, 2015) (Ridlon et al., 2016) (Yu et al., 2019). However, the gut microbiota has several different mechanisms to be resistant to bile salts, and microbes that have such mechanisms will naturally have a higher probability of survival. Microbes are believed to have a fundamental defense mechanism that involves the transformation of bile acids into less harmful compounds. For instance, primary bile acids are deconjugated as an initial step in bile acid biotransformation, which reduces their solubility and enables them to be utilized as substrates for further modifications (Ridlon et al., 2016) (Staley et al., 2017). This defense mechanism is evidenced by the presence of a bile salt hydrolase (BSH) encoding gene in all major bacterial species found in the gut (Jones et al., 2008). Gut microbes are also able to regulate bile acids pool composition, which means that a delicate balance between microbiota and bile acid composition exists (Ridlon et al., 2006).

The intestinal microbiota is also important for the biosynthesis of many vitamins. It has been shown that intestinal microbiota is, besides dietary intake, an important source of vitamin K, biotin, cobalamin, folic acid, riboflavin, and other vitamins of the vitamin B group (Hill, 1997) (LeBlanc et al., 2013). Microorganisms are also known to affect drug metabolism by biotransformation, and therefore different reactions to drugs in different individuals are thought to be at least partially caused by different sets of microbes in these individuals (Zimmermann et al., 2019).

Another important function of gut microbes is the degradation of complex carbohydrates indigestible by our own enzymes, such as cellulose, lignin, hemicellulose, pectin or some oligosaccharides, into various metabolic products that can positively or negatively affect our health (Gomaa, 2020). When the microbiota in our gut is exposed to fiber, microbial fermentation occurs and the resulting metabolites include SCFAs, represented by butyric, propionic and acetic acids. SCFAs have a positive effect on human physiology serving as important energy sources, immunomodulators and signaling molecules and also improving glucose and lipid metabolism (Koh et al., 2016) (Cummings et al., 1987) (Kumar et al., 2020) (Morrison & Preston, 2016) (Venegas et al., 2019). It is therefore not surprising that the possible positive links of butyrate-forming bacteria to improved human health are now being intensively investigated.

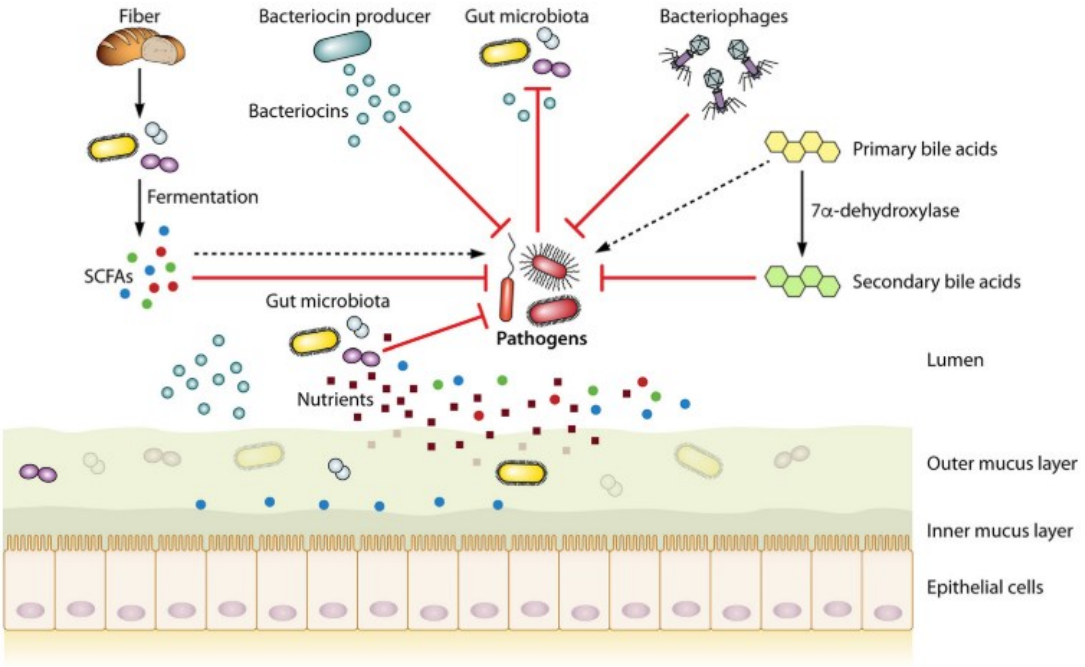
However, when the fermentable fiber is absent or much less abundant in our diet, bacteria ferment less energetically beneficial sources such as proteins or amino acids and the resulting metabolites include phenol, indole, p-cresol, ammonia, the branched-chain amino acids (BCAAs) valine, leucine, isoleucine and their products, branched-chain fatty acids (BCFAs) isobutyrate, 2-methylbutyrate and isovalerate (Mohajeri et al., 2018) (Oliphant & Allen-Vercoe, 2019). Recent evidence showed that gut microbiota may be a source of essential amino acids including BCAAs (Gojda & Cahova, 2021). Higher concentrations of BCAAs in plasma are associated with obesity and diabetes (Arany & Neinast, 2018), suggesting that elevated levels of these substances are not desirable, although their deficiency is also devastating. Another compound with potential negative effects is trimethylamine of microbial origin, derived from choline or L-carnitine found in foods such as red meat, eggs or fish, which can be further converted to trimethylamine N-oxide (TMAO) in the liver. TMAO is associated with some non-communicable diseases (NCDs) frequent in westernized societies although the causality is yet to be proved (Agus et al., 2021). Clearly, the gut microbiota has the ability to switch from making metabolites that are beneficial to our health into metabolites that can have harmful effects, and it all depends largely on how we feed them.

1.3.3.3 Protective and immunological properties

Commensal microbiota inhabiting the intestine protect the host against pathogen invasion and substantially contribute to the maturation of the immune system. Several metabolites produced by these bacteria have antimicrobial effects including secondary bile acids, bacteriocins and SCFAs (Ducarmon et al., 2019). Moreover, the intestinal microbes contribute most to the defense against pathogenic microorganisms through colonization resistance, production of toxic substances for some pathogens or nutrient competition (Figure 4). The homeostasis of the mucus barrier that covers the surface of the epithelial cells inside the intestines is essential for maintaining health. In the colon, the mucosal layer is divided into two distinct parts, the outer layer, which provides nutrients, binding sites and a home for some gut microbes, and the dense inner layer, which is impenetrable to microbes and is attached to epithelial cells keeping them bacteria-free (Johansson et al., 2011). The microbiota that lives in the outer part of the mucus layer is called mucus-associated microbes and include genera such as *Lachnospiraceae*, *Bifidobacterium*, *Akkermansia*, *Faecalibacterium*, *Clostridium* and *Eubacterium* (Ouwerkerk et al., 2013).

The mucosal layer has special viscoelastic properties due to its main compositional and functional building blocks, glycosylated proteins called mucins, which combine with other components such as water, lipids, proteins and other substances (Paone & Cani, 2020). Mucins are secreted by the goblet cells, which are present throughout the digestive tract and lubricate its inner surface, thus aiding the passage of food and protecting epithelial cells against pathogens, toxic substances or digestive enzymes (Paone & Cani, 2020). Interestingly, the site with the highest proportion of goblet cells is the colon, which is probably related to the presence of commensal microbes that can directly influence the thickness of the mucosal layer by various glycosidases and proteases (Kim & Ho, 2010). The development of the mucus layer is a very dynamic process with gut microbes being involved (Johansson et al., 2015).

Figure 4. The schematics of microbially mediated colonization resistance in the gut. Figure adapted from (Ducarmon et al., 2019).



The immune system in the gut must be highly balanced to tolerate and cooperate with the commensal microbiota, but at the same time be able to respond properly to pathogenic microbes. This special relationship between microbiota and the immune system is influenced by many factors and probably begins to form during pregnancy and follows the first contact of the commensals with the immune system during birth (Kalbermatter et al., 2021). The gut is interspersed with a high number of different immune cells connected to the epithelium, which together with commensal microbes and mucosal barrier protect the host from pathogens and possibly harmful substances. The mucosal layer contains several components of the innate immune system, such as defensins, Paneth cells that are capable of secreting various AMPs, and plasma cells that produce secretory immunoglobulin type A (sIgA) which is now thought to be key in the intestinal humoral immune system (Hamada et al., 2002) (Kalbermatter et al., 2021) (Macpherson et al., 2001). After birth, breast milk containing maternal antibodies provides protection for the baby and also shapes the developing immune system and gut microbial communities; more specifically, mucosal immune memory is transferred to the baby *via* maternal sIgA shaped by the mother's microbiota (Maynard et al., 2012). As a result, microbial antigens in conjunction with sIgA will be tolerated by the innate immune system, which will shape the overall commensal microbiota and also help in the formation of the regulatory immune system (Maynard et al., 2012).

1.3.3.4 Development and functioning of organ systems

Thanks to extensive evidence, it is now clear that the gut microbiota can influence the function of organs outside the gut mainly through its metabolites. Scientists refer to these relationships by the term "axis", which specifies various relationships and pathways between the gut and other organs. To allow proper communication between the gut and other organs, microbial signals must be first transmitted through epithelial cells in the intestines (Schroeder & Bäckhed, 2016). These microbial-derived signals include lipopolysaccharides (LPS), flagellin, peptidoglycan, secondary bile

acids, tryptophan metabolites, and SCFA-related signals. As suggested in previous chapters, the microbiota plays a key role in the development of the immune system and the protective mucosal layer, so it is not so surprising that it extends its influence to other organs and contributes to their development and function.

To be more specific, intestinal microbes are also essential for the development of the digestive tract itself, where they interact and influence digestion, immunity, and many other biological processes from the beginning of colonization. The intestinal epithelium is known for its remarkable adaptive and self-renewal functions, which are mediated mainly by the proliferative activity of intestinal stem cells (IECs) located in the epithelial crypts which can differentiate into many types of cells, including enterocytes, Paneth cells, goblet cells, tuft cells, enteroendocrine cells, or M-cells (Ye & Rawls, 2021). In GF mice, crypt depth is reduced compared to normal mice, but after colonization, crypt depth deepens and ISCs proliferation increases (Peck et al., 2017). Similarly, ISCs proliferation is increased in wild-type mice after *Lactobacillus plantarum* supplementation (Lee et al., 2018). The microbiota also strongly influences the brush border at the apical luminal surface of enterocytes, where GF animals have reduced microvilli thickness (Sommer & Bäckhed, 2013). In addition, the number of Paneth cells and goblet cells in GF animals is much lower than in conventional animals (Schoenborn et al., 2019)(Sharma et al., 1995).

As outlined in this chapter, gut microbes are involved in countless processes in our bodies, which should not be surprising given that humans co-evolved along with the microbes they harbor. The extensive functions that microorganisms perform are also supported by the fact that bacteria, unlike our own cells, can produce new generations of themselves within an hour, giving themselves a great advantage in that they can adapt quickly to changes in the environment. Environmental factors such as diet are important for maintaining the positive functions of the gut microbiota and supporting the production of potentially beneficial metabolites produced by the microbes.

1.3.4 Metabolome as a functional readout of the gut microbiota

Although the interindividual variability of gut microbial communities in terms of taxonomy is a well-known phenomenon, the gene composition and functional capacity of intestinal bacteria are highly conserved. Many phylogenetically distant bacteria carry similar genes and are therefore able to perform similar functions and produce similar metabolites (Tian et al., 2020). These metabolites provide an additional level of understanding of the relationship between host and microbiota beyond the classical characterization of microbial taxonomy or microbial genes. Metabolomics is therefore often involved in microbiome studies and provides a fingerprint of microbial functional status (Marcobal et al., 2013). Based on the Twins UK Study published by Zierer *et al*., the gut microbial composition explained approximately 68% of the observed variance of several hundred fecal metabolites on average (Zierer et al., 2018). This observation strongly suggests the connection between intestinal microbiota and intestinal metabolome. Based on a study performed on GF and colonized ex-GF mice, out of 179 identified colonic luminal metabolites, approximately 70% of the metabolites were significantly different in amount between the two groups of mice pointing out to their microbial origin (Matsumoto et al., 2012). Moreover, differences in fecal and urine metabolomes were also observed between GF mice, conventional mice and mice colonized by adult human fecal contents (Matsumoto et al., 2012). A surprisingly large influence of gut microbes can be observed even in the blood metabolome, where hundreds of metabolites in plasma were unique to conventional mice compared to GF mice, and in addition, approximately 10% of all features that were common to both groups differed significantly in signal intensity (Wikoff et al., 2009). A more

recent study addressed the global impact of microbiota by analyzing both microbial composition and metabolome using a total of 768 samples from 96 sampling sites of 29 organs from GF and colonized mice. The strongest differences were observed in the GIT; however, unique biochemical signatures originating from microbiota were identified in all organs, providing further evidence that microbiota affects all organs (Quinn et al., 2020). McHardy *et al*. conducted a study on human subjects and described the connections between the microbiota and metabolome in the cecum and sigmoid colon. Their analysis of microbial ecology led to the imputed Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway abundances, which were found to be mostly consistent with metabolic data. This indicates a reciprocal relationship between the microbiota and metabolome (McHardy et al., 2013). Overall, studies that take into account the structure of microbial communities, as well as the identification of metabolites, support the idea that the gut microbiota is indeed involved in establishing the biochemical environment in both proximal and distant niches within the human body and that the human metabolome can consequently impact the composition of gut microbes (Lee-Sarwar et al., 2020).

1.4 Diet as one of the main environmental factors modulating gut microbial ecosystem

It is increasingly recognized that there is no one-size-fits-all diet and each individual responds differently to a particular dietary intervention, mainly due to the unique relationships between the host and its microbes. However, this connection is bi-directional and the possibility of shaping gut microbial communities and their functional potential through diet is emerging. Future studies must take into account these complex relationships and focus on the various cornerstones that determine personal responses – firstly, the diet of the individual with its essential food components and their vast array of products; secondly, the gut microbiota of the host, which consists of hundreds of different species that influence a multitude of biological processes; and thirdly, the physiology and metabolism of the host itself, which is no easy task given the interdependence of these three key elements (Kolodziejczyk et al., 2019).

1.4.1 Differences between herbivores, omnivores and carnivores

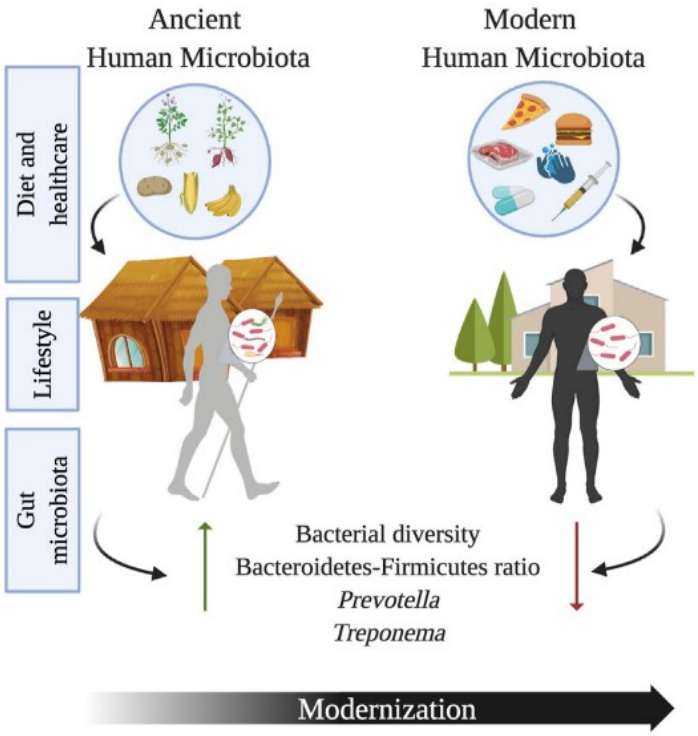
It is important to note that diet has a greater influence on the formation of microbiome than phylogeny, as found in a study examining the distribution of microbial species and their function in several mammalian animals belonging to three dietary groups, namely herbivores, omnivores and carnivores (Muegge et al., 2011). This study also elucidated the relationships between sets of functional genes and dietary habits, showing a clear separation of functional genes by diet. The authors suggested that the observed differences in microbiomes between the three dietary groups are likely due to differences in functional characteristics, such as having more specific enzymes associated with the digestion of substrates from different diets (Muegge et al., 2011). It is logical that herbivores need more enzymes associated with the breakdown of complex plant polysaccharides and also need specific enzymes involved in amino acid biosynthesis, which was confirmed by the study (Muegge et al., 2011). In contrast, a significant increase in enzymes associated with nine amino acid degradation pathways was found in carnivores, whereas only the BCAAs degradation pathway involving the degradation of valine, leucine and isoleucine was enriched in herbivores (Muegge et al., 2011). Thus, it is clear that the functional potential of the microbiota is reversed in herbivores and carnivores, with carnivore microbes specializing in the breakdown of proteins from meaty diets and using them as the main source of energy, while herbivore microbiota specializes in the synthesis of amino acids as

building blocks of proteins (Muegge et al., 2011). This research has helped the scientific community to better understand the relationship between host diet and gut microbial communities and their functions, as well as to develop new hypotheses focusing on key factors in the coevolution of the gut microbiota and its host.

1.4.2 Differences between "traditional" and "westernized" societies

Over the past millennia, major advances in medical and hygienic practices, including the use of vaccinations or antibiotics, have rapidly reduced the incidence of many infectious diseases (e.g., malaria or tuberculosis). On the other hand, this may have led to a loss of ancient microbes in humans and to a higher incidence of other diseases found exclusively in developed countries, such as obesity, inflammatory diseases or epidemics of methicillin-resistant *Streptococcus aureus* (MRSA) infections (Blaser & Falkow, 2009). Although industrialization and improved healthcare have led to an increase in life expectancy, human microbial diversity appears to have declined and many other changes in the gut microbiota have come with modernization (Figure 5) (Reynoso-García et al., 2022). Therefore, studies need to be conducted on groups of people unaffected by industrialization and urbanization, which are now the last image reminiscent of the "ancient" microbiota, to better understand which changes have occurred in modern society and how this relates to the new epidemics.

Figure 5. The differences in gut microbiota resulting from industrialization, urbanization and changes associated with modern lifestyle in general. Figure adapted from (Reynoso-García et al., 2022).



For example, the Hadza people in Tanzania, who still live as hunter-gatherers, have been shown to have a higher diversity of gut microbiota and a different set of taxa compared to other populations, probably as a result of consuming more fiber as opposed to the typical diet in industrialized countries (Fragiadakis et al., 2019). Furthermore, Bacteroidaceae has been shown to be the predominant taxon across industrialized societies, in contrast to populations with traditional lifestyles including

hunter-gatherers or rural farmers, where Prevotellaceae is much more common (Smits et al., 2017). Another study on Hadza hunter-gatherers also showed that they had higher bacterial richness and diversity compared to an Italian cohort with a modern lifestyle (Schnorr et al., 2014). This study also confirmed that the family Prevotellaceae is among the most abundant families in the gut microbiota of the Hadza cohort, along with Ruminococcaceae and Lachnospiraceae. An interesting finding in this study was that the genus *Bifidobacterium* was completely absent from the digestive tract of all hunter-gatherers (Schnorr et al., 2014).

It has been shown that European children consuming a modern Western diet and children from the African village of Burkina Faso consuming a rural diet rich in fiber have different gut microbiota (De Filippo et al., 2010). More specifically, in line with the above studies, a unique occurrence of taxa from the Prevotellaceae family, specifically the genera *Prevotella* and *Xylanibacter*, which were not present in European children, was found in children from rural Africa (De Filippo et al., 2010). A study focusing on an isolated Amerindian tribe with no prior contact with Western societies reported the highest bacterial and functional diversity ever recorded in human studies, with a high prevalence of taxa in the genus *Prevotella* similar to other findings observed in non-industrialized societies (Clemente et al., 2015). Interestingly, it is not only bacterial communities that are affected; specifically, the presence of *Entamoeba* and *Blastocystis* protozoa has been shown to be associated with an increase in overall microbial diversity, suggesting that these eukaryotes are likely associated with a "healthy" gut microbiota (Audebert et al., 2016) (Morton et al., 2015).

However, it remained unclear to what extent microbes were changing as a result of geography or industrialism itself. Based on a study conducted on four groups of Himalayan societies with different levels of traditional lifestyles, it was found that differences in lifestyles correlated strongly with differences observed in microbial communities (Jha et al., 2018). Moreover, the microbiota of all these traditional societies differed from the microbiota of industrialized societies in America, and the Himalayan group with the most advanced level of agriculture has the most similar gut microbiomes to Americans (Jha et al., 2018). Overall, the changes in gut microbes that have occurred in the modern era are clear, and their cause is probably mainly related to the changes in lifestyle and diet that followed the industrial revolution.

1.4.3 Manipulation of gut microbiome via diet

Adult human gut microbiota is a relatively stable community under normal conditions, although its composition may fluctuate due to environmental factors and especially diet. A well-known study on the dynamics of the human gut microbiota induced by diet was conducted by L. A. David and his colleagues, who demonstrated that even short-term changes in diet, whether entirely of plant or animal origin, alter gut microbial communities. Interestingly, this group also showed that the altered microbial communities returned to their original state just a few days after the subjects returned to their long-term dietary habits (David et al., 2014). This study provides evidence of the rapid flexibility of the gut microbiota due to the different energy sources provided to it. Despite this rapid diet-induced dynamic, long-term dietary habits are the main driving force in the modulation of the microbial composition of each individual (Sonnenburg & Bäckhed, 2014).

Our group recently studied the effect of a plant-based diet on the microbial community, and although some small differences were observed in the gut microbiota composition of vegans compared with omnivores, the main and significant difference was in metabolic activity, suggesting that the same microbiota can adapt to different diets and change their fermentation pathways accordingly (Prochazkova et al., 2022). This finding is supported by other studies; for example, it was shown that

a long-term high-fiber diet led to an increase in glycan-degrading carbohydrate-active enzymes (CAZymes) of microbial origin, although the diversity of the microbiota remained stable (Wastyk et al., 2021). Plant-based diets have also been associated with the highest abundance of microbial genes or proteins related to carbohydrate and protein hydrolyzing enzymes, cell motility, transport pathways, and biosynthesis of certain vitamins, essential amino acids, and some other beneficial compounds such as SCFAs (De Angelis et al., 2020).

Based on the results of the American Gut Project, a favorable microbiota also appears to be associated with plant-based diet diversity, with consumption of more than 30 different plants per week leading to a higher prevalence of *Faecalibacterium* and *Oscillospira* genera and a lower prevalence of antibiotic resistance genes compared to a diet with less than 10 different plants per week (McDonald et al., 2018). This suggests that a diet rich in a wide variety of plants is likely to support the growth of more bacterial groups (McDonald et al., 2018). Other findings from the American Gut Project recently reported an association between fecal microbial diversity and dietary patterns based on dietary recommendations, with the Healthy Eating Index (HEI) as a measure of diet quality being associated with greater microbial diversity, specifically higher total scores for vegetables, greens and beans, whole grains, refined grains, and dairy products (Baldeon et al., 2023).

Extreme dietary regimes, such as the keto diet, can lead to more significant changes in the gut microbiota. For example, the keto diet resulted in a reduction in the number of *Bifidobacterium* genera and also a reduction in the abundance of important butyrate-producing bacterial taxa, leading to a reduction in stool SCFAs concentrations (Rew et al., 2022). In their review, Singh et al. described that not only dietary fiber but also protein plays a significant role in changes in gut microbiota function and diversity (Singh et al., 2017). Consumption of a diet high in beef resulted in a decrease in the number of *Bifidobacterium* genera and an increase in the number of *Bacteroides* and *Clostridia* genera compared to the group on a meat-free diet (Singh et al., 2017). In mice, a Western-type diet high in fat and sugar has been shown to lead to an inflammatory environment in the gut, reduced microbial diversity and species richness, an overgrowth of pro-inflammatory *Escherichia/Shigella* genera in the intestinal mucosa, a decrease in the number of protective species, and a decrease in SCFAs concentration (Agus et al., 2016). The authors believe that the observed changes in the gut microbiota composition increase the host's susceptibility to chronic inflammatory bowel disease. The findings are also consistent with studies performed in Western populations and non-industrialized societies mentioned above where similar differences have been observed.

In conclusion, the human gut microbiota is relatively stable under normal conditions, but its composition can change rapidly due to dietary perturbations, albeit this shift is only transitional. However, a diet rich in a wide variety of plants is likely to support the growth of more bacterial groups, i.e., to increase diversity. Extreme dietary changes, such as the keto diet, can lead to significant changes in the gut microbiota and affect its function and diversity. The metabolic activity of the gut microbiota reflects the diet, i. e. the substrate provided, which is reflected by a change in the spectrum of metabolites produced. In summary, the diet plays a significant role in modulating the composition of the gut microbiota, and a diverse plant-based diet could be beneficial for promoting healthy microbiota and overall health.

1.5 Challenges of multi-omics data

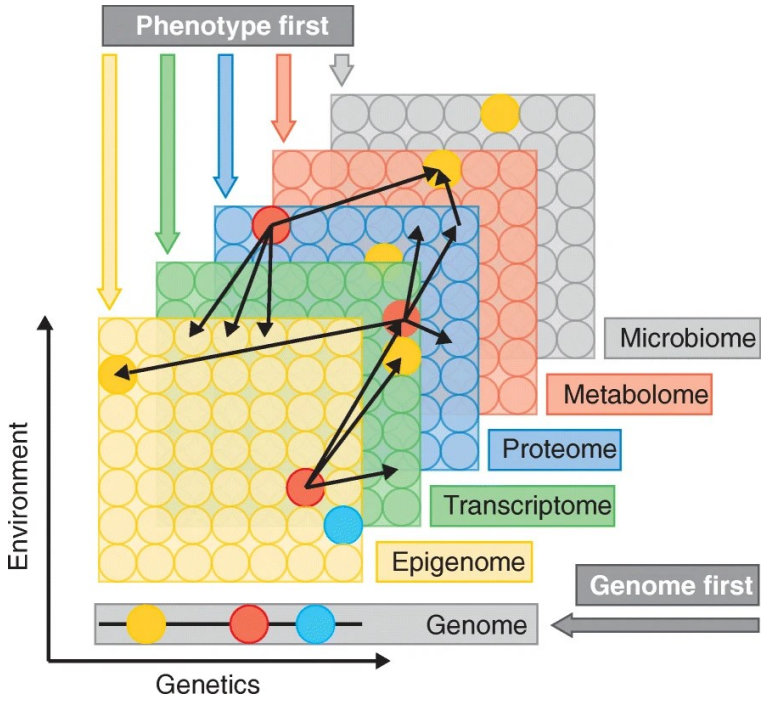
So-called "multi-omics" data has revolutionized contemporary exploratory research by integrating more than one type of dataset into a single analysis using multiple approaches to understand one

particular problem. This approach generates more data and therefore more information about a particular research topic can be obtained. This allows scientists to perform deeper analysis, see a more complete picture, and generate new insights into one complex biological problem because most biological processes are naturally interconnected. However, the biggest challenge is how to process this kind of data, which generates thousands of variables measured in a limited number of cases, resulting in highly multi-dimensional data. Different data sets need to be combined in a standardized way and appropriate computational tools have to be used to extract relevant variables that will be used to draw conclusions and generate new hypotheses. Another issue to keep in mind is the storage of huge amounts of data generated by multi-omics approach and also the use of appropriate statistical tests, which is, as expected, quite complex (Conesa & Beck, 2019). Nevertheless, with recent advances in bioinformatics and statistics including machine learning and regularization techniques, the analysis of high-dimensional data has been greatly simplified and continues to improve. By combining information from several multi-omics disciplines, scientists are able to find possible causal influences leading to a particular type of disease and can also use this information to search for disease-specific markers and possibly improve prevention (Hasin et al., 2017). It is no exaggeration to say that multi-omics studies are the future.

1.5.1 Overview of omics data types

The addition of the term "omics" means that almost all measurable variables of certain molecular types are evaluated in one single procedure or assay (Conesa & Beck, 2019). There are six basic "omics" data types – genomics, epigenomics, transcriptomics, proteomics, metabolomics a microbiomics (Hasin et al., 2017) as shown in Figure 6.

Figure 6. Overview of the six main types of omics data displayed as layers. Some of these include genetic and environmental influences. Each circle represents one sample. The interaction between layers is obvious and there are also interactions within layers. Figure adapted from (Hasin et al., 2017).



Genomics is the oldest of the omics fields, having been recognized in the 1970s when scientists discovered that differences in the DNA code are the source of most genetic variation between individuals (Bustamante et al., 2011). Epigenomics studies the broader associations of DNA and histones and all possible epigenetic modifications in the cell. These covalent modifications are master regulators of gene transcription and may influence the biological process and disease development (Hasin et al., 2017). In the context of genomics and epigenomics, several types of assays are currently available, either using DNA microarrays, methods targeting DNA modifications such as restriction endonucleases, or NGS methods in general (Shendure et al., 2019).

The goal of transcriptomics is not just to describe all known transcripts including messenger ribonucleic acids (RNAs), small RNAs, and non-coding RNAs but also to examine the level of gene expression under different conditions or pathological states and help researchers to deeper understand post-transcriptional modification such as alternative splicing variants or different starting sites (Wang et al., 2009). Currently, two principles are applied in transcriptomic studies - one is based on hybridization using specially designed microarrays, and the other involves a newer approach, RNA sequencing. RNA sequencing does not depend on knowledge of the genome sequence as the microarray approach does, but it is more expensive (Wang et al., 2009).

The basis of proteomics is the identification and quantification of proteins and peptides, their modifications and interactions. Today, the most frequently used approach for studying proteins is the combination of gel- or chromatography-based separation techniques coupled with mass spectrometry (MS) analysis and bioinformatic methods (Nikolov et al., 2021). There are two primary methods of measuring the mass-to-charge ratio of ionized molecules in the gas phase using MS. The first is targeted MS, which involves the use of standard peptides for absolute quantification. The second is non-targeted MS, which provides a semi-quantitative readout by measuring the intensity of peptide ions (Suhre et al., 2021). At present, the field of proteomics is witnessing a surge of innovative techniques that are revolutionizing the way we study biological systems. One of the most promising approaches that has gained significant attention in recent times is the integration of single-cell proteomics with single-cell RNA sequencing. Metabolomics shares some similarities with proteomics, but it concentrates on a broad range of small molecules present in the biological system, with an attempt to link these metabolites to biological pathways. In addition to MS-based techniques, nuclear magnetic resonance spectroscopy (NMR) is also employed in metabolomics to detect and measure small molecules (Martins-de-Souza, 2014). The field of microbiomics investigates the composition, functionality, and intricate interrelationships among microbial communities, as elaborated in previous sections.

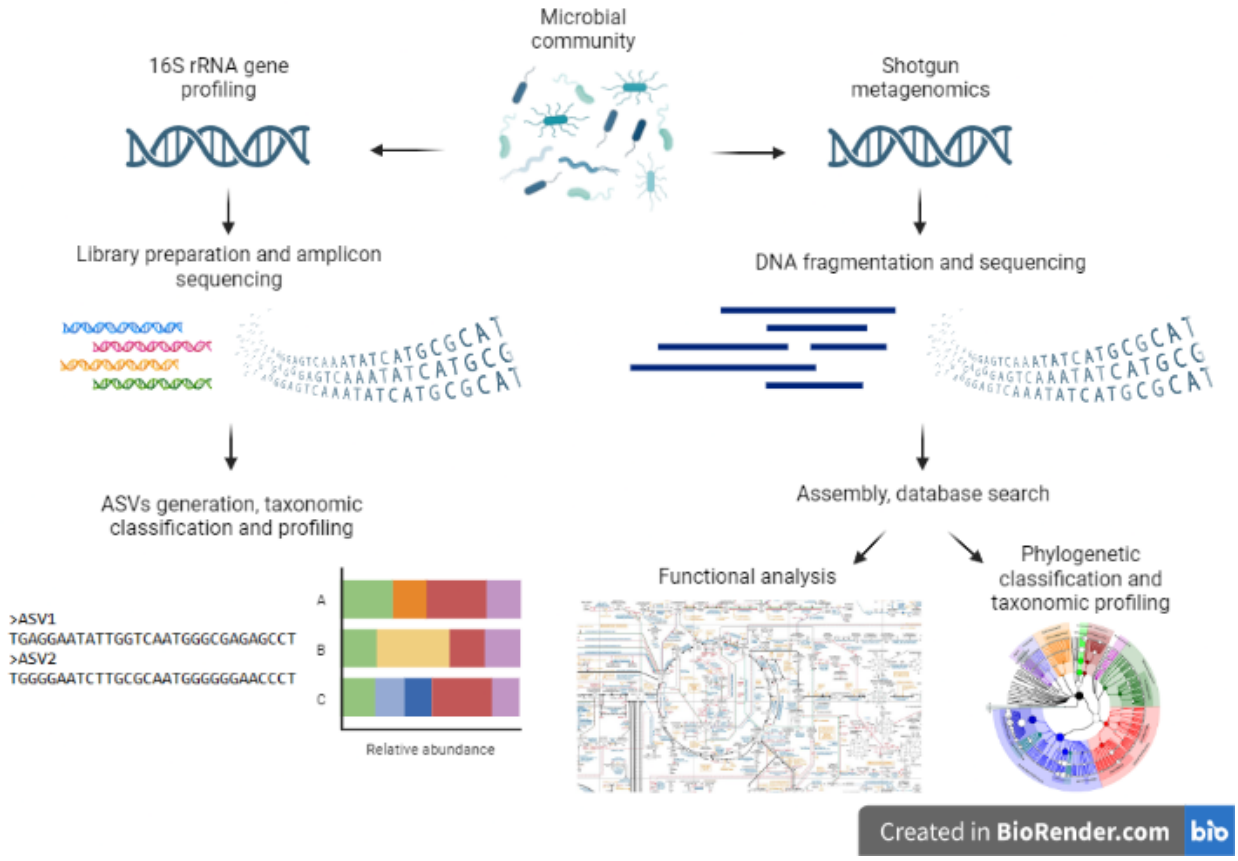
1.5.2 Methodology for microbiota determination

In the field of microbial research, there are currently two predominant approaches for the determination of the microbiota composition of a given sample and subsequent bioinformatic analysis. These methods are known as 16S ribosomal RNA (rRNA) gene amplicon sequencing and shotgun metagenomics. Despite the fact that both of these techniques rely on NGS technologies, they differ significantly in terms of their methodology, advantages, and disadvantages (Figure 7). In this chapter, the nuances of each of these approaches will be described, their unique features, strengths and limitations explored and the various factors that researchers must take into consideration when selecting the appropriate method for their specific research question highlighted.

The 16S rRNA gene is a fundamental component of all bacteria and Archaea and serves as a crucial tool in microbiological studies, facilitating a deeper understanding of the complex microbial communities

that exist in our world (Janda & Abbott, 2007) (Woese et al., 1990). The gene encodes for a transcriptional product called the 16S ribosomal RNA, which constitutes an essential part of the small subunit of the ribosome and is thus critical for protein synthesis (Woese & Fox, 1977). At a length of approximately 1550 base pairs, the 16S rRNA gene consists of both highly conserved regions and nine variable regions (Clarridge, 2004) (Neefs et al., 1993). It is the variability of these regions that enables researchers to differentiate between different bacterial taxa usually at the genius level by analyzing their unique sequences (Clarridge, 2004). In addition to the ability to distinguish between bacterial taxa, one of the major benefits of this method is its relatively low cost, making it accessible to a wide range of research laboratories. Moreover, 16S rRNA amplicon sequencing is highly sensitive and can detect bacterial populations at very low levels, which is especially useful for analyzing environmental samples with low microbial biomass. This has made the 16S rRNA gene a widely used target in microbial ecology and taxonomy studies. However, this approach has also its limitations. The variable regions within the 16S rRNA gene are inadequate for classifying all microbes at the species level, and it is not possible to distinguish strains using this method (Clarridge, 2004). Furthermore, this method does not provide information about microbial functionality, so it is recommended to combine it with other omics data types such as metabolomics.

Figure 7. Simplified pipeline schemes for microbial 16S rRNA gene profiling and shotgun metagenomics. Created by using https://www.biorender.com/.



One of the most commonly used methods in microbial profiling involves 16S rRNA gene amplicon sequencing. This process starts by extracting DNA from a sample and using it to create a DNA library. A desired section of the 16S rRNA gene is then amplified through polymerase chain reaction (PCR), with PCR primers designed to include sequencing adapters and barcodes (Celis et al., 2022). The adapters enable the DNA to be bound to a sequencing machine's flow cell, while barcodes identify

each sample's DNA sequences, which is important when analyzing large sets of samples. The amplified DNA is cleaned, size-checked, and pooled before quantification and sequencing, where each sequencing run results in thousands of "reads" per sample indicating how many times each DNA amplicon was sequenced (Slatko et al., 2018). The number of reads of each DNA fragment is proportional to its relative abundance in the sequenced mixture, which allows quantitative analysis.

There are two main approaches commonly used for bioinformatical analysis but there is no universal consensus about their usage. Both of them aim to assign taxonomy correctly based on amplicon sequences and to obtain relative abundances based on read counts.

The older method is founded on the concept that similar DNA sequences indicate the same species or genera. This method involves clustering the sequences together to form Operational Taxonomic Units (OTUs) based on a particular similarity threshold, most commonly at 97% (Caporaso et al., 2011) (Chiarello et al., 2022). However, this similarity cut-off creates an external bias that influences the entire analysis. Furthermore, the clustering process alone cannot be replicated, and the choice of clusters is subjective, resulting in difficulties when comparing results from different laboratories or researchers. Therefore, this approach is being gradually replaced by more advanced methods based on denoising sequences to create exact sequence variants, such as Amplicon Sequence Variants (ASVs) (Callahan et al., 2017). Denoising techniques aim to identify and correct sequencing errors by generating an error model for each sequencing run. This method helps in obtaining high-quality and biologically relevant sequences, which can be compared between different laboratories and studies. In addition to its advantage of providing comparable results, denoising techniques are more sensitive in detecting bacterial taxa, as highlighted by Caruso et. al and others (Caruso et al., 2019) (Prodan et al., 2020) (Xue et al., 2018).

Shotgun metagenomic sequencing represents an alternative method to cataloging the human gut microbiota compared to the 16S rRNA gene microbial profiling mentioned above. In this method, instead of targeting a specific gene, all the DNA present in the sample is extracted and fragmented into smaller pieces. Then, adapters with tags are usually added to these fragments to enable PCR amplification and sequencing (Quince et al., 2017). After size-checking, cleaning and pooling the PCR products, the resulting reads are processed using bioinformatic tools to explore both taxonomic and functional aspects of the microbiota. One advantage of shotgun metagenomics is that it generates much more sequencing data than 16S rRNA profiling, allowing for a more comprehensive and detailed analysis of the microbial community. The bioinformatics analysis required for shotgun metagenomics is, therefore, more complex and challenging, as it involves either assembling the reads into whole or partial genomes of the organisms present in the sample or aligning the reads to reference databases to identify functional genes (Quince et al., 2017). By using shotgun metagenomics, researchers can obtain a more complete picture of the microbiota's composition and functional potential. This method enables not only the identification of microbial species and strains but also the characterization of their metabolic potential determined by the coding enzymes. However, it should be noted that shotgun metagenomics is more expensive and time-consuming than 16S rRNA profiling, and requires more computational resources and expertise.

In summary, while 16S rRNA profiling and shotgun metagenomics are both valuable tools for studying the human gut microbiota, each has its own strengths and limitations. Shotgun metagenomics provides a more comprehensive and detailed view of the microbiota, but at a higher cost and with more computational challenges.

1.5.3 Integration of omics data types

In the age of big multi-omics data, with technology constantly evolving and datasets growing in size and diversity, it is of utmost importance to develop a strong bioinformatics framework that can effectively support the potential advantages of personalized medicine by combining data sets of each individual. To address this challenge, various computational approaches have been developed to integrate and analyze omics data, the state-of-the-art methods being various machine learning algorithms and dimension reduction methods (Tebani et al., 2016). Rather than analyzing individual types of biological data in isolation, integrating multiple data types can provide a more comprehensive and nuanced understanding of complex biological processes (Olivier et al., 2019). While this approach does increase the complexity of the bioinformatics analysis required, it has the potential to reveal previously unseen relationships and interactions between different biological components. In essence, by combining diverse data sources, a more complete picture of the underlying biological mechanisms can be achieved.

At the outset of multi-omics studies, correlation and network analyses are often used as basic methods to obtain a broad overview of the data. These methods aim to identify all possible relationships between variables from different omics datasets. Such variables can exhibit similar patterns or may be in opposition to each other, which can reveal how they interact across multiple omics layers and even help to explain certain biological phenomena or formulate hypotheses. Although these techniques are relatively simple, they can provide important insights into the complex relationships between different omics data types (Hasin et al., 2017). However, the limitations of this approach become evident when dealing with complete multi-omics studies, which can involve measuring hundreds of thousands of variables, resulting in significant computational memory requirements (Olivier et al., 2019).

Visual representations of correlations, such as correlation networks, can be a valuable tool in data analysis (Figure 8). These networks offer a way to map relationships between variables from multiple data sets and can reveal hidden patterns in the flow of information (Jiang et al., 2019). In a correlation network, nodes typically represent various variables, such as bacterial taxa, genes, metabolites, dietary features, and other metrics, while edges between the nodes indicate the presence of an interaction, often weighted by the strength of that interaction (Jiang et al., 2019). Positive and negative interactions are usually distinguished by color, making it easier to identify complex relationships beyond just numerical values. When analyzing a network, one approach is to count the degree of the nodes, which refers to the number of edges connected to each node. Nodes with a higher degree are likely to have a greater influence on the system being depicted (e.g., a biological process or metabolic pathway) as they are more connected to other nodes (variables) in the network (Jiang et al., 2019). Heatmap correlograms with specifically designed annotations can be also a useful graphical tool for analyzing high-dimensional data (Figure 9). These correlograms display a heatmap of the correlation matrix between variables, with annotations added to highlight possible interactions and patterns. These annotations can provide additional insights into the relationships between variables, such as identifying clusters of highly correlated variables or patterns of positive and negative correlations (Gu et al., 2016). This approach can help to identify underlying structures and relationships within complex data sets, making it easier to interpret and draw meaningful conclusions.

Figure 8. An example of a correlation network based on clinical parameters.

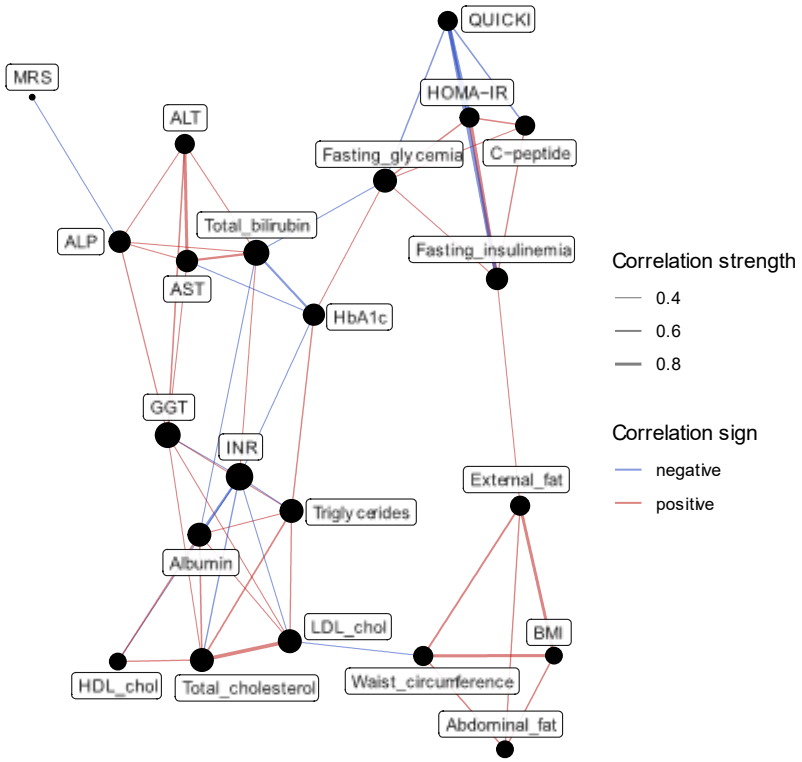
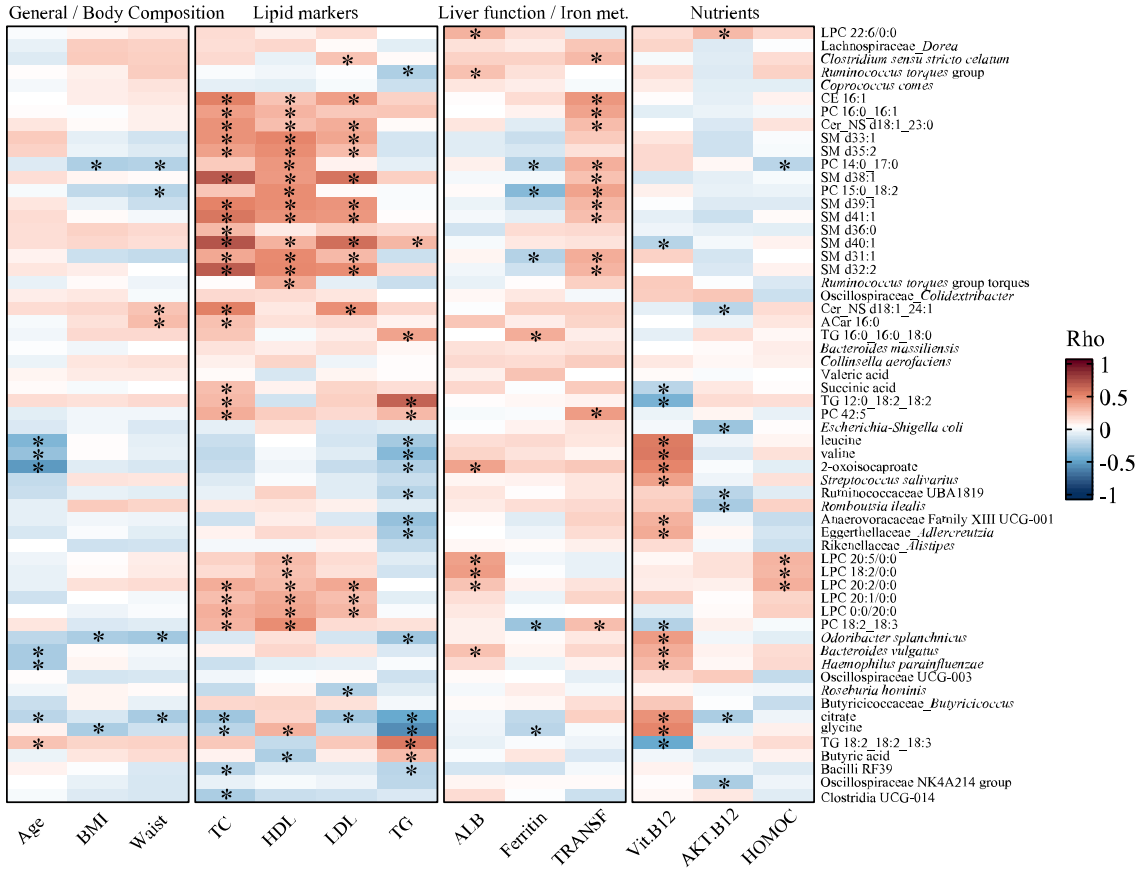
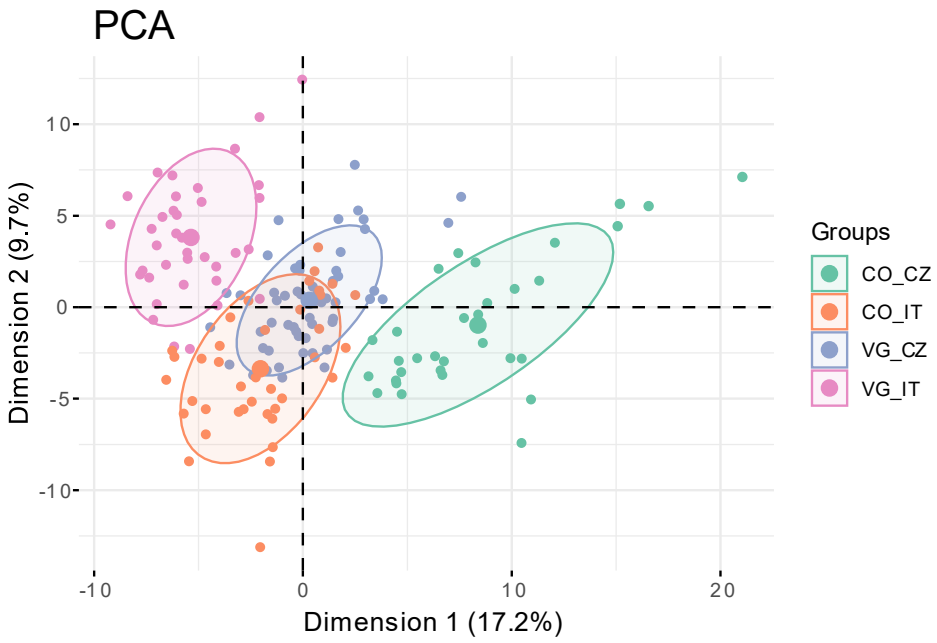


Figure 9. An example of annotated heatmap correlogram combining several omics data types and some body and clinical parameters.



It is important to bear in mind that correlations are also susceptible to false positives, and as a result, multiple testing corrections must be carried out. To mitigate this challenge, several clustering or dimension reduction methods have been developed to complement correlation-based analyses (Chong & Xia, 2017). These techniques can help to simplify the analysis and reduce the computational burden by grouping or reducing the number of variables while still retaining important information about the relationships between different omics data types. These methods are widely used in multi-omics data analysis and are typically based on commonly used dimension-reduction techniques, such as principal component analysis (PCA) (Figure 10). PCA is an unsupervised method for reducing the dimensionality of a data set (Chong & Xia, 2017). It involves creating new variables, called principal components, which are linear combinations of the original variables (Park et al., 2020). The principal components are designed to capture the maximum amount of variance present within the data set (Meng et al., 2016). While PCA is primarily used for dimensionality reduction, another popular multivariate technique, Canonical Correlation Analysis (CCA) is used to analyze the relationship between two sets of variables (Rodosthenous et al., 2020). PCA aims to capture the maximum variance in a single dataset, and CCA seeks to find patterns of covariation between two different datasets.

Figure 10.An example of PCA visualization.



As computational power continues to increase and artificial intelligence technologies are being successfully implemented in various fields, machine learning (ML) is gaining popularity in health sciences (Reel et al., 2021). However, one of the challenges in analyzing multi-omics data is the problem of multidimensionality, which refers to the presence of more variables than observations in the data matrix. Furthermore, this data matrix often contains many correlated features, which can mislead results during algorithm training (Reel et al., 2021). Therefore, special care must be taken in selecting appropriate ML techniques and preprocessing the data to address these challenges.

Regularization (penalization) approaches such as elastic net, Least Absolute Shrinkage and Selection Operator (LASSO) and Ridge regression are currently popular methods for feature selection, dimension reduction and/or classification in multi-omics studies (C. Wu et al., 2019). Regularization methods are able to handle the problem of multidimensionality by introducing penalty terms to the model equation and shrinking the coefficients of the features resulting in a more predictive model with a lower risk

of overfitting (Tibshirani, 1996). In the context of omics studies, feature selection aims to remove noisy and redundant features, while retaining only those that contain the most relevant information, thereby reducing the total number of features and overall dimensionality of the data (Picard et al., 2021). This is important because high-dimensional data can result in the overfitting of machine learning models, leading to poor performance on new, unseen data (Picard et al., 2021). Therefore, selecting the most informative features is crucial for the accurate prediction and interpretation of multi-omics data.

Deep learning methods, also known as deep neural methods, are a group of powerful algorithms that are gaining popularity in analyzing big data. These methods fall under the umbrella of machine learning and are capable of discovering hidden patterns in complex datasets without relying on mathematical formulas (Kang et al., 2022). One of the main advantages of deep learning algorithms is that they are self-teaching and can learn from the data without human intervention. However, one disadvantage is that they require a large training dataset with many observations to learn across multiple layers; otherwise, they are prone to overfitting (Kang et al., 2022). Additionally, deep learning algorithms are often considered a black box since their operations are not easily interpretable. Despite these limitations, deep learning methods have enormous potential in personalized medicine, such as detecting early stages of disease or classifying disease types, predicting drug responses, and identifying disease biomarkers.

In conclusion, integrating and analyzing multi-omics data is a complex and challenging task, but it has the potential to provide a more complete understanding of biological systems and the development of personalized medicine. Correlation and network analyses, as well as dimension reduction techniques, are commonly used to address this challenge. Correlation networks offer a way to map relationships between variables from multiple data sets, while dimension reduction techniques such as PCA and CCA can help to simplify the analysis and reduce the computational burden. Machine learning techniques are also gaining popularity in multi-omics data analysis, but appropriate algorithms and preprocessing of data must be used to handle the challenges of multidimensionality and correlated features. Overall, by combining diverse data sources, a more comprehensive and nuanced understanding of complex biological processes can be achieved, leading to the development of precision medicine and improved patient outcomes.

2 AIMS AND HYPOTHESES

The current state of knowledge points to a causal relationship between the composition of the gut microbiota and the development of many diseases of apparently different origin. Targeting the gut microbiota, either in composition or functional manifestations, could represent an effective therapeutic strategy. However, the wide implementation of this approach in therapeutic practice is still limited by the lack of knowledge about the behavior of such a complex system like the gut microbiome and its interaction with external stimuli and the host organism. The goal of this thesis is to enrich the knowledge in this area from several perspectives described below.

AIM 1:

To describe the microbiome and metabolome signature associated with a vegan diet.

Hypothesis

Long-term adherence to a vegan diet is associated with less incidence of NCDs like obesity, T2D or cardiovascular disease. We hypothesize that at least some of the health benefits of a vegan diet could be explained by the composition and/or activity of gut microbiota.

AIM 2:

To develop an alternative tool for the estimation of specific function(s) of gut microbiota.

Hypothesis

Real-time quantitative PCR (qPCR) based method may serve as an alternative tool for the quantification of the specific gene across the whole bacterial population in the tested sample and therefore provide an insight into the functional capacity of the microbiota.

AIM 3:

To explore the possibilities of the manipulation of the gut microbiota by the dietary fiber inulin in the personalized treatment of T2D.

Hypothesis

The amount of fiber in the diet is one of the strongest environmental factors shaping the composition of gut microbiota but the results of clinical trials evaluating the effects of dietary fiber intervention in NCDs treatment are highly individually variable. We hypothesized that the outcome of the fiber intervention depends on the ability of the individual´s microbiota to process it and the potential beneficiaries of this treatment could be predicted based on the initial microbiome and metabolome characteristics.

AIM 4:

To assess the protective effect of the vegan microbiota against the influence of the obesogenic diet.

Hypothesis

We hypothesized that vegan microbiota may be protective against the effects of a western-type obesogenic diet and that its effect could be potentiated by the addition of dietary fiber inulin into the diet.

3 RESULTS AND COMMENTARY

3.1 Description of the microbiome and metabolome signature associated with a vegan diet

As highlighted in the introduction, diet is a crucial factor that shapes gut microbiota. On the other hand, the gut microbial community is known for its resilience. Plant-based diets belong to nutritional trends gaining increasing attention both among the general population and nutrition specialists. These diets differ significantly from traditional omnivorous diets in many aspects. Due to their high content of microbiota-accessible carbohydrates, plant-based diets may lead to a shift in the composition of the gut microbiota towards that seen in traditional societies. Therefore, we performed a cross-sectional study comparing healthy vegans' and omnivores' microbiome and metabolome profiles and explored how the microbial composition or functional potential of gut microbiota differs between the groups with contrasting dietary habits.

This study compared the subjects of lean and healthy vegans (VG, n = 62) and omnivores (OM, n = 33). It involved collecting dietary records and measuring the macronutrient composition and fiber content. Stool samples were obtained from the participants for the untargeted metabolomic analysis (gas chromatography-mass spectrometry, GC-MS), bile acid spectrum determination, and microbial 16S rRNA sequencing. The plasma was also analyzed for SCFAs concentrations and untargeted metabolomics using liquid chromatography-mass spectrometry (LC-MS) and NMR, respectively. Glucose and lipid homeostasis parameters were assessed as well. The level of systemic inflammation was estimated according to the serum concentration of C-reactive protein (CRP). These additional analyses provide a more comprehensive understanding of the metabolic effects of the diets being compared in the study.

The results of the 3-day prospective dietary records showed that omnivores had a higher daily intake of protein and lipids, while vegans had a higher intake of carbohydrates and dietary fiber. Compared with omnivores, vegans exhibited more favorable glucose homeostasis parameters, as evidenced by a lower concentration of glycated hemoglobin and lower secretion of insulin during the oral glucose tolerance test (OGTT). Additionally, vegans had lower serum concentrations of total and low-density lipoprotein (LDL) cholesterol. Median serum CRP concentration was lower in vegans, although the values remained within the physiological range in both groups. These results suggest that a plant-based diet may offer benefits for glucose and lipid metabolism, as well as inflammation, compared to an omnivorous diet.

In terms of the microbial composition, permutational analysis of variance (PERMANOVA) tests revealed significant differences in β-diversity between vegans and omnivores at the order, family, and genus levels. However, the differences were relatively small, with only 15% of all bacteria being affected by diet at the genus level as determined by univariable analysis. When it comes to fecal metabolomics, we identified 146 different volatile organic compounds determined by GC-MC. We found that the vegan fecal metabolome was enriched in products of polysaccharide fermentation, such as SCFAs, while amino acid fermentation products were lower in the VG group. On the other hand, amino acid fermentation products such as indole, scatole, and p-cresol were higher in the OM group. VG and OM groups did not differ in primary bile acid spectrum composition, but vegans had significantly lower fecal content of one secondary bile acid, LCA, in feces compared with omnivores.

In the urine metabolome, we found higher concentrations of metabolites related to protein/amino acid metabolism in the OM group. The most significant changes between groups were observed in serum metabolomics, where we found a clear separation between the vegan and omnivore groups. The vegan serum metabolome was characterized by a higher content of SCFAs, dimethyl sulfone, and amino acids such as glycine, glutamine, asparagine, proline, and threonine, while the concentrations of branched-chain amino acids, their derivatives, and essential amino acid lysine were lower in the VG group.

These findings suggest that the differences in the diets of vegans and omnivores have a significant impact on their metabolome profiles, particularly in serum metabolome. The vegan diet was associated with a higher occurrence of potentially beneficial metabolites from dietary fiber fermentation products and a lower abundance of potentially harmful metabolites from amino acid fermentation products.

This article was published on the 7th of January 2022 in Frontiers in Nutrition, IF = 6.59

frontiers in Nutrition

ORIGINAL RESEARCH
published: 07 January 2022
doi: 10.3389/fnut.2021.783302

Vegan Diet Is Associated With Favorable Effects on the Metabolic Performance of Intestinal Microbiota: A Cross-Sectional Multi-Omics Study

Magdalena Prochazkova[1], Eva Budinska[2], Marek Kuzma[3], Helena Pelantova[3], Jaromir Hradecky[4], Marie Heczkova[5], Nikola Daskova[5,6], Miriam Bratova[5], Istvan Modos[5], Petra Videnska[2], Petra Splichalova[2], Solomon A. Sowah[7,8], Maria Kralova[9], Marina Henikova[1], Eliska Selinger[1], Krystof Klima[10], Karel Chalupsky[10], Radislav Sedlacek[10], Rikard Landberg[11], Tilman Kühn[12,13†], Jan Gojda[1†] and Monika Cahova[5†]*

[1] Department of Internal Medicine, Kralovske Vinohrady University Hospital and Third Faculty of Medicine, Charles University, Prague, Czechia, [2] Research Centre for Toxic Compounds in the Environment, Faculty of Science, Masaryk University, Brno, Czechia, [3] Laboratory of Molecular Structure Characterization, Institute of Microbiology, Czech Academy of Sciences, Prague, Czechia, [4] Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Prague, Czechia, [5] Center of Experimental Medicine, Institute for Clinical and Experimental Medicine, Prague, Czechia, [6] First Faculty of Medicine, Charles University, Prague, Czechia, [7] Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Heidelberg, Germany, [8] Medical Faculty and University Hospital, Heidelberg University, Heidelberg, Germany, [9] Department of Applied Mathematics and Computer Science, Masaryk University, Brno, Czechia, [10] Czech Centre for Phenogenomics, Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czechia, [11] Division of Food and Nutrition Science, Department of Biology and Biological Engineering, Chalmers University of Technology, Goteborg, Sweden, [12] Institute of Global Food Security, Queen's University Belfast, Belfast, United Kingdom, [13] Heidelberg Institute of Global Health (HIGH), Medical Faculty and University Hospital, Heidelberg University, Heidelberg, Germany

OPEN ACCESS

Edited by:
Mirjana Rajilić-Stojanović, University of Belgrade, Serbia

Reviewed by:
Manja Zec, University of Arizona, United States
Olga Sakwinska, Nestlé Research Center, Switzerland

***Correspondence:**
Monika Cahova
monika.cahova@ikem.cz

[†] These authors have contributed equally to this work and share last authorship

Specialty section:
This article was submitted to Nutrition and Microbes, a section of the journal Frontiers in Nutrition

Received: 25 September 2021
Accepted: 25 November 2021
Published: 07 January 2022

Citation:
Prochazkova M, Budinska E, Kuzma M, Pelantova H, Hradecky J, Heczkova M, Daskova N, Bratova M, Modos I, Videnska P, Splichalova P, Sowah SA, Kralova M, Henikova M, Selinger E, Klima K, Chalupsky K, Sedlacek R, Landberg R, Kühn T, Gojda J and Cahova M (2022) Vegan Diet Is Associated With Favorable Effects on the Metabolic Performance of Intestinal Microbiota: A Cross-Sectional Multi-Omics Study. Front. Nutr. 8:783302. doi: 10.3389/fnut.2021.783302

Background and Aim: Plant-based diets are associated with potential health benefits, but the contribution of gut microbiota remains to be clarified. We aimed to identify differences in key features of microbiome composition and function with relevance to metabolic health in individuals adhering to a vegan vs. omnivore diet.

Methods: This cross-sectional study involved lean, healthy vegans ($n = 62$) and omnivore ($n = 33$) subjects. We assessed their glucose and lipid metabolism and employed an integrated multi-omics approach (16S rRNA sequencing, metabolomics profiling) to compare dietary intake, metabolic health, gut microbiome, and fecal, serum, and urine metabolomes.

Results: The vegans had more favorable glucose and lipid homeostasis profiles than the omnivores. Long-term reported adherence to a vegan diet affected only 14.8% of all detected bacterial genera in fecal microbiome. However, significant differences in vegan and omnivore metabolomes were observed. In feces, 43.3% of all identified metabolites were significantly different between the vegans and omnivores, such as amino acid fermentation products p-cresol, scatole, indole, methional (lower in the vegans), and polysaccharide fermentation product short- and medium-chain fatty acids (SCFAs, MCFAs), and their derivatives (higher in the vegans). Vegan serum metabolome differed markedly from the omnivores (55.8% of all metabolites), especially in amino acid composition, such as low BCAAs, high SCFAs (formic-, acetic-, propionic-, butyric acids),

and dimethylsulfone, the latter two being potential host microbiome co-metabolites. Using a machine-learning approach, we tested the discriminative power of each dataset. Best results were obtained for serum metabolome (accuracy rate 91.6%).

Conclusion: While only small differences in the gut microbiota were found between the groups, their metabolic activity differed substantially. In particular, we observed a significantly different abundance of fermentation products associated with protein and carbohydrate intakes in the vegans. Vegans had significantly lower abundances of potentially harmful (such as p-cresol, lithocholic acid, BCAAs, aromatic compounds, etc.) and higher occurrence of potentially beneficial metabolites (SCFAs and their derivatives).

Keywords: vegan diet, omics signature, protein fermentation, short-chain fatty acids (SCFAs), metabolic health

INTRODUCTION

Recent studies suggest that the composition and function of the gut microbiome play a fundamental role in the development of non-communicable diseases (1). Diet is a key determinant of the relationship between humans and their microbial residents, as it affects the composition of gut microbial ecosystem, which, in turn, impacts on human physiology *via* direct interaction with the immune system and metabolic outputs (2). Adherence to plant-based diets (vegetarian or vegan) was shown to be associated with potential health benefits (3). Epidemiological studies show a lower incidence of several chronic diseases, such as type 2 diabetes (T2D), cardiovascular diseases, and cancer (4–7). When compared to lacto-ovo-vegetarian diets, vegan diets may lower the risk of obesity, hypertension, T2D, and cardiovascular mortality. Moreover, with respect to certain cancers, a strict vegan diet may be more beneficial than a lacto-ovo-vegetarian one, although further studies are needed (8, 9). Intervention studies comparing vegan or vegetarian diets vs. omnivorous diets have shown beneficial effects of these diets on cardiometabolic risk factors (10), T2D (11), and obesity (12).

Whether the beneficial effects of plant-based diets can be attributed to their nutritional composition alone or whether they are mediated, at least partly, by different microbiota and their metabolites remains to be clarified. There is no clear consensus concerning the effect of a profound dietary switch to a strict plant-based diet on gut microbiota, microbial fermentation products, and their impact on host metabolism. This issue gains in importance with the increasing interest in microbiota manipulation in the therapy of noncommunicable diseases, and with the simultaneous trend for plant-based diets (13).

Given the association among diet, gut microbiome/metabolome (MIME), and metabolic health, we hypothesized that there are specific compositional and functional characteristics of MIME that link different eating habits to metabolic phenotype. To this end, we explored two metabolically healthy groups defined by distinct dietary habits, i.e., lean healthy long-term vegans (i.e., those who have been adhering to a vegan diet for at least 3 years) and lean healthy omnivores. Our aim was to analyze the fecal microbiome as well as serum, urine, and fecal metabolomes of these groups in order to identify key features associated with the different diets and provide potential functional links among them.

MATERIALS AND METHODS

Study Population

Sixty-two self-reported vegans (VGs) and 33 omnivores (Os) were screened and enrolled between October 2018 and October 2019 for cross-sectional comparison. The VGs strictly avoided all animal products for at least 3 years, and the omnivore group comprised subjects without any dietary restrictions who consumed meat and other animal products on a daily basis. In both groups, the exclusion criteria were age under 18 years, obesity defined as BMI $>$ 30, chronic diseases related to metabolism, diseases of the digestive tract, antibiotic therapy in the past 3 months, pregnancy, any chronic medication (excluding hormonal contraception), and regular alcohol consumption defined as any alcoholic drink on a daily basis. A clinical visit was scheduled after enrollment. After 12-h overnight fast, blood and urine were sampled, and clinical examination was performed. Afterward, oral glucose tolerance test (OGTT, 75g glucose) was performed with blood sampling at 0, 30, 60, 90, and 120 min. All blood samples were immediately centrifuged and snap frozen at -80°C before analyses. Glucose homeostasis indices were derived from serum glucose and insulin changes in OGTT: AUCs for glucose and insulin using trapezoid rule, Matsuda index of insulin sensitivity as described elsewhere (14).

Study Approval

All the participants signed informed consent prior to enrollment. The research protocol was approved by the Ethics Committee of the Third Faculty of Medicine of the Charles University and the Ethics Committee of University Hospital Kralovske Vinohrady (EK-VP/26/0/2017) in accordance with the Declaration of Helsinki.

Dietary Intake Assessment

Dietary records and stool samples were obtained no longer than a week after the clinical visit. A 3-day prospective record supervised by a trained dietitian was used to assess the macronutrient composition and fiber content of the diet.

Each participant filled in a prospective record, where dietary data from 3 typical days were collected (2 working days, 1 weekend day). The volunteers were educated. Instructions were given for portion size estimation and recording of foods in sufficient detail to obtain an accurate estimate of consumed portions, and a portion estimation guide was given as a reference. Moreover, examples of complete and incomplete diaries were explained to show how to appropriately record the intake. After collection, the records were retrospectively checked by an independent researcher. The USDA database was used for assessment of food composition, NutriServis PROFI, and CR, and a program was used for dietary intake calculations. Daily intake of carbohydrates, lipids, proteins, and dietary fiber was calculated separately.

Fecal Sample Collection, Storage, and Processing

Fecal samples collected at home had been immediately stored at −20°C until transported in the frozen state to the laboratory within 7 days of collection. Once thawed on ice, the samples were homogenized using stomacher (BioPro, Czechia); one aliquot was used for DNA extraction, one aliquot was used for dry mass estimation, and the rest was aliquoted and stored at −50°C. For metabolome analyses, the aliquots were thawed and diluted with sterile water to 1% dry mass equivalent. For bile acid composition analyses, the samples were lyophilized.

Gut Microbiome Analysis

DNA from the fecal samples was isolated with QIAmp PowerFecal DNA Kit (Qiagen, Germany), and the V4 region of the bacterial 16S rRNA gene was amplified by PCR. A library was prepared according to the Illumina 16S Metagenomic sequencing Library Preparation protocol with some deviations described below. Each PCR was performed with an EMP primer pair consisting of Illumina overhang nucleotide sequences, an inner tag, and gene-specific sequences. The sequences of EMP primers, overhang, and tag sequences are shown in **Supplementary Table 1**. The Illumina overhang served to ligate the Illumina index and adapter. Each inner tag, i.e., a unique sequence of 7–9 bp, was designed to differentiate the samples into groups. The total reaction volume of PCR was 30 μl, and cycling parameters included initial denaturation at 98°C for 30 s, followed by 30 cycles of 10 s denaturation at 98°C, 15 s annealing at 55°C and 30-s extension at 72°C, followed by final extension at 72°C for 2 min. Samples with different inner tags were equimolarly pooled, and pools were used as a template for second PCR with Nextera XT (Illumina, United States) indexes. Differently indexed samples were equimolarly pooled. The final library was diluted to a concentration of 8 pM, and 20% of PhiX DNA (Illumina, United States) was added. Sequencing was performed with the Miseq reagent kit V2 using a MiSeq instrument according to the manufacturer's instructions (Illumina, Hayward, CA, United States). Raw sequences were processed using an in-house pipeline based on a DADA2 amplicon denoiser (15), and a standard bioinformatic procedures within the QIIME 1.9.1 package (16).

Availability of Materials

Sequencing data are available in the European Nucleotide Archive database under the accession number PRJEB43938. Publication of our dietary data, as well metabolomics data, was not possible, as it was not covered by the participants' informed consent used for the study. However, pseudonomized data will be made available by the corresponding authors upon reasonable request.

Determination of *But* Gene Expression

The abundance of butyryl-CoA:acetate CoA-transferase (*but*) gene in the DNA isolated from stool samples was determined, as described in Daskova et al. (17) Briefly, bacteria containing the *but* gene in their genome were identified using FunGene Database. The selection was narrowed only to bacteria already found in human gut microbiota. The sequence of *but* gene coding for butyryl-CoA:acetate CoA-transferase is highly variable among gut butyrate producers;, therefore, degenerate primers targeting different variants of the *but* gene were designed. Even when degenerate primers were used, six different primer pairs had to be designed in order to cover all *but* gene sequences (17) (**Supplementary Table 2**). The copy number of *but* gene in the DNA isolated from the stool samples was determined by quantitative PCR (qPCR) and normalized to spike DNA (*C. elegans* UNC-6 gene; forward primer GAAGAGCAAGATCAGTGTTC, reverse primer CTTGCAAATGACACCTTG).

Short-Chain Fatty Acid in Plasma

Short-chained fatty acids (SCFAs) were analyzed in the plasma by LC-MS according to a method described before (18). Standards for SCFAs used were: formic acid (C1) (Scharlau, Spain), acetic acid (C2) (Honeywell, United States), propionic acid (C3) (Alfa Aesar, United States), butyric acid (C4) (Sigma Aldrich, United States), isobutyric acid (C4) (Alfa Aesar, United States), succinic acid (C4) (Acros, United States), isovaleric acid (C5) (Sigma Aldrich, United States), valeric acid (C5) (Alfa Aesar, United States), and caproic acid (C6) (Sigma Aldrich, United States). Analytical reagent-grade 3-nitrophenylhydrazine (3NPH)-HCl (97%), 2-nitrophenylhydrazine N-(3-dimethylaminopropyl)-N0-ethylcarbodiimide (EDC) HCl, quinic acid, HPLC-grade pyridine, and Lichrosol reagent-grade MeOH and water were obtained from Sigma–Aldrich. Acetonitrile Optima LCMS Grade was obtained from Thermo Fisher Scientific (United States). ^{13}C6-3NPH-HCl was custom synthesized to us by IsoSciences Inc. (King of Prussia, PA, United States) (catalouge 13309). This custom-synthesized compound was structurally confirmed by 1H NMR spectroscopy and by MS/MS on a triple-quadruple mass spectrometer.

Volatile Compound (VOC) Analysis on Feces

Volatile fingerprinting of the fecal samples was performed using an Agilent 7890B (Agilent Technologies, United States) gas chromatograph coupled to a Pegasus 4D (LECO, United States) time of flight mass spectrometer. Volatiles were collected

using a solid-phase microextraction (SPME) fiber with divinylbenzene/carboxen/polydimethylsiloxane coating from Supelco (United States). Data acquisition and initial data processing were carried out using instrumental SW ChtomaTOF by LECO (United States).

NMR Analyses

Analyses were performed on fecal extracts prepared from homogenized stool aliquot corresponding to 1% of dry mass. All the samples were measured on a 600 MHz Bruker Avance III (Bruker BioSpin, Rheinstetten, Germany) spectrometer equipped with a 5-mm TCI cryogenic probe head. 1D-NOESY, CPMG, and *J*-resolved experiments were performed using standard manufacturers' software Topspin 3.5. Concentrations of individual metabolites, identified by comparison of proton and carbon chemical shift with HMDB database, were expressed as PQN-normalized intensities of corresponding signals in 1D-NOESY (urine), CPMG (serum extracts), and 1D projections of *J*-resolved (fecal extracts) spectra. The list of quantified metabolites in the urine, serum, and fecal extracts with corresponding ^{1}H and ^{13}C chemical shifts is given in **Supplementary Table 3**. The representative ^{1}H NMR spectra are shown in **Supplementary Figures 1–3**.

Bile Acid Analysis on Feces

Methanol extract was prepared from lyophilized fecal homogenate (1 ml of 1% homogenate). Liquid chromatography separation of the extracts was performed using 1290 Infinity LC (Agilent Technologies, United States) followed by mass spectrometry using 6550 iFunnel LCQ- TOF-MS (Agilent Technologies, United States) equipped with a Dual AJS ESI probe in negative-ion mode. System control and data acquisition were performed with Agilent MassHunter Quadrupole Time of Flight Acquisition Software (B.06) with Qualitative Analysis (B.07 SP2) Software.

Statistics

Statistical analyses were performed in R software packages and in-house scripts (19). For individual tasks, the following R packages were used: composition (clr transformation), zCompositions (zero multiplicative replacement) vegan (PERMANOVA), ropls (PLS-DA metrics), mixOmics (VIP identification, 2D score plot PLS-DA), effsize (Cliffs delta), and caret (machine learning library). Clinical characteristics of the observational sample were compared by standard tests. The microbiome and VOCs data were treated as compositional (proportions of total read count in each sample, non-rarefied or proportion of total area under curve), and prior to all the statistical analyses were transformed by centered log-ratio (clr) transformation, and zero values were handled using count using R package zCompositions. According to their abundance and prevalence, the bacteria were classified as "core microbial taxa" when they fulfilled the following conditions, i.e., abundance $>$ 0.1% and prevalence $>$ 75% at least in one experimental group. Other microbial taxa were classified as rare. NMR data were normalized by probabilistic quotient normalization (PQN). All the data were scaled (z-score) before applying PERMANOVA, PCA, PLS-DA, or random forest method. The genera were filtered by minimal prevalence, i.e., present at least in three samples per cohort, with minimum of nine reads each. Principal component analysis (PCA) was performed to investigate possible sample clustering in each dataset. For each data type, multivariable statistics (PERMANOVA) were applied to test the differences between the groups; for gut microbiome, PERMANOVA was performed on each of the five taxonomy levels (phylum, class, order, family, and genus) separately. Univariable statistical analyses were performed by Mann-Whitney-Wilcoxon test. The results were adjusted for multiple-hypothesis testing by Benjamini-Hochberg procedure with a cut-off level of false discovery rate equal to 0.1. A multivariable statistic evaluation was performed by partial least square discriminant analysis (PLS-DA). We analyzed the discriminating power of each omics dataset using machine learning; specifically, we used a random forest method. The validity of a model was verified by permutation test with 300 repetitions. Correlation networks based on Spearman's correlation coefficient were used to assess the correlation between the studied variables.

All methods are described in detail in Supplemental Experimental Procedures.

RESULTS

Subject Characteristics

Clinical characteristics of the study participants are given in **Table 1**. Dietary consumption quantified using 3-day prospective dietary records showed no significant difference in total energy intake between the groups. In the omnivores, higher daily intake of protein and lipids was recorded, while the vegans' diet consisted of more carbohydrates and dietary fiber. We observed more favorable indices of glucose homeostasis, i.e., lower concentration of glycated hemoglobin and lower secretion of insulin during OGTT in the vegans. Regarding lipid metabolism, the vegans had lower plasma levels of total as well as LDL- and HDL-cholesterol. Serum CRP, which serves as an inflammatory marker, was significantly lower in the vegans, albeit in both groups it remained within physiological range. Fecal pH was significantly lower in the vegans, whose stool samples contained more water.

Fecal Microbiome Composition

Ninety fecal samples were available for microbiome analysis (57 from Os; 33 from VGs). In all the 90 samples, we identified 62,683 amplicon sequence variants (ASVs). Median sequencing coverage was 22,957 ASVs per sample (min 7,385; max 38,528). We detected 10 phyla, 19 classes, 24 orders, 44 families, and 144 genera; 55 of the genera belong to the core microbiome. For the purpose of this study, the core microbiome was defined as taxa (genus level) that meet the following criteria: median abundance of 0.1% and

TABLE 1 | Group characteristics for vegans and omnivores.

	Omnivore	Vegan	*p*-value
General characteristics			
Sex [F/M]	17/16	25/37	
Weight [kg]	73.0 (24.4)	67.9 (16.6)	n.s.
Age [years]	31.3 (11.2)	30.9 (10.5)	n.s.
BMI [kg/m2]	22.8 (4.4)	21.6 (3.6)	n.s.
WHR	0.8 (0.1)	0.8 (0.1)	n.s.
Body composition			
Fat [kg]	13.9 (5.8)	11.6 (9.3)	n.s.
FFM [kg]	54.2 (23.4)	57.1 (19.3)	n.s.
TBW [kg]	39.7 (17.1)	41.8 (14.1)	n.s.
Macronutrients intake			
Total energy [kcal/day]	2 100 (683)	2 072 (706)	n.s.
Proteins [g/day]	81 (29)	69 (38)	0.020
Lipids [g/day]	83 (49)	70.0 (35)	0.030
Carbohydrates [g/day]	232 (98)	250 (105)	0.030
Dietary fiber [g/day]	18 (10)	33 (20)	<0.001
Glucose metabolism			
Fasting glucose [mmol/l]	4.8 (0.3)	4.7 (0.4)	n.s.
2h OGTT glucose [mmol/l]	5.9 (1.4)	5.5 (1.3)	0.070
AUC for OGTT glucose [mmol/l x 120min^{-1}]	255 (137)	184 (159)	n.s.
AUC for OGTT insulin [mIU/l x 120min^{-1}]	4,416 (1938)	3,143 (2603)	0.004
Insulin [mIU/l]	3.9 (2.7)	3.4 (1.7)	n.s.
C-peptide [pmol/l]	232 (103)	229 (79)	n.s.
HbA1c [mmol/mol]	32.0 (2.5)	30.0 (4.0)	0.010
Matsuda index	10.2 (6.6)	9.9 (5.2)	n.s.
Lipid metabolism			
Total cholesterol [mmol/l]	4.3 (1.1)	3.3 (0.8)	<0.001
HDL-C [mmol/l]	1.7 (0.7)	1.4 (0.4)	<0.001
LDL-C [mmol/l]	2.4 (1.2)	1.7 (0.8)	<0.001
Triacylglycerols [mmol/l]	0.7 (0.5)	0.7 (0.4)	n.s.
Inflammatory markers			
CRP (mg/l)	0.074 (0.081)	0.045 (0.028)	<0.001
Stool characteristics			
pH in feces	7.3 (0.7)	6.9 (0.8)	0.005
dry mass (%)	25.1 (9.9)	20.3 (8.8)	0.002

Data are given as median (interquartile range). BMI, body mass index; WHR, waist-hip ratio; FFM, fat-free mass; TBW, total body water; OGTT, oral glucose tolerance test; AUC, area under the curve during oral glucose tolerance test; CRP, C-reactive protein; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein–cholesterol; LDL-C, low-density lipoprotein–cholesterol.

prevalence >75% at least in one group. The normalized α-diversity of the gut microbiota was estimated using indexes measuring richness (observed species) and evenness (Chao1, Shannon, Simpson, Pielou). In all the parameters, diversity was higher in the omnivore group (**Supplementary Table 4**). We identified 10 phyla dominated by *Firmicutes* (median abundance 46 and 52% in the vegans and omnivores, respectively) and *Bacteroidetes* (median abundance 44 and 41%) followed by much less abundant *Proteobacteria* (median abundance 1.6 and 1.6%), *Actinobacteria* (median abundance 1.8 and 4.2%), and *Verrucomicrobia* (median abundance 0.2 and 0.4%). The abundance of other phyla was below 0.01%. Multivariable statistics (PERMANOVA) revealed significant differences in β-diversity at the level of order ($p = 0.023$), family ($p = 0.013$), and genus ($p > 0.001$) between the groups. The separation of vegans and omnivores at the genus level is visualized in **Figures 1A,C** using unsupervised (PCA) and supervised (PLS-DA) methods, respectively. Univariable differential abundance analysis followed by effect size analysis (Cliff's delta) identified 34 genera with significantly different abundance between the groups (FDR $\geq$ 0.1) (**Figure 1B**, **Supplementary Table 5**). As next step, we employed a PLS-DA

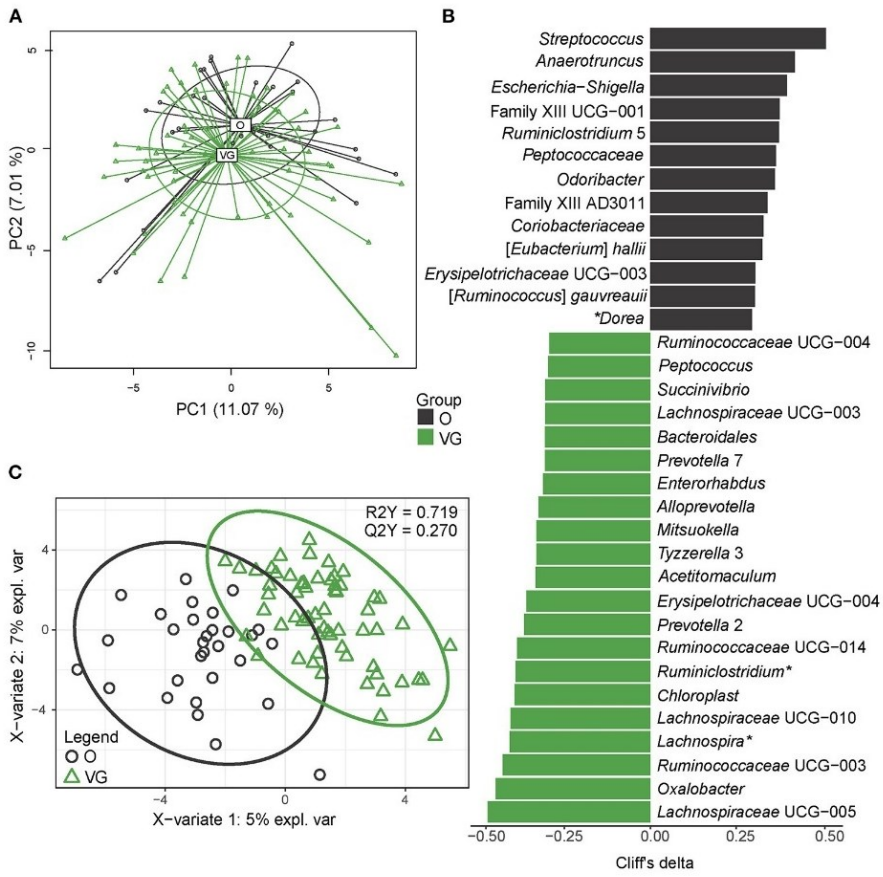


FIGURE 1 | Gut microbiome composition. **(A)** Two-dimensional (2D) principal component analysis (PCA) score plot with the explained variance of each component. **(B)** Biomarker taxa generated from univariable discrimination analysis (FDR ≥ 0.1), effect size estimated by Cliff's delta; **(C)** 2D score plots of PLS-DA. R2Y fit goodness, Q2Y predictive power. *genera belonging to the core microbiome (abundance > 0.1%, prevalence > 75%). Effect sizes, FDR values, and variable importance in projection (VIP) values can be found in **Supplementary Table 3**. Data are presented as compositional (proportion of the particular bacteria of total sum of bacteria) after clr transformation.

model in order to address mutual relationships among the variables. According to this model, characterized by R2Y = 0.719 (goodness of fit) and Q2Y = 0.27 (goodness of prediction) metrics, we selected 55 genera with VIP value >1 (**Supplementary Table 5**). The combined set of variables selected by both approaches comprised 58 genera, representing 14.8% of total bacteria detected in both the vegans and omnivores. Ten of them belong to the core microbiome; three being enriched (*Lachnospira, Lachnospiraceae NK4A136* group, and *Ruminiclostridium*) and seven (*Alistipes, Bifidobacterium, Blautia, Fusicatenibacter, Dorea, Anaerostipes,* and *Ruminococcaceae_uncultured)* being depleted in the vegans compared with the omnivores. The remaining genera belong to the low abundant (0.1%) and very low abundant (0.01%) and rare taxa; the former being enriched rather in the omnivores, while the latter mostly in the vegans. To explore whether we could discriminate between vegans and omnivores according to microbiome composition, we employed a machine learning approach, specifically, a random forest algorithm. As we had only 90 subjects, we adopted 10-fold cross-validation to avoid reporting insignificant results for an overfitted model (the same method was used for the calculation of Q2Y metrics for PLS-DA). For fecal microbiome, we reached 83% accuracy of discrimination between vegans and omnivores, $p < 0.01$, obtained by permutation test. Nevertheless, this model tended to misclassify the vegans as controls with a false positive rate of 36.7%.

Functional Capacity of the Gut Microbiota: But Gene Abundance

Having in mind the limitations of 16S rRNA gene sequencing regarding resolution power, we tried to characterize gut microbiota independently on taxonomic classification by

TABLE 2 | Normalized *but* gene copy number.

Cluster	Copy number		*p*-value	FDR	Cliff's delta
	Omnivore	Vegan			
A	4.0 (2.3)	3.5 (2.0)	0.056	0.337	−0.3
B	0.20 (2.25)	0.44 (5.09)	0.135	0.404	−0.2
C	190 (310)	211 (243)	0.289	0.432	−0.1
D	31 (111)	63 (118)	0.360	0.432	0.1
E	0.48 (0.55)	0.28 (0.48)	0.269	0.432	0.1
F	22 (40)	12 (25)	0.647	0.647	0.1

Data are given as median (interquartile range). The copy number of the but gene was normalized to spike DNA (C. elegans UNC-6 gene) and calculated using the ΔCt method. Clusters represent groups of bacteria sharing sufficient but gene similarity allowing for the use of one degenerate primer pair. Bacteria belonging to individual clusters are listed in ***Supplementary Table 9****.*

searching for markers of its functional capacity. We determined the abundance of butyryl-CoA:acetate CoA-transferase (*but*) gene, encoding the key enzyme of butyrate synthesis. We employed the qPCR method based on degenerate primers that allow for covering a wide spectrum of *but* gene variants. As shown in **Table 2**, we did not identify any difference in *but* gene abundance in gut microbiome in the vegan and omnivore groups.

Fecal Metabolome

We analyzed the fecal metabolome using two approaches, each of them covering a different spectrum of metabolites. By SPME-GC-TOF-MS, we identified 146 different VOCs, of which 80 were very low abundant (>0.1%), 52 were low abundant (0.1–1%), 10 were medium abundant (1–5%), and 4 (p-cresol, indole, scatole, ethyl butyrate) were highly abundant compounds (>5 %). The NMR spectrum comprised 34 quantified analytes. Only two compounds, i.e., butyric acid/butyrate and valeric acid/valerate, were identified by both approaches, and in both cases the values obtained by different methods correlated. For further statistical analysis, both datasets were combined and analyzed together. The separation of vegans and omnivores is visualized in **Figure 2A**. Multivariable statistics (PERMANOVA) revealed significant differences in β-diversity ($p = 0.0045$) between the groups. Univariable analysis revealed that abundances of 32 analytes differed significantly between the vegan and the omnivore groups (**Figure 2B**), and that the PLS-DA model (R2Y = 0.698; Q2Y = 0.403) selected 70 compounds (**Figure 2C**, **Supplementary Table 6**). The set of variables identified by both approaches comprised 77 compounds, representing 43.3% of all the fecal metabolites detected in both the vegans and omnivores. Vegan fecal metabolome was enriched by products of polysaccharide fermentation, i.e., short-chain fatty acids (SCFAs), such as butyrate and acetate and their derivatives ($n = 18$), medium-chain fatty acids (MCFAs) and their derivatives ($n = 5$), and further by methanol, monosaccharides, and several other compounds. In contrast, amino acid fermentation products, such as three most abundant metabolites (p-cresol, indole, and scatole) were approximately 50% lower in the vegans. The vegan fecal metabolome was also characterized by lower content of aromatic compounds benzacetaldehyde or 2-pentyl thiophene, medium- or long-chain alcohols, ketones, and aldehydes. The classification accuracy of the random forest model reached 76.2% ($p < 0.01$, obtained by permutation test). The false positive rate was 43.3%.

Bile Acid Profile in Feces

By targeted LCQ-TOF-MS, we identified 11 bile acids. PERMANOVA analysis did not provide significant differences between the vegans and omnivores. Univariable analysis revealed significantly lower lithocholic acid (LCA) content in the samples of vegans. LCA was also the most abundant bile acid (**Supplementary Table 7**).

Serum/Plasma Metabolome

To identify the composition of serum metabolome, we employed an untargeted NMR approach and LC-MS analysis allowing for the exact determination of SCFA concentration in the plasma. Altogether, we identified 34 quantified analytes by NMR and nine SCFAs by LC-MS, and only acetate/acetic acid was identified by both methods. For further statistical analysis, both datasets were combined and analyzed together. PCA shows clear separation of vegan and omnivore serum metabolome (**Figure 3A**), which was confirmed by PERMANOVA ($p > 0.001$). Univariable analysis identified 24 metabolites (of which 15 were amino acids or their derivatives and five were SCFAs) significantly differentially abundant between the groups (**Figure 3B**). The PLS-DA model (R2Y = 0.726, Q2Y = 0.573) selected 15 metabolites with VIP > 1 (**Figure 3C**, **Supplementary Table 8**). The set of variables selected by both approaches comprised 24 compounds, representing 55.8% of all the detected serum metabolites. Vegan serum metabolome is characterized by higher content of SCFAs (formic, acetic, propionic, and butyric acids), dimethylsulfone, and amino acids glycine, glutamine, asparagine, proline, and threonine, while the concentrations of branched-chain amino acids (BCAAs), their derivatives, and essential amino acid lysine were lower. Some of these metabolites, i.e., SCFAs, dimethylsulfone, and BCAA derivatives are potential co-metabolites of host and bacterial metabolism. The classification accuracy of the random forest model built on serum metabolome data reached 91.6% ($p < 0.001$, obtained by permutation test); false positive rate was 18.2%.

Urine Metabolome

Urine metabolome, determined by untargeted NMR analysis, comprised 18 quantified metabolites and significantly differed between the groups (PERMANOVA, $p > 0.001$). The distribution of vegans and omnivores is shown in **Figure 4A**. Univariable analysis identified 10 metabolites that differed in abundance in the vegan and omnivore groups (**Figure 4B**), while the PLS-DA model (R2Y = 0.476, Q2Y = 0.309) selected seven discriminatory compounds (**Figure 4C**, **Supplementary Table 9**). The list of variables selected by both approaches comprised 12 compounds, representing 66.7% of all the detected metabolites. Ten of these compounds were depleted in the vegan urine metabolome, and all of them were related to protein/amino acid metabolism. Only glycine and trigonelline were higher in the vegans. The discrimination power of the random forest algorithm was 78.3%

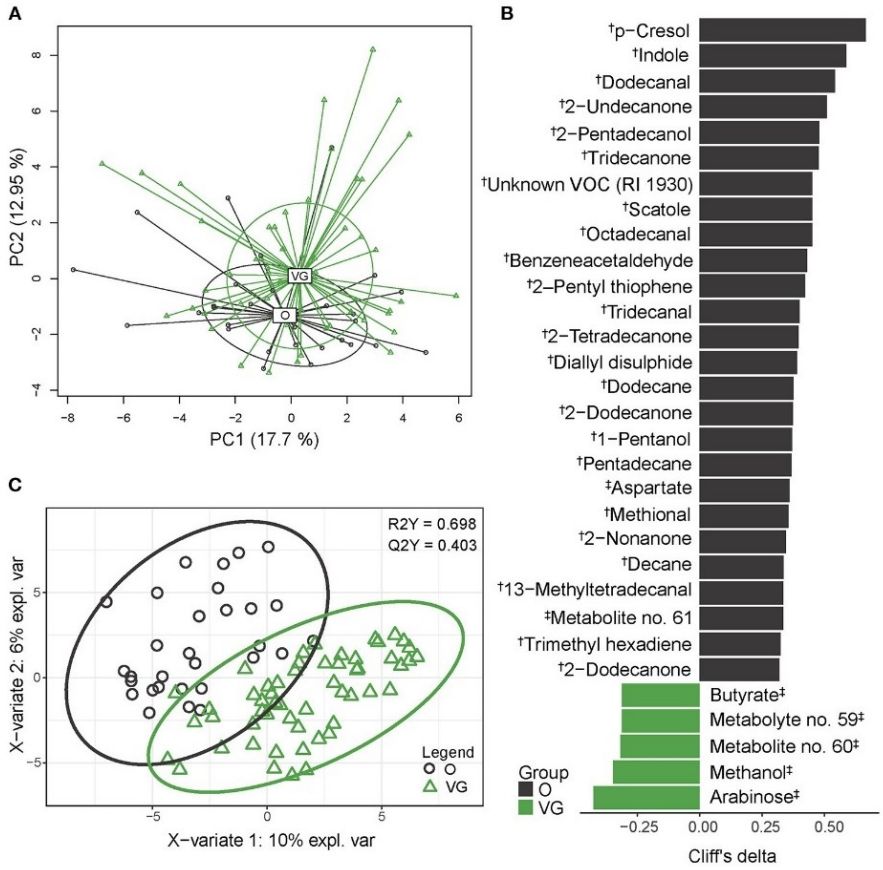


FIGURE 2 | Fecal metabolome composition. **(A)** 2D PCA analysis score plot with the explained variance of each component. **(B)** Biomarker metabolites generated from univariable discrimination analysis (FDR ≥0.1), effect size estimated by Cliff's delta. **(C)** 2D score plots of PLS-DA. R2Y fit goodness, Q2Y predictive power. †metabolites identified by GC-MS; ‡metabolites identified by NMR. Effect sizes, FDR values and VIP values can be found in **Supplementary Table 4**. Volatile compound (VOC) abundances are presented as compositional after clr transformation, NMR data were normalized by probabilistic quotient normalization (PQN).

($p < 0.01$, obtained by permutation test); false positive rate was 46.7%.

Network Analysis

Finally, we looked for possible relationships between microbiome composition and metabolomic biomarkers, pooling data from both groups to gain contrast and power. Looking at the relationships between fecal microbiome and metabolome (**Figure 5**), we identified several motifs. First, the dominant tyrosine metabolites p-cresol and scatole positively correlated with *Anaerotruncus, Alistipes, Family XIII* AD3011 group, and *Ruminococcaceae* UCG-002. These metabolites further negatively correlated with methanol and several SCFA esters. Second, amino acids, such as BCAAs, lysine, tyrosine, phenylalanine, and methionine, positively correlated with *Bactreoides, Blautia, Dorea, Lachnoclostridium*, and *Fusicatenibacter*. With the exception of *Bacteroides*, all these bacteria were enriched in the omnivores. Third, a cluster of rare genera more represented in the vegan microbiome (*Tyzzerella, Succinivibrio, Shuttleworthia,* etc.) positively correlated with SCFAs (acetic, propionic, and butyric acids).

Examination of the relationships between fecal bile acids and microbiota identified LCA as the dominant compound at the interface of both datasets. LCA concentration in feces positively correlated with the abundance of *Ruminococcaceae* UCG-002 and *Family XIII* AD3011 group, both of them also correlated positively with protein fermentation products. In contrast, LCA negatively correlated with the abundance of nine bacteria enriched in the vegans, with two of them (*Lachnospira, Ruminiclostridium*) belonging to the core microbiota (**Figure 6**).

The network analysis further unraveled the central role of dietary fiber in the modulation of gut microbiome and metabolome. As expected, fiber positively correlated with many bacteria enriched in the vegan microbiome and negatively with

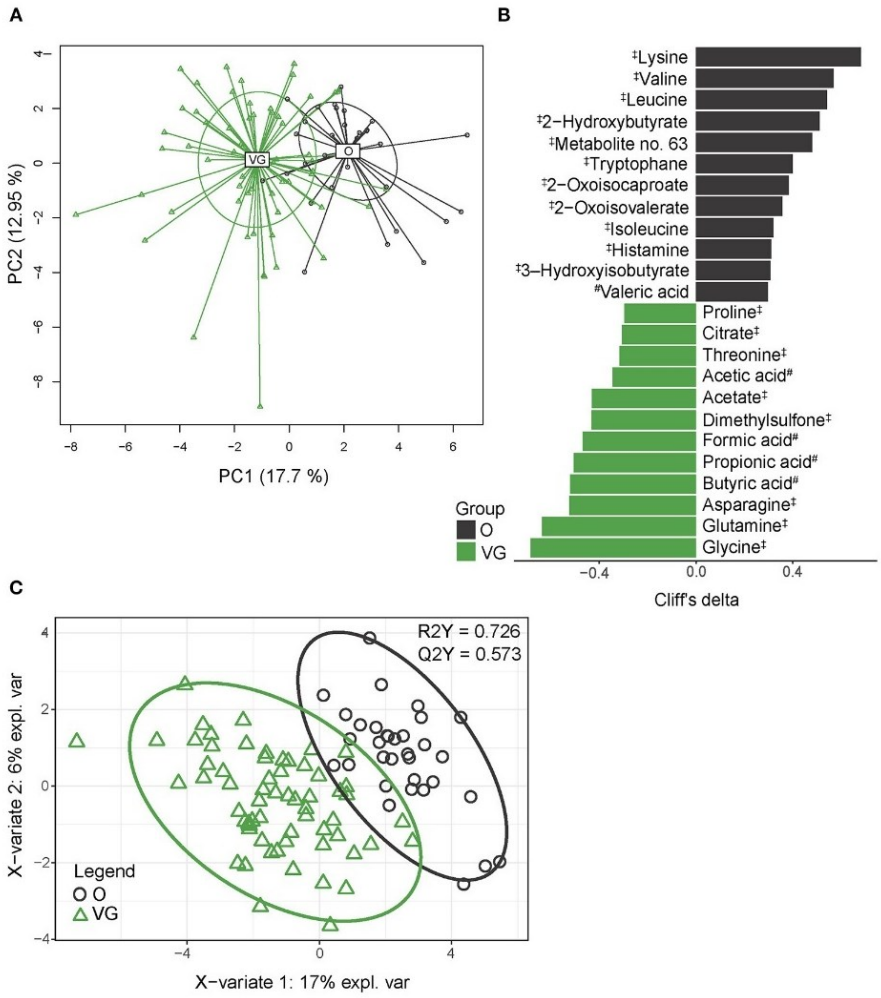


FIGURE 3 | Serum metabolome composition. **(A)** 2D PCA analysis score plot with the explained variance of each component. **(B)** Biomarker metabolites generated from univariable discrimination analysis (FDR ≥ 0.1), effect size estimated by Cliff's delta. **(C)** 2D score plots of PLS-DA. R2Y fit goodness, Q2Y predictive power. #metabolites identified by LC-MS; ‡metabolites identified by NMR. Effect sizes, FDR values, and VIP values can be found in **Supplementary Table 6**. NMR data were normalized by PQN.

some of those typical for the omnivores (**Figure 7**). Interestingly, negative associations with omnivore-characteristic bacteria were less frequent than positive associations with vegan-characteristic bacteria. Dietary protein correlated positively with *Streptococcus* and dietary fat with *Peptococcaceae*. Dietary fiber correlated negatively with numerous products of protein fermentation, and positively with methanol, acetate, butyrate, and SCFA esters (**Figure 8**).

Because some of the serum metabolites contributing to the separation of vegans and omnivores may be of microbial origin, we further looked for associations between gut microbiome and serum metabolome (**Figure 9**). The main findings may be summarized as follows: first, BCAAs and their derivatives positively correlated with bacteria enriched in the omnivore microbiome (*Family XIII* UCG-001, *Erysipelotrichaceae* UCG-003, *Streptococcus, Eubacterium hallii* group, *Dorea,* and *Blautia*) and negatively with vegan-characteristic bacteria *Lachnospiraceae* NC2004 group. Second, tryptophan positively correlated with *Streptococcus, Ruminclostridium 6,* and the *Ruminococcus gauvreauii* group, all of which were higher in the omnivores. Third, proline positively correlated with numerous vegan-characteristic microbes and negatively with the *Family XIII* AD3001 group, omnivore-characteristic bacteria. Fourth, we found positive correlations between serum SCFAs and some of the vegan-characteristic bacteria, particularly propionate/*Ruminococcaceae*

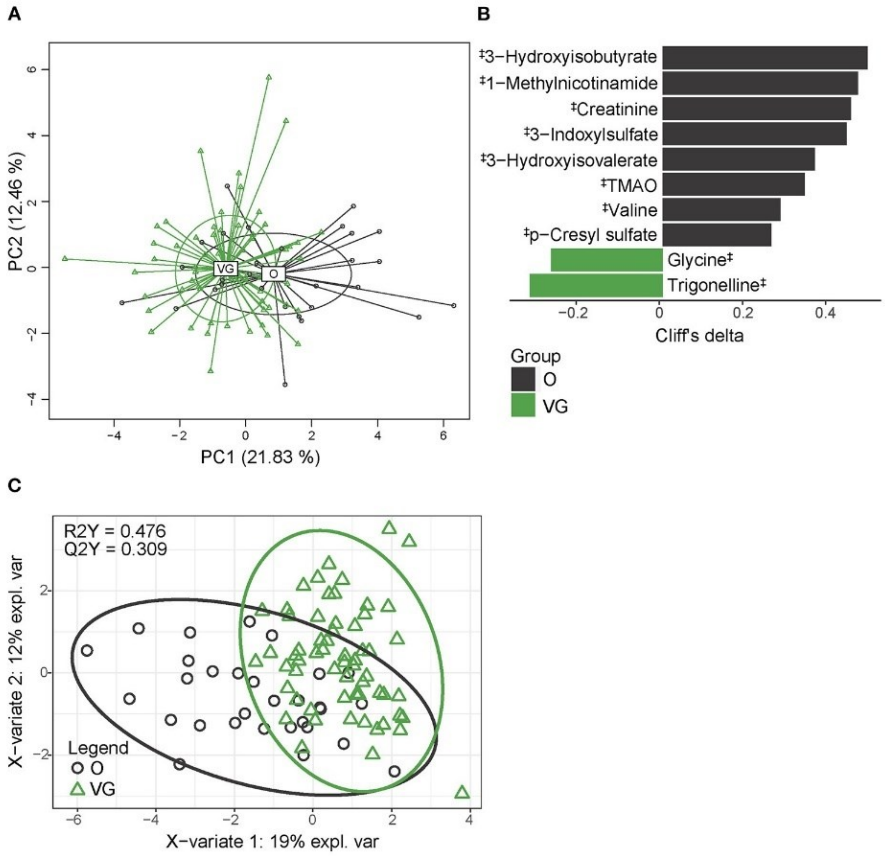


FIGURE 4 | Urine metabolome composition. **(A)** 2D PCA analysis score plot with the explained variance of each component; **(B)** Biomarker metabolites generated from univariable discrimination analysis (FDR ≥ 0.1), effect size estimated by Cliff's delta. **(C)** 2D score plots of PLS-DA. R2Y fit goodness, Q2Y predictive power. ‡metabolites identified by NMR. Effect sizes, FDR values, and VIP values can be found in **Supplementary Table 7**. NMR data were normalized by PQN.

UCG-003 and butyrate/*Haemophilus,* and *Lachnospiraceae* UCG-005. *Dorea* (omnivore-characteristic bacteria) negatively correlated with acetate. Finally, dimethylsulfone negatively correlated with omnivore-characteristic genera (*Escherichia-Shigella,* and *Lachnoclostridium*) and positively with vegan-characteristic bacteria *Ruminococcaceae* UCG-014 and *Oxalobacter*.

Taken together, our findings indicate not only relationships among diet, fecal metabolome, and fecal microbiome but also more far-reached links connecting gut microbiota with metabolites in the serum.

DISCUSSION

To fill knowledge gaps in mechanisms linking the effects of diet and intestinal microbiota, we explored differences in the intestinal microbiota and related metabolites in a model population of vegans vs. omnivores. The groups clearly differ in their eating habits. The major finding of this study is that dietary composition relates to distinct gut microbiota metabolic performance and metabolomic features despite highly similar established proxies of metabolic health across the groups. Our finding of slightly greater alpha-diversity of gut microbiota among the non-vegans was unexpected but may be related to the fact that the control group consisted of younger volunteers with a rather favorable pattern of lifestyle factors.

Vegan Diet and Microbiome Composition

The vegan diet is characterized by a different nutrient composition when compared to the omnivore diet, i.e., lower amounts of fat and protein, different patterns of amino acids, and higher amounts of dietary fiber. All of these features have a potential to promote important alterations of the gut microbiota (20) but available studies, recently reviewed in detail by Trefflich (21) and Losno (22), reported variable outcomes regarding changes in the overall composition of the microbiome associated with adherence to a vegan diet. The most reported

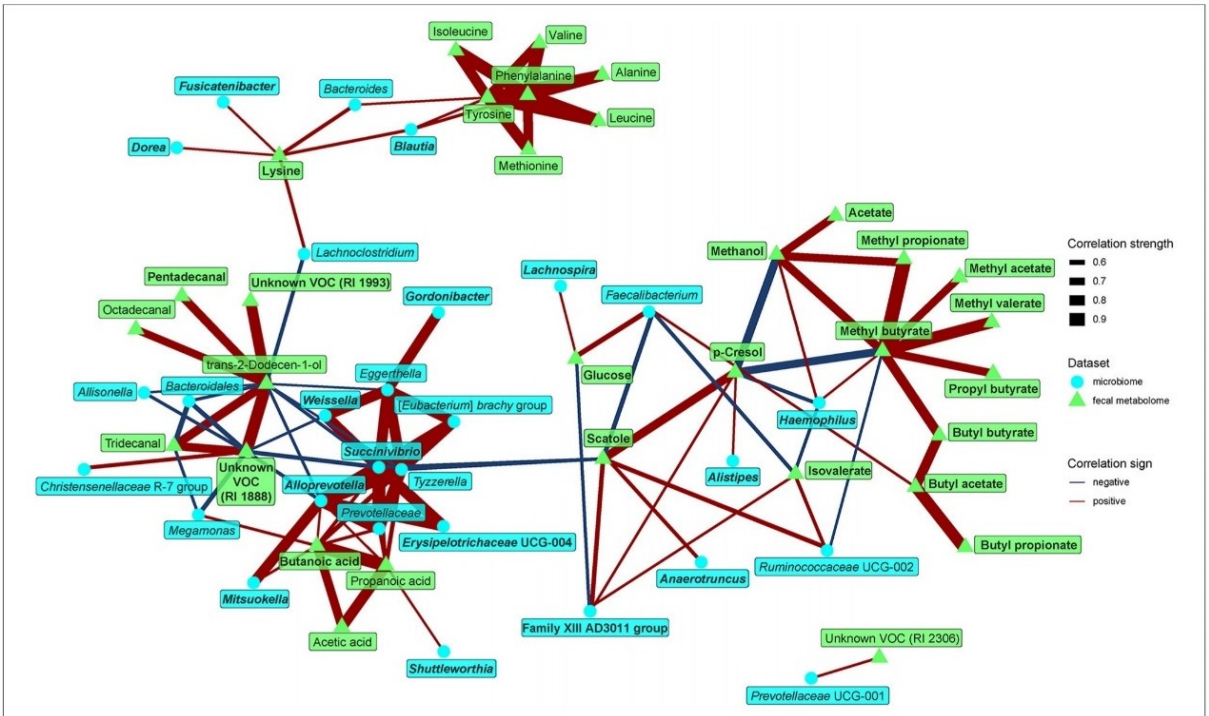


FIGURE 5 | Spearman's correlations between bacteria and fecal metabolome components. The compositional data (values of bacteria and VOC abundance) were clr transformed prior to the construction of the networks, whereas data obtained from NMR were normalized by PQN. The edge width and color are proportional to the value of the correlation (red: positive; blue: negative). The features highlighted in bold were selected as significantly contributing to the discrimination between groups.

groups affected by the vegan diet were Bacteroidetes and Firmicutes at the phylum level and *Bacteroides, Prevotella*, and *Bifidobacterium* at the genus level (22). In our study, we found only modest differences in microbiome composition associated with a vegan vs. omnivore diet, as only 14.8% of all the identified bacteria were affected by the diet, and the machine-learning algorithm based on microbiome data was quite inefficient in discriminating between vegans and omnivores. Nevertheless, it is important to mention that our results, as well as majority of other studies focused on vegan microbiota, are based on 16S rRNA hypervariable amplicon sequencing. Compared to shotgun sequencing, this method does not allow for more detailed taxonomy classification at the level of species and strain. We definitely cannot exclude the possibility that there are substantial differences between both groups at these levels. De Filippis et al. (23) demonstrated that different oligotypes within the same genus showed distinctive correlation patterns with dietary components and metabolome. *Prevotella* is a typical representative of plant-based diet-associated microorganism, while *Bactreoides* is linked to animal-based nutrients. Nevertheless, within both genera, oligotypes exist that associate with the opposite dietary component than the majority. Therefore, the diet/microbiome associations based only on genus-level taxonomy may be oversimplified and may not catch more subtle relationships.

On the other hand, taxonomic assignment only has limited ability to describe the functional capacity of the microbiota community. In addition to the non-optimal resolution of 16S rRNA sequencing, even well-assigned bacteria may possess unexpected characteristics because of the horizontal gene transfer that readily occurs among bacteria. Shotgun sequencing is the gold-standard method for the identification of full set of genes present in the bacterial community, but it is also costly and demanding for bioinformatics capacity. We developed an alternative approach based on qPCR quantification of gene of interest in stool DNA. In this study, we quantified the abundance of the *but* gene coding a key enzyme of butyrate synthesis. We chose this gene, as butyrate synthesis is one of the potential final steps of fiber fermentation, and butyrate is an important fermentation product with a significant impact on host health. We speculated that it could be a good readout of the effect of a diet rich or poor in fiber on microbiota composition. Somewhat surprisingly, we did not prove a significant difference in *but* abundance in the vegan and omnivore groups. This finding supports the results of taxonomic analysis.

Alpha diversity was higher in the omnivores than in the vegans, which is in disagreement with previously published results that showed no difference (24, 25) or higher diversity in vegans (26) but in line with a recent report comparing

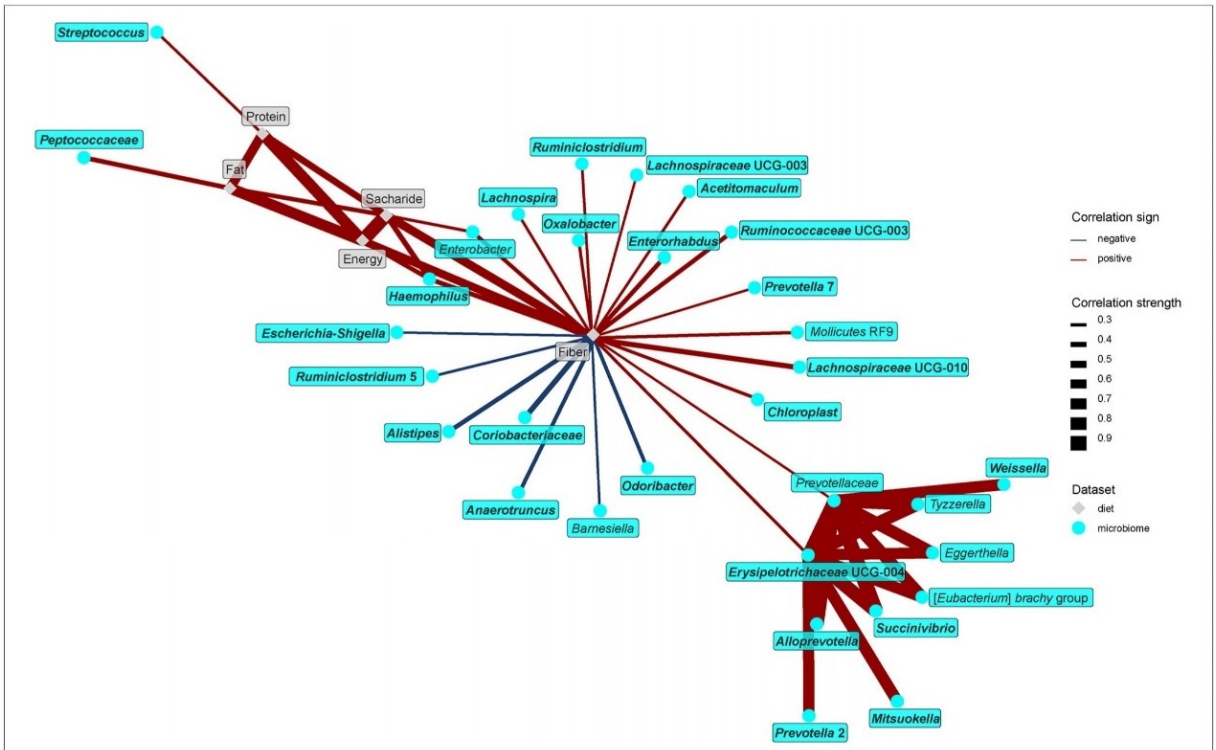


FIGURE 6 | Spearman's correlations between bacteria and dietary components. The compositional data (values of bacteria abundance) were clr transformed prior to the construction of the networks. The edge width and color are proportional to the value of the correlation (red: positive; blue: negative). The features highlighted in bold were selected as significantly contributing to the discrimination between groups.

vegan and omnivore cohorts in Germany (27). The lack of significant effects of a long-term vegan diet or diet with high amounts of dietary fiber from cereals on gut microbiome composition observed by us and others (25, 28) is in contrast with significant differences that have been demonstrated between people from traditional agrarian societies dependent on mostly plant-based diets and Westernized societies consuming low-fiber high-protein diets (29, 30). Nevertheless, this observation still does not contradict the profound effect of diet on microbiota composition. Evaluation of archaic native American coprolith remains suggests that pre-agricultural fiber intake exceeded 100 g/day (31). This corresponds to estimated daily intake of fiber in surviving traditional societies still sticking to hunter-gatherer way of life, which is 80–150g/day (32, 33). In contrast to it, the median fiber intake in our vegan cohort was 33 g/day, and the value 100 g/day was exceptional. Furthermore, recent research indicates that microbiome composition is established in early childhood (34) and is relatively stable during adulthood. None of the vegans included into our study has been vegan since childhood, so the formation period of her/his microbiota occurred in omnivore setting. Taking into account all these circumstances, our findings support the hypothesis that the core gut microbiota (abundance > 0.1%, prevalence > 75%), at least at the genus level, are stable and resilient to compositional change, even during a long-term dietary shift in adulthood. However, we cannot rule out that compositional differences between the vegans and non-vegans would become apparent at the metagenomics level.

Vegan Diet and Fecal Metabolome

Although DNA-based fingerprint procedures provide information about the composition of the microbial community, they do not reflect the metabolic activity of the populations (35). In contrast, fecal metabolome has been proposed as a functional readout of the human microbiome (36), reflective of microbiome–host interactions with immediate impact on host health. Bacterial genome often encodes genes for alternative metabolic pathways allowing for high metabolic flexibility. Bacteria are able to switch among different metabolic programs depending on available substrate in order to reach maximal energy extraction efficacy. The same bacteria are, therefore, capable to produce a very different spectrum of metabolites. Thus, we performed untargeted fecal metabolome analyses as a functional readout. Among the important characteristics of vegan fecal metabolome was significantly lower content of amino acid fermentation products p-cresol, indole, scatole, and some aromatic compounds that were consistently identified by two

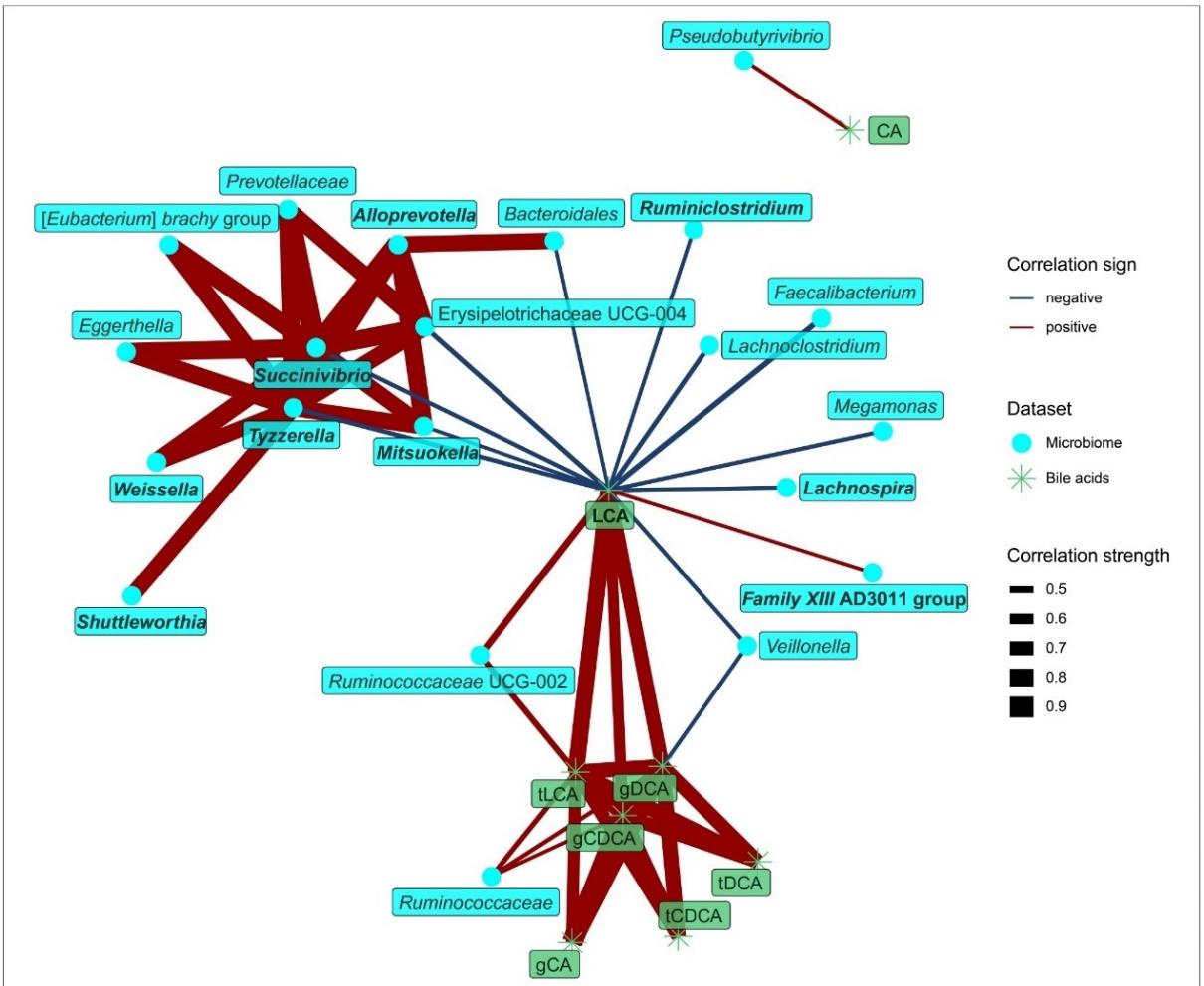


FIGURE 7 | Spearman's correlations between bacteria and fecal bile acids. The compositional data (values of bacteria abundance) were clr transformed prior to the construction of the networks. The edge width and color are proportional to the value of the correlation (red: positive; blue: negative). The features highlighted in bold were selected as significantly contributing to the discrimination between groups.

independent statistical methods. These metabolites also belong to the most abundant components of fecal metabolome. The second group of compounds discriminating between vegans and omnivores are SCFA and SCFA-derived esters. Most bacteria possess multiple metabolic programs that may be switched on and off according to the available substrate and environmental conditions. The composition of the fecal metabolome, therefore, reflects the preferential carbohydrate fermentation in vegans and shift to protein fermentation in omnivores because of the different diet macronutrient composition of their diets. The vegan diet contains less protein but more fiber than the omnivore one. Amino acids are less efficient as energy source for human gut microbes; therefore, gut microbiota preferentially consume carbohydrates over proteins (37). This shift in fecal metabolome composition has implications for human health, as products of protein fermentation, like p-cresol, have considerable health effects. It is associated with adverse effects such as genotoxicity, oxidative stress, compromised integrity of the gut epithelium, and decreased viability as well as proliferation of intestinal epithelial cells (37). Our study clearly demonstrated that a vegan dietary pattern is associated with distinct metabolomic profiles compared to a meat-containing diet, even when comparing vegan vs. non-vegan individuals with otherwise similar characteristics, e.g., regarding age and BMI. However, whether the observed differences underlie potential long-term health benefits of a vegan diet needs to be further investigated, for example, by comprehensive metabolomics assessments in epidemiological long-term studies.

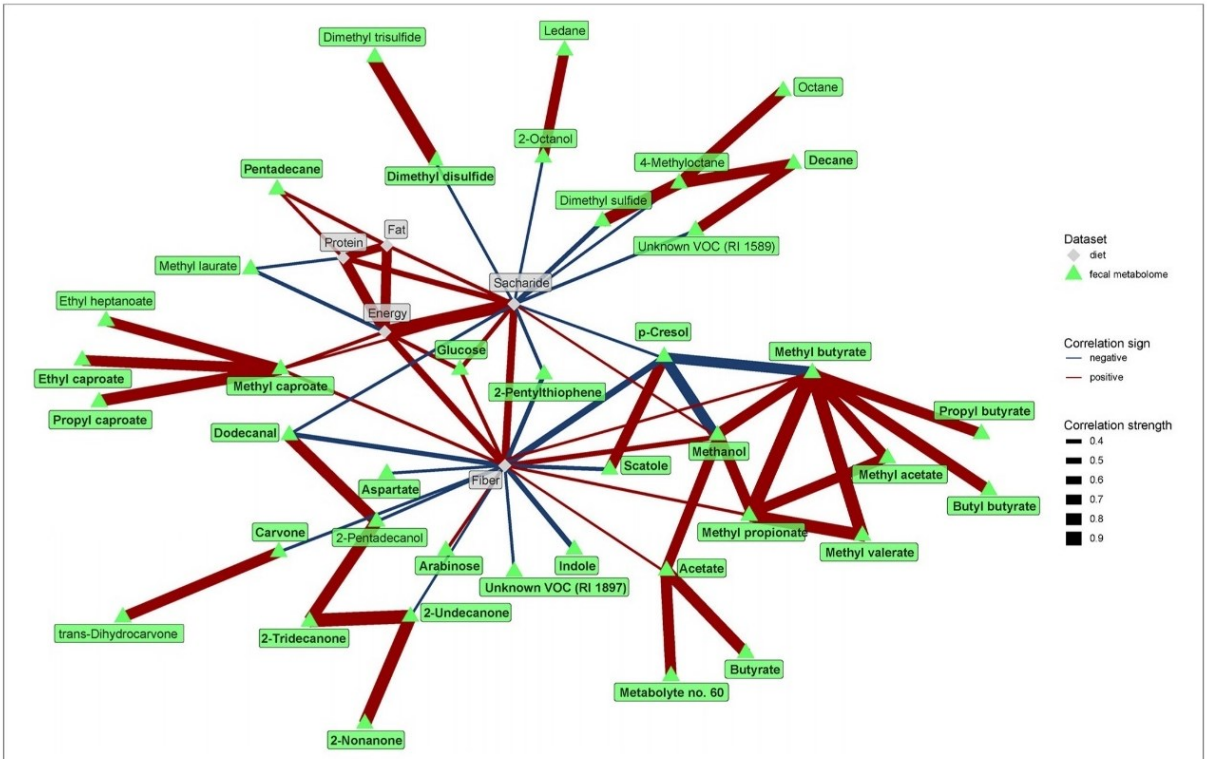


FIGURE 8 | Spearman's correlations between fecal metabolome and dietary components. The compositional data (values of VOC abundance) were clr transformed prior to the construction of the networks. The edge width and color are proportional to the value of the correlation (red: positive; blue: negative). The features highlighted in bold were selected as significantly contributing to the discrimination between groups.

Gut Microbiota and Fecal Metabolome

Several potentially interesting associations between bacterial abundances and metabolites were revealed in this study. *Alistipes,* the *Family XIII*AD3011 group, and *Anaerotruncus,* all higher in omnivores, positively correlate with p-cresol and scatole. Three other bacteria significantly higher in omnivores (*Dorea, Blautia,* and *Fusicatenibacter*) positively correlate with amino acids tyrosine, BCAAs, methionine, lysine, phenylalanine, and valine. The association of these bacteria with metabolically adverse phenotypes was reported (38, 39). Not surprisingly, dietary fiber correlated (both positively and negatively) with most of bacteria differently represented in both groups. Accordingly, we found positive correlations between dietary fiber and SCFAs and their derivatives, as well as negative correlations between dietary fiber and protein fermentation products. Nevertheless, the fecal metabolome still did not discriminate well between both groups, probably because of factors such as metabolic cross-feeding between different bacterial groups, utilization of bacterial metabolites by the host, and technical issues concerning the difficult normalization of fecal metabolite concentration.

Gut Microbiota and Fecal Bile Acids

Primary bile acids secreted from the liver to the intestine are subjects of extensive microbial transformation within the gut, and the spectrum of secondary bile acids reflects the composition and metabolic performance of gut microbiota. In our study, we did not observe any difference between the VG and O groups in the fecal concentration of primary bile acids and their derivatives but significantly lower concentration of one secondary bile acid, LCA, in VGs than in Os. Secondary bile acids are solely the product of microbial transformation of deconjugated primary bile acids (40); therefore, the significant difference in LCA concentration between vegans and omnivores with comparable primary BA synthesis may indicate different microbial activity. Recent research revealed the important role of bile acids as signals in the regulation of lipid and glucose metabolism (41) or in cancerogenesis (42). Type 2 diabetes is associated with higher plasma levels of LCA (43), and with significantly altered bile acid signature in feces (44). Our study only comprised metabolically healthy omnivores and vegans, so we cannot draw any strong conclusions regarding LCA fecal content and metabolic health, but even within physiological

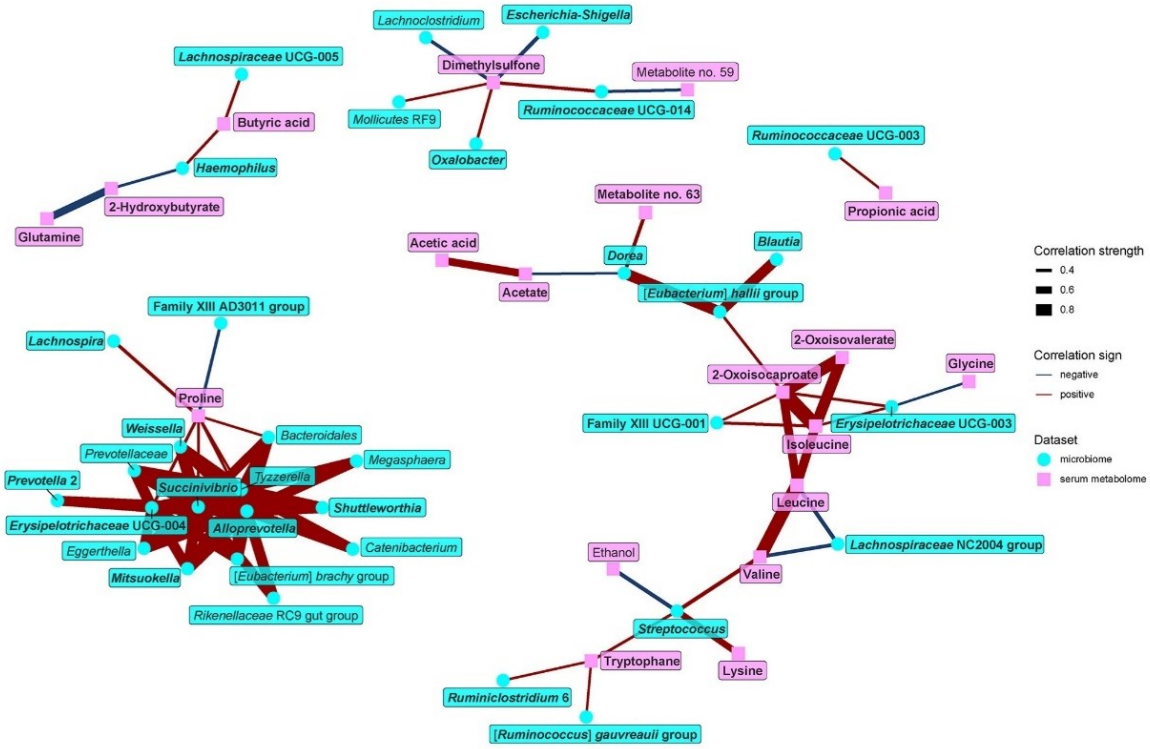


FIGURE 9 | Spearman's correlations between bacteria and serum metabolome components. The compositional data (values of bacteria abundance) were clr transformed prior to the construction of the networks, whereas the data obtained from NMR were normalized by PQN. The edge width and color are proportional to the value of the correlation (red: positive; blue: negative). The features highlighted in bold were selected as significantly contributing to the discrimination between groups.

limits, the vegans had more favorable glucose metabolism-related parameters than the omnivores. Further research is needed to confirm or deny the hypothesis that microbial metabolism of bile acids contributes to the impairment of glucose homeostasis of a host.

Gut-To-Circulation Crosstalk

The composition of serum/plasma metabolome is the most discriminative characteristic between vegans and omnivores. Our data indicate that it reflects both dietary pattern and gut microbiota activity. Without a doubt, the macronutrient, particularly protein intake and amino acid composition, is different in vegans and omnivores. The different diet composition has a direct effect on physiology, and absorption of nutrients in the upper gastrointestinal tract, therefore, had a limited impact on colonic microbiota. However, some of the metabolites differently abundant in vegan and omnivore serum are co-metabolites formed both by the host and gut microbiota advocating direct gut-to-circulation crosstalk. This is the case for dimethylsulfone, a product of microbial metabolism of methionine as well as BCAAs and SCFAs.

Reduced circulating BCAA in vegans has been observed previously (25, 45) and may relate to lower BCAA intake (46). Nevertheless, Wang et al. proved that the BCAA degradation pathway is upregulated in gut microbiota of vegans and vegetarians compared to those of omnivores (45). Therefore, upregulated BCAA degradation in the gut may contribute to the observed lower serum BCAA concentrations in vegans. The reduction of circulating BCAA may represent one of the microbiome-related mechanism contributing to the health-promoting effects of plant-based diet, as it has been repeatedly shown that increased circulating BCAA decline in metabolic health and diabetes development (47). We identified a less favorable metabolic phenotype of omnivores despite the fact that both groups in this study comprised volunteers with healthy normal weight and normal glucose tolerance.

Short-chained fatty acids (SCFAs) are products of bacterial fermentation of fiber in the gut, and while demonstrating their higher concentration in vegan fecal as well as serum metabolome we provide a direct link connecting microbiome activity in the gut and circulating metabolome.

Strengths and Limitations

Our aim was to perform rigorous matching of participant characteristics across vegans and omnivores, and to focus on young and healthy population to minimize the risk of potential

confounders influencing metabolic health. The main limitation of our study is that the results were obtained on rather small vegan and omnivore groups, and that the outcomes were not validated in an independent cohort. However, the results were internally validated through permutation tests. Thus, the main purpose of the methods employed was to demonstrate that certain domains of the metabolome provide better discrimination of vegans and omnivores, and possibly a better picture of differential functional consequences of the diets, compared to bacterial abundances alone. The outcomes deserve further validation in independent well-matched cohorts. Another potentially limiting aspect that must be taken into consideration is the methodology used for the characterization of microbiota composition. We based our analysis on the sequencing of the V4 region of 16S rRNA gene, which provides lower resolution than shotgun sequencing. This may lead to the underestimation of the effect imposed by vegan diet on gut microbiota composition especially at the sub-genus level. Finally, the diet of both groups was analyzed only at the macronutrient level, as the aim of this study was comparative analysis of microbiome composition and metabolomic footprints of vegans and non-vegans. While our analyses strongly suggest that omitting animal foods has a distinct effect on the metabolome, further studies are needed on the potential mediating role of nutrient intakes, which may underlie the observed metabolic differences between vegans and non-vegans, beyond macronutrient and fiber intake.

CONCLUSION

We showed that the composition of gut microbiota of long-term vegans and omnivores is not dramatically different. In contrast, vegans and omnivores significantly differ in the composition of the fecal, serum, and urine metabolomes as an effect of different availability of substrates (dietary fiber vs. protein). Consequently, the vegan diet was associated with a lower abundance of the potentially harmful (protein fermentation products) and a higher occurrence of potentially beneficial (dietary fiber fermentation products) metabolites. While our study suggests that a shift toward a vegan diet may be an avenue to personalized manipulation of microbiome function, targeting metabolites with health implications such as indole or cresole (48), we acknowledge that our study was observational and that proof-of-concept RCTs are needed to investigate the potential of dietary interventions in this context. In general, while our study had the purpose of identifying metabolites that are differentially abundant between vegans and non-vegans, the clinical utility of the measured biomarkers for risk prediction or clinical monitoring is yet to be proven.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB43938.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Multicentric Ethic Committee of Kralovske Vinohrady University Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MP: resources, investigation, and writing of the original draft. EB and SS: methodology and formal analysis. MKu: investigation and writing-review and editing. HP, JH, PV, PS, and KK: investigation. MHec: investigation and project administration. ND: investigation and visualization. MB: data curation and project administration. IM: methodology, formal analysis, and visualization. MKr: methodology, formal analysis, and supervision. MHen and ES: resources and formal analysis. KC: investigation, supervision, and formal analysis. RS: writing-review and editing, and supervision. RL: methodology, investigation, and writing-review and editing. TK: methodology, supervision, and writing-review and editing. JG and MC: conceptualization, methodology, data curation, funding acquisition, and writing of the original draft. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the Ministry of Health of the Czech Republic (Grant NV-18-01-00040), Charles University (Grant 1280218), institutional support PROGRES Q36, and MH CR - DRO Institute for Clinical and Experimental Medicine -IKEM (Grant IN00023001). The authors used services of the Czech Centre for Phenogenomics supported by the Czech Academy of Sciences RVO 68378050 and by the project LM2018126 Czech Centre for Phenogenomics provided by MYES CR.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.783302/full#supplementary-material

REFERENCES

1. Appleby PN, Key TJ. The long-term health of vegetarians and vegans. *Proc Nutr Soc.* (2016) 75:287–93. doi: 10.1017/S0029665115004334
2. Gentile CL, Weir TL. The gut microbiota at the intersection of diet and human health. *Science.* (2018) 362:776–80. doi: 10.1126/science.aau5812
3. Marrone G, Guerriero C, Palazzetti D, Lido P, Marolla A, Di Daniele F, et al. Vegan diet health benefits in metabolic syndrome. *Nutrients.* (2021) 13:817. doi: 10.3390/nu13030817

4. Dinu M, Abbate R, Gensini GF, Casini A, Sofi F. Vegetarian, vegan diets and multiple health outcomes: A systematic review with meta-analysis of observational studies. *Crit Rev Food Sci Nutr.* (2017) 57:3640–9. doi: 10.1080/10408398.2016.1138447
5. Huang T, Yang B, Zheng J, Li G, Wahlqvist ML Li D. Cardiovascular disease mortality and cancer incidence in vegetarians: a meta-analysis and systematic review. *Ann Nutr Metab.* (2012) 60:233–40. doi: 10.1159/000337301
6. Kahleova H, Levin S, Barnard ND. Vegetarian dietary patterns and cardiovascular disease. *Prog Cardiovasc Dis.* (2018) 61:54–61. doi: 10.1016/j.pcad.2018.05.002
7. Kahleova H, Pelikanova T. Vegetarian diets in the prevention and treatment of type 2 diabetes. *J Am Coll Nutr.* (2015) 34:448–58. doi: 10.1080/07315724.2014.976890
8. Penniecook-Sawyers JA, Jaceldo-Siegl K, Fan J, Beeson L, Knutsen S, Herring P, et al. Vegetarian dietary patterns and the risk of breast cancer in a low-risk population. *Br J Nutr.* (2016) 115:1790–7. doi: 10.1017/S0007114516000751
9. Tantamango-Bartley Y, Knutsen SF, Knutsen R, Jacobsen BK, Fan J, Beeson WL, et al. Are strict vegetarians protected against prostate cancer? *Am J Clin Nutr.* (2016) 103:153–60. doi: 10.3945/ajcn.114.106450
10. Djekic D, Shi L, Calais F, Carlsson F, Landberg R, Hyotylainen T, et al. Effects of a lacto-ovo-vegetarian diet on the plasma lipidome and its association with atherosclerotic burden in patients with coronary artery disease-a randomized, open-label, cross-over study. *Nutrients.* (2020) 12:3586. doi: 10.3390/nu12113586
11. Jardine MA, Kahleova H, Levin SM, Ali Z, Trapp CB, Barnard ND. Perspective: plant-based eating pattern for type 2 diabetes prevention and treatment: efficacy, mechanisms, and practical considerations. *Adv Nutr.* (2021) 12:2045–55. doi: 10.1093/advances/nmab063
12. Kahleova H, Rembert E, Alwarith J, Yonas WN, Tura A, Holubkov R, et al. Effects of a low-fat vegan diet on gut microbiota in overweight individuals and relationships with body weight, body composition, and insulin sensitivity: a randomized clinical trial. *Nutrients.* (2020) 12:2917. doi: 10.3390/nu12102917
13. Willett W, Rockstrom J, Loken B, Springmann M, Lang T, Vermeulen S, et al. Food in the Anthropocene: the EAT-Lancet Commission on healthy diets from sustainable food systems. *Lancet.* (2019) 393:447–92. doi: 10.1016/S0140-6736(18)31788-4
14. Matsuda M, DeFronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diab Care.* (1999) 22:1462–70. doi: 10.2337/diacare.22.9.1462
15. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods.* (2016) 13:581–3. doi: 10.1038/nmeth.3869
16. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods.* (2010) 7:335–6. doi: 10.1038/nmeth.f.303
17. Daskova N, Heczkova M, Modos I, Videnska P, Splichalova P, Pelantova H, et al. Determination of butyrate synthesis capacity in gut microbiota: quantification of but gene abundance by qPCR in fecal samples. *Biomolecules.* (2021) 11:1303. doi: 10.3390/biom11091303
18. Han J, Lin K, Sequeira C, Borchers CH. An isotope-labeled chemical derivatization method for the quantitation of short-chain fatty acids in human feces by liquid chromatography-tandem mass spectrometry. *Anal Chim Acta.* (2015) 854:86–94. doi: 10.1016/j.aca.2014.11.015
19. R Core Team. *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. (2017).
20. Redondo-Useros N, Nova E, Gonzalez-Zancada N, Diaz LE, Gomez-Martinez S, Marcos A. Microbiota and lifestyle: a special focus on diet. *Nutrients.* (2020) 12:1776. doi: 10.3390/nu12061776
21. Trefflich I, Jabakhanji A, Menzel J, Blaut M, Michalsen A, Lampen A, et al. Is a vegan or a vegetarian diet associated with the microbiota composition in the gut? Results of a new cross-sectional study and systematic review. *Crit Rev Food Sci Nutr.* (2020) 60:2990–3004. doi: 10.1080/10408398.2019.1676697
22. Losno EA, Sieferle K, Perez-Cueto FJA, Ritz C. Vegan diet and the gut microbiota composition in healthy adults. *Nutrients.* (2021) 13:2402. doi: 10.3390/nu13072402
23. De Filippis F, Pellegrini N, Laghi L, Gobbetti M, Ercolini D. Unusual sub-genus associations of faecal Prevotella and Bacteroides with specific dietary patterns. *Microbiome.* (2016) 4:57. doi: 10.1186/s40168-016-0202-1
24. Jia W, Zhen J, Liu A, Yuan J, Wu X, Zhao P, et al. Long-term vegan meditation improved human gut microbiota. *Evid Based Complement Alternat Med.* (2020) 2020:9517897. doi: 10.1155/2020/9517897
25. Wu GD, Compher C, Chen EZ, Smith SA, Shah RD, Bittinger K, et al. Comparative metabolomics in vegans and omnivores reveal constraints on diet-dependent gut microbiota metabolite production. *Gut.* (2016) 65:63–72. doi: 10.1136/gutjnl-2014-308209
26. Losasso C, Eckert EM, Mastrorilli E, Villiger J, Mancin M, Patuzzi I, et al. Assessing the influence of vegan, vegetarian and omnivore oriented westernized dietary styles on human gut microbiota: a cross sectional study. *Front Microbiol.* (2018) 9:317. doi: 10.3389/fmicb.2018.00317
27. Trefflich I, Dietrich S, Braune A, Abraham K, Weikert C. Short- and branched-chain fatty acids as fecal markers for microbiota activity in vegans and omnivores. *Nutrients.* (2021) 13:1808. doi: 10.3390/nu13061808
28. Roager HM, Vogt JK, Kristensen M, Hansen LBS, Ibrugger S, Maerkedahl RB, et al. Whole grain-rich diet reduces body weight and systemic low-grade inflammation without inducing major changes of the gut microbiome: a randomised cross-over trial. *Gut.* (2019) 68:83–93. doi: 10.1136/gutjnl-2017-314786
29. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci U S A.* (2010) 107:14691–6. doi: 10.1073/pnas.1005963107
30. Sonnenburg JL, Backhed F. Diet-microbiota interactions as moderators of human metabolism. *Nature.* (2016) 535:56–64. doi: 10.1038/nature18846
31. Konner M, Eaton SB. Paleolithic nutrition: twenty-five years later. *Nutr Clin Pract.* (2010) 25:594–602. doi: 10.1177/0884533610385702
32. Pontzer H, Wood BM, Raichlen DA. Hunter-gatherers as models in public health. *Obes Rev.* (2018) 19:24–35. doi: 10.1111/obr.12785
33. Pontzer H, Wood BM. Effects of evolution, ecology, and economy on human diet: insights from hunter-gatherers and other small-scale societies. *Annu Rev Nutr.* (2021) 41:363–85. doi: 10.1146/annurev-nutr-111120-105520
34. Mesa MD, Loureiro B, Iglesia I, Fernandez Gonzalez S, Llurba Olive E, Garcia Algar O, et al. The evolving microbiome from pregnancy to early infancy: a comprehensive review. *Nutrients.* (2020) 12:133. doi: 10.3390/nu12010133
35. Ferrocino I, Di Cagno R, De Angelis M, Turroni S, Vannini L, Bancalari E, et al. Fecal microbiota in healthy subjects following omnivore, vegetarian and vegan diets: culturable populations and rRNA DGGE profiling. *PLoS ONE.* (2015) 10:e0128669. doi: 10.1371/journal.pone.0128669
36. Zierer J, Jackson MA, Kastenmuller G, Mangino M, Long T, Telenti A, et al. The fecal metabolome as a functional readout of the gut microbiome. *Nat Genet.* (2018) 50:790–5. doi: 10.1038/s41588-018-0135-7
37. Oliphant K, Allen-Vercoe E. Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. *Microbiome.* (2019) 7:91. doi: 10.1186/s40168-019-0704-8
38. Bailen M, Bressa C, Martinez-Lopez S, Gonzalez-Soltero R, Montalvo Lominchar MG, San Juan C, et al. Microbiota features associated with a high-fat/low-fiber diet in healthy adults. *Front Nutr.* (2020) 7:583608. doi: 10.3389/fnut.2020.583608
39. Thingholm LB, Ruhlemann MC, Koch M, Fuqua B, Laucke G, Boehm R, et al. Obese individuals with and without type 2 diabetes show different gut microbial functional capacity and composition. *Cell Host Microbe.* (2019) 26:252–64.e10. doi: 10.1016/j.chom.2019.07.004
40. Ridlon JM, Kang DJ, Hylemon PB. Bile salt biotransformations by human intestinal bacteria. *J Lipid Res.* (2006) 47:241–59. doi: 10.1194/jlr.R500013-JLR200
41. Jones ML, Martoni CJ, Ganopolsky JG, Labbe A, Prakash S. The human microbiome and bile acid metabolism: dysbiosis, dysmetabolism, disease and intervention. *Expert Opin Biol Ther.* (2014) 14:467–82. doi: 10.1517/14712598.2014.880420
42. Ohtani N, Hara E. Gut-liver axis-mediated mechanism of liver cancer: a special focus on the role of gut microbiota. *Cancer Sci.* (2021) 12:133. doi: 10.1111/cas.15142

43. Wang S, Deng Y, Xie X, Ma J, Xu M, Zhao X, et al. Plasma bile acid changes in type 2 diabetes correlated with insulin secretion in two-step hyperglycemic clamp. *J Diabetes.* (2018) 10:874–85. doi: 10.1111/1753-0407.12771
44. Hegyi P, Maleth J, Walters JR, Hofmann AF, Keely SJ. Guts and gall: bile acids in regulation of intestinal epithelial function in health and disease. *Physiol Rev.* (2018) 98:1983–2023. doi: 10.1152/physrev.00054.2017
45. Wang F, Wan Y, Yin K, Wei Y, Wang B, Yu X, et al. Lower circulating branched-chain amino acid concentrations among vegetarians are associated with changes in gut microbial composition and function. *Mol Nutr Food Res.* (2019) 63:e1900612. doi: 10.1002/mnfr.201900612
46. Gojda J, Strakova R, Plihalova A, Tuma P, Potockova J, Polak J, et al. Increased incretin but not insulin response after oral versus intravenous branched chain amino acids. *Ann Nutr Metab.* (2017) 70:293–302. doi: 10.1159/000475604
47. Newgard CB. Interplay between lipids and branched-chain amino acids in development of insulin resistance. *Cell Metab.* (2012) 15:606–14. doi: 10.1016/j.cmet.2012.01.024
48. Descamps HC, Herrmann B, Wiredu D, Thaiss CA. The path toward using microbial metabolites as therapies. *EBioMedicine.* (2019) 44:747–54. doi: 10.1016/j.ebiom.2019.05.063

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.2 Development of an alternative method for the estimation of specific function(s) of gut microbiota

Although 16S rRNA sequencing is widely used to investigate the composition of bacterial communities in the gut, it does not provide insight into the functional aspects of these microorganisms and taxonomic resolution is in some cases insufficient. This knowledge gap has been addressed by the use of shotgun sequencing, which can provide a comprehensive view of the gut microbiota and its functional capacity. However, the high cost of this technique and relatively high requirements for bioinformatic skills has made it inaccessible to many laboratories. To address this problem, we focused on developing a simple and cost-effective method to estimate the functional capacity of butyrate synthesis by the gut microbiota. This method focuses on RT-qPCR quantification of the bacterial gene encoding butyryl-CoA:acetate CoA-transferase, a key enzyme involved in butyrate synthesis. As the importance of butyrate in overall health is increasingly recognized, the proposed method may serve as a valuable tool for investigating the role of gut microbiota in health and disease.

As a first step towards developing a simple and inexpensive method to estimate the functional capacity of butyrate synthesis by the gut microbiota, we searched for human butyrate-producing gut bacteria whose genome contains *but* gene coding sequences. Thirty-six bacterial genomes containing the *but* gene were selected for further analysis, but due to the large variation in *but* coding sequences among the selected bacteria, it was not possible to design a single primer targeting all sequences at once. Therefore, six sets of degenerate primers targeting selected groups of bacteria were designed and validated based on bacterial phylogenetic distance and similarity of *but* gene sequences. All primers were validated based on the length of their PCR products, where the predicted and observed lengths matched.

To quantify the qPCR results, a reference (housekeeping) gene had to be selected, which was a difficult task given the complexity of human stool. We compared two strategies. First, we used the 16S rRNA gene, which is universal to all bacteria and therefore inherently present in any sample. The target gene is quantified relative to the copy number of the 16S rRNA gene. The disadvantage of this approach is the variable number of 16S rRNA genes per genome in different bacteria, which may influence the results. The second strategy was based on a DNA spike whose sequences are not found in humans, such as the gene originating from the worm *Caenorhabditis elegans*. This method should be more precise but more demanding on labor and material. The target gene is quantified relative to the amount of spike DNA originating from the *C. elegans* worm that was added prior to fecal DNA isolation. Surprisingly, copy numbers normalized against both the 16S rRNA gene and the *C. elegans* gene were correlated for all primer sets, which was also verified by the Bland-Altman method.

The developed method was then applied to DNA extracted from stool samples of a cohort of healthy lean vegans (VG, n = 63) and healthy obese omnivores (OB, n = 62) with known information about their fecal microbiota and metabolome composition. In both groups, the highest abundance of the *but* gene was found when using primers targeting cluster C of selected bacteria. Cluster C included the bacterial taxa *Faecalibacterium prausnitzii*, *Clostridium symbiosum*, *Clostridium* sp. M62/1 and three species belonging to the genus *Eubacterium*, and the VG group in this cluster differed significantly in *but* gene abundance from the OB group (Mann-Whitney U-test, $p < 0.001$). The abundance of the *but* gene determined by qPCR targeting all bacterial clusters correlated with results obtained previously from 16S rRNA sequencing. In addition, the higher copy number of the *but* gene in the VG group corresponded to the significantly higher amount of butyrate (Mann-Whitney U-test, $p = 0.002$)

in respective fecal samples determined by NMR. Thus, our results support the hypothesis that the *but* gene copy number determination in bacterial DNA reflects its taxonomic composition, especially in the case of the more abundant bacteria, as well as a functional readout, in this case, the butyrate content of the feces.

In conclusion, this method may represent a powerful tool for estimating the functional capacity of the gut microbiota for butyrate synthesis based on qPCR quantification of bacterial butyryl-CoA:acetate CoA-transferase, provides deeper insight into the functional capacity of a particular sample, and could be useful for individual estimation of the utility of prebiotic therapy. This approach requires only equipment and skills commonly available in diagnostic laboratories and does not require advanced bioinformatic data analysis, making it a useful method for rapid screening of the specific functional capacity of the gut microbiota.

This article was published on the 2nd of September 2021 in Biomolecules, IF = 4.93

biomolecules

MDPI

Article

Determination of Butyrate Synthesis Capacity in Gut Microbiota: Quantification of *but* Gene Abundance by qPCR in Fecal Samples

Nikola Daskova [1,2,*], Marie Heczkova [1], Istvan Modos [1], Petra Videnska [3], Petra Splichalova [3], Helena Pelantova [4], Marek Kuzma [4,5], Jan Gojda [6] and Monika Cahova [1]

[1] Institute for Clinical and Experimental Medicine, Videnska 1958, 140 21 Prague 4, Czech Republic; mahz@ikem.cz (M.H.); modi@ikem.cz (I.M.); moca@ikem.cz (M.C.)
[2] First Faculty of Medicine, Charles University, Katerinska 1660/32, 121 08 Prague, Czech Republic
[3] RECETOX, Faculty of Science Masaryk University, Kamenice 753, 625 00 Brno, Czech Republic; petra.videnska@recetox.muni.cz (P.V.); splichalova@recetox.muni.cz (P.S.)
[4] Institute of Microbiology, AS CR, Videnska 1083, 142 20 Prague 4, Czech Republic; pelantova@biomed.cas.cz (H.P.); kuzma@biomed.cas.cz (M.K.)
[5] Department of Analytical Chemistry, Faculty of Science, Palacky University Olomouc, 17. Listopadu 1192/12, 779 00 Olomouc, Czech Republic
[6] Department of Internal Medicine, Kralovske Vinohrady University Hospital and Third Faculty of Medicine, Charles University, Srobarova 1150, 100 34 Prague 10, Czech Republic; jan.gojda@lf3.cuni.cz
* Correspondence: daon@ikem.cz

Citation: Daskova, N.; Heczkova, M.; Modos, I.; Videnska, P.; Splichalova, P.; Pelantova, H.; Kuzma, M.; Gojda, J.; Cahova, M. Determination of Butyrate Synthesis Capacity in Gut Microbiota: Quantification of *but* Gene Abundance by qPCR in Fecal Samples. *Biomolecules* **2021**, *11*, 1303. https://doi.org/10.3390/biom11091303

Academic Editor: Piotr Ceranowicz

Received: 28 July 2021
Accepted: 1 September 2021
Published: 2 September 2021

Abstract: Butyrate is formed in the gut during bacterial fermentation of dietary fiber and is attributed numerous beneficial effects on the host metabolism. We aimed to develop a method for the assessment of functional capacity of gut microbiota butyrate synthesis based on the qPCR quantification of bacterial gene coding butyryl-CoA:acetate CoA-transferase, the key enzyme of butyrate synthesis. In silico, we identified bacteria possessing *but* gene among human gut microbiota by searching *but* coding sequences in available databases. We designed and validated six sets of degenerate primers covering all selected bacteria, based on their phylogenetic nearness and sequence similarity, and developed a method for gene abundance normalization in human fecal DNA. We determined *but* gene abundance in fecal DNA of subjects with opposing dietary patterns and metabolic phenotypes—lean vegans (VG) and healthy obese omnivores (OB) with known fecal microbiota and metabolome composition. We found higher *but* gene copy number in VG compared with OB, in line with higher fecal butyrate content in VG group. We further found a positive correlation between the relative abundance of target bacterial genera identified by next-generation sequencing and groups of *but* gene-containing bacteria determined by specific primers. In conclusion, this approach represents a simple and feasible tool for estimation of microbial functional capacity.

Keywords: gut microbiota; butyrate; functional capacity

1. Introduction

The gut microbiota is now recognized as a "new organ", and its role in health and disease has become widely acknowledged. Emerging evidence supports the hypothesis that gut microbiota dysbiosis is closely related to the development of non-communicable diseases, including cardiovascular diseases, colorectal cancer, obesity, or type 2 diabetes (T2D) [1–5]. Host–microbiome interactions are heterogeneous and multifaceted, some of them being mediated by microbial fermentation products.

Short-chain fatty acids (SCFA), acetate, butyrate, and propionate, are primary products of microbial fermentation of dietary fiber [6–8] in the colon. Acetate is the most abundant product of fiber fermentation and serves as a suppressor of adipocyte lipolysis. It has been proposed to stimulate leptin secretion in adipocytes [9] and may also regulate appetite

and satiety [10]. Propionate is metabolized in the liver, where it seems to be the inhibitor of de novo lipogenesis as well as synthesis of cholesterol [11]. Major attention is focused on butyrate. In contrast to propionate and acetate, which are transported to systemic circulation, butyrate is predominantly used by colonocytes, where it serves as a major source of energy. In addition to this trophic function, it exerts several other beneficial effects. It helps to control malignant transformation of colonocytes; in healthy cells, it promotes proliferation, while in transformed cells, it induces apoptosis [6,9,12–14]. Butyrate ameliorates inflammation as it binds GPR109a receptor on dendritic cells associated with intestinal mucosa and stimulates production of IL-10 and subsequent activation of anti-inflammatory Treg cells [15]. Activation of GPR109a receptor also suppresses production of pro-inflammatory cytokines TNFα and IL-6 [16]. Butyrate, as well as other SCFAs, stimulates endocrine L-cells to release GLP-2, which regulates the expression of tight junction proteins essential for maintaining intestinal barrier integrity [17]. Furthermore, butyrate stimulates MUC2 gene expression and affects mucus production [18]. Butyrate and propionate contribute to the regulation of energy homeostasis and eating behavior of the host via binding to GRP41/43 receptors, which, in turn, stimulates the production of GLP-1 and peptide YY [19].

The knowledge of functional capacity of gut microbiota is essential for personalized medicine, allowing for efficient targeted treatment tailored to a particular patient's needs. For example, dietary fiber is often recommended due to its potential to stimulate production of SCFAs. Nevertheless, very high variability in individual response to fiber supplementation exists [20]. This variability could be explained, at least partly, by the different representation of key species of microorganisms and/or absence of the whole functional microbial communities responsible for fiber fermentation [21]. Therefore, the quantitative assessment of gut microbiota functional capacity is of great interest not only in a research context but also in terms of personalized medicine and nutrition.

The current microbiome research was enabled by the development of next generation sequencing (NGS) methods that allow sequencing of a wide variety of samples in a short time and for a reasonable price. At present, there are two main approaches to the microbiome composition determination—16S rRNA gene sequencing and shotgun sequencing [22,23]. Both methods face some limitations. Shotgun sequencing provides full information about the DNA sequence of the tested sample, i.e., DNA isolated from feces, and it is possible to derive full information about the bacteria functional capacity from the data. On the other hand, the routine implementation of this method is prevented by still relatively high costs and by high demands on bioinformatics capacity.

The analysis of variable regions of 16S ribosomal RNA gene (16S rRNA gene sequencing) is the most popular method for determination of microbial communities originating from various niches. At the end of July 2021, there were 63,875 results in PubMed search engine [24] for key word "16S rRNA sequencing". This method is widely available and able to provide results quickly and for a reasonable price, but its main disadvantage is insufficient taxonomic resolution, which can be problematic if a deep evaluation of species or strains is needed. It is inevitable that some species are overlooked or wrongly identified, which may significantly compromise the prediction of microbiota functional capacity and result in the disagreement with conclusions derived from metatranscriptomic or metabolomic analyses.

In this study, we focused on the development of a method for the assessment of functional capacity of a selected process, in our case, butyrate synthesis in gut microbiota. Gut microbes produce butyrate through two main pathways, the butyryl-CoA:acetate CoA-transferase pathway (*but*) and the butyrate kinase (*buk*) [25,26]. Using the collection of colonic isolates obtained from healthy individuals, Louis et al. (2004) [27] demonstrated that conversion of butyryl-CoA to butyrate catalyzed by butyryl-CoA:acetate CoA-transferase (*but*) is a dominant route for butyrate formation in the human colonic ecosystem while butyrate kinase/phosphotransbutyrylase pathway was present only in a minor portion of isolates. Therefore, we developed a method based on the real-time qPCR quantification of the bacterial gene coding butyryl-CoA:acetate CoA-transferase in DNA isolated from

human feces. We further show the application of this method on populations with different metabolic phenotypes, i.e., vegans and obese omnivores. Each participant signed an informed consent to the study.

2. Materials and Methods

2.1. Description of the Study Population

In this study, we used fecal samples obtained from self-reported vegans (VG, $n = 63$) who avoided all animal products for at least three years and healthy obese omnivores (OB, $n = 62$) without any dietary restrictions. In VG group, median BMI was 21.6 (min 17.6; max 32.5), in OB group, median BMI was 30.9 (min 23.3; max 55.1). VG group was comprised of younger subjects (median 31; min 18; max 58 years) compared to OB group (median 51; min 21; max 66 years). In both groups, the exclusion criteria were: age under 18 years, chronic diseases related to glucose metabolism, diseases of the digestive tract, antibiotic therapy in the past three months, pregnancy, any chronic medication (excluding hormonal contraception), and regular alcohol consumption. All data were obtained within an observational study TRIEMA supported by grant no. NV18-01-00040 MH CR. The research protocol was approved by the Ethics Committee of the Third Faculty of Medicine of the Charles University and the Ethics Committee of University Hospital Kralovske Vinohrady (EK-VP/26/0/2017) in accordance with the Declaration of Helsinki.

2.2. Fecal Samples Handling and Storage

Fecal samples collected at home were immediately stored at −20 °C until transported in the frozen state (<0 °C) to the laboratory within 7 days from collection. The samples were stored at −50 °C until processed. Once thawed on ice, 5–10 g of the samples were diluted in sterile water (1:4) and then homogenized using stomacher (BioPro, Prague, Czech Republic). One aliquot was used for DNA extraction, one aliquot was used for dry mass estimation, and the rest was aliquoted and stored at −50 °C.

2.3. DNA Isolation from Fecal Samples

DNA isolation was performed immediately after thawing and homogenization of the sample using QIAmp PowerFecal DNA Kit (Qiagen, Hilden, Germany). For each sample, two DNA isolations (each using 600 µL of the homogenate) were performed and DNA yields were combined. Then, DNA from every fecal sample was diluted to 10 ng/µL and used further on.

2.4. Preparation of Spike DNA

Caenorhabditis elegans worms were grown on agar plates that were covered with *Escherichia coli* and harvested as described in [28]. DNA was extracted as described above. Primers for UNC-6 gene ("inner primer") and a wider region of *C. elegans* DNA containing fragment transcribed from UNC-6 (referred to as "outer primer") were designed in Primer 3 software [29]. The preparation of spike DNA fragment is shown on Figure 1. Sequences for both primer pairs are shown in Table 1. Using Nucleotide BLAST tool [30], we confirmed that UNC-6 gene target sequence is unique and was not found in any organism other than *C. elegans*. Using the outer primer pair, we amplified the DNA fragment containing UNC-6 sequence and separated it by gel electrophoresis. The resulting fragment was eluted using Gel Extraction Kit (Qiagen, Hilden, Germany). We obtained a fragment of *C. elegans* DNA allowing for the "inner" UNC-6 primers annealing. *C. elegans* DNA was diluted to 2 ng/µL, and after optimization, the fragment was used further on as a spike to our samples (sample DNA was mixed with *C. elegans* DNA).

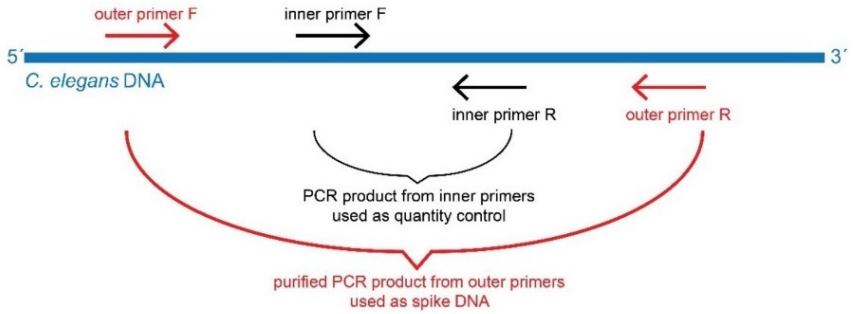


Figure 1. Graphical representation of spike DNA preparation.

Table 1. Sequences of inner and outer primer pairs used in spike preparation.

Primer Name	Primer Sequence (5′ to 3′)
inner primer forward	GAAGAGCAAGATCAGTGTTC
inner primer reverse	CTTGCAAATGACACCTTG
outer primer forward	GTAATCGTTGTGCCAAAGG
outer primer reverse	TCCTCCCATTCCACCAATAC

2.5. Design of the Degenerate Primers for but Gene and Analysis of PCR Products

Degenerate primers targeting *but* gene were designed as described in Section 3. The resulting products were analyzed on Fragment Analyzer 5200 (Agilent, Santa Clara, CA, USA) using Agilent dsDNA 910 reagent Kit (35–1500 bp). The results were processed and checked in PROSize software (version 4.0.1.4).

2.6. qPCR

The copy number of *but* gene in DNA isolated from stool samples was determined by quantitative PCR (qPCR). qPCR reaction was performed with Syber Green master mix solution (Quanti-Tect, Qiagen, Hilden, Germany) in a volume of 20 µL with primer concentration varying from 200 nM to 1 µM (according to degeneracy number of each primer pair). Final concentration of stool DNA was 0.5 ng/µL and *C. elegans* DNA 0.1 ng/µL in the reaction mixture. Reaction was run on ViiA7 Real-Time PCR System with 96-well plates (Applied Biosystems, Waltham, MA, USA) according to the following protocol: (i) initial denaturation: 95 °C, 12 min; (ii) propagation (40 cycles): denaturation 95 °C, 15 s; annealing 60 °C, 30 s; elongation 72 °C, 20 s. The results were analyzed by SDS software version 2.3 (Applied Biosystems, Waltham, MA, USA). The copy number of genes of interest was normalized to spike DNA (*C. elegans* UNC-6 gene) or to the 16S rRNA gene (forward primer ACACTGACGACATGGTTCTACAGAGTTGATCNTGGCTCAG, reverse primer TACGGTAGCAGAGACTTGGTCTGTNTTANGCGGCKGCTG) and calculated using ΔCt method.

2.7. Gut Microbiome Taxonomic Analysis

DNA from fecal samples was isolated as mentioned before and V4 region of the bacterial 16S rRNA gene was amplified by PCR [31]. Sequencing analysis was performed on MiSeq (Illumina, Hayward, CA, USA) as described previously [32]. Raw sequences were processed using an in-house pipeline based on DADA2 amplicon denoiser [33]. Raw sequences were processed using standard bioinformatic procedures within the QIIME 1.9.1 package [34].

2.8. Quantification of Butyrate in Fecal Samples by NMR Spectroscopy

Fecal extracts for NMR analysis were prepared from homogenized stool aliquots corresponding to 1.5% of dry mass. NMR experiments (1D-NOESY and *J*-resolved with presaturation) were performed on a Bruker AVANCE III 600 MHz spectrometer (Bruker, Billerica, MA, USA) at 25 °C according to the standard protocols [35]. Butyrate signals were identified by the comparison of proton chemical shifts with HMDB database. Butyrate was quantified from 1D projections of *J*-resolved spectra to overcome the problem of signal overlap. The concentration was expressed as PQN normalized intensity of butyrate signal at 0.90 ppm.

2.9. Statistical Evaluation

All statistical analyses were performed using R software version 4.1.0 with in-house scripts [36]. The normality of distribution of the *but* gene abundance was tested by the Shapiro–Wilk test for normality using the shapiro.test function from the stats package (on each phylogenetically related bacteria and subject group), where the null hypothesis corresponds to data normality. Because the normal data distribution was not confirmed ($p < 0.05$), univariate statistical analyses were performed by Mann–Whitney–Wilcoxon test using the wilcox.test function from the stats package with significance of 0.05. The microbiome data were treated as compositional (proportions of total read count in each sample, nonrarefied) and, prior to all statistical analyses, were transformed using centered log-ratio transformation [37]; zero values were handled using count zero multiplicative replacement (using the cmultRepl function from the zCompositions package). The correlation between variables was assessed by using Spearman's rank correlation coefficient utilizing cor and corrplot functions. Bland–Altman plots were used to analyze the difference between the two qPCR normalization methods (using the blandr.draw function from the blandr package).

3. Results

3.1. Identification of the Target Bacteria

As the first step, we identified bacteria containing the *but* gene (coding the enzyme butyryl-CoA:acetate CoA-transferase) in their genome using FunGene Database [38]. Every bacterium possessing this gene according to the FunGene database was searched for individually through available literature. If the bacterium was found in human gut microbiota and if it was previously confirmed as a butyrate producer [7,26,39,40], we used the bacterium further on. Every other bacterium was disregarded. In total, we identified thirty-six bacterial genomes possessing the *but* gene and meeting criteria mentioned above. Next, the specific gene sequence, as well as the amino acid sequence of the enzyme in a particular genome, was cross checked in National Centre for Biotechnology Information (NCBI) database and used further on (Table 2).

3.2. Design of the Degenerate Primers for but Gene

The sequence of *but* gene coding for butyryl-CoA:acetate CoA-transferase is highly variable among gut butyrate producers, and therefore, it is not possible to design one primer fitting all target species [39,41]. Before designing any primer, the CLUSTALW tool [42] was used for a multiple sequence alignment. The diversity of the *but* coding sequence did not allow us to design one, even degenerate, primer. Therefore, we grouped the bacteria according to their phylogenetic distance, aiming to obtain groups of more similar target sequences. We used the bioinformatic web service Phylogeny.fr [43] and obtained a phylogenetic tree of closely related bacteria (Figure 2). We used the following tools: (i) MUSCLE 3.8.31 for the alignment, (ii) Gblocks 0.19b for the alignment refinement, (iii) PhyML 3.1/3.0 aLRTFor for utilizing the phylogeny analysis itself, and (iv) TreeDyn 198.3 to display the tree.

Table 2. Butyrate producers in human gut microbiota possessing the *but* gene. A total of thirty-six *but* gene nucleotide and protein sequences were identified (stated as an NCBI accession numbers).

Taxonomy (including the Strain)	Nucleotide Sequence Accession Number	Protein Sequence Accession Number
Anaerostipes caccae DSM 14662	ABAX03000012	WP_006566634
Anaerobutyricum hallii DSM 3353	ACEP01000025	EEG37758
Anaerostipes hadrus DSM 3319	AMEY01000089	EKY19441
Clostridiales bacterium KA00134	LTAF01000006	KXO16903
Clostridium sp. JN-9	CP035280	QAT39812
Clostridium sp. M62/1	ACFX02000051	EFE10856
Clostridium sp. SS2/1	ABGC03000034	EDS21983
[*Clostridium*] *propionicum* DSM 1682	FQUA01000004	SHE65336
[*Clostridium*] *symbiosum* ATCC 14940	AWSU01000039	ERI80067
[*Clostridium*] *symbiosum* WAL-14673	ADLR01000107	EGB17928
Coprococcus eutactus 2789STDY5608829	CYYZ01000002	CUN77211
Coprococcus eutactus 2789STDY5608843	CYYJ01000005	CUO17024
Coprococcus eutactus 2789STDY5608888	CYYE01000001	CUN69525
Coprococcus eutactus 2789STDY5834963	CYXU01000007	CUN05838
Eubacterium callanderi FD	FRBP01000012	SHM18802
Eubacterium limosum ATCC 8486	CP019962	ARD67787
Eubacterium limosum SA11	CP011914	ALU15403
Eubacterium maltosivorans YI	CP029487	QCT73558
Eubacterium sp. 14-2	ASSS01000012	EOT23498
Faecalibacterium prausnitzii 942/30-2	CP026548	AXA81262
Faecalibacterium prausnitzii A2165 CG447_00005	CP022479	ATO98751
Faecalibacterium prausnitzii A2-165 FAEPRAA2165_01562	ACOP02000044	EEU96797
Faecalibacterium prausnitzii APC918/95b	CP030777	AXB28579
Faecalibacterium prausnitzii Indica	CP023819	ATL89114
Faecalibacterium prausnitzii KLE1255	AECU01000083	EFQ07628
Flavonifractor plautii 2789STDY5834892	CZAS01000006	CUP57950
Flavonifractor plautii 2789STDY5834932	CZBD01000023	CUQ37563
Lachnospiraceae bacterium 3-1	ASST01000018	EOS23550
Lachnospiraceae bacterium 3-1	ASST01000032	EOS21051
Lachnospiraceae bacterium A2	ASSX01000004	EOS48506
Lachnospiraceae bacterium A4	ASSR01000007	EOS36856
Lachnospiraceae bacterium MD335	ASSW01000016	EOS51721
Lachnospiraceae bacterium TF01-11	LLKB01000001	KQC86641
Pseudoflavonifractor capillosus ATCC 29799	AAXG02000004	EDN01706
Roseburia intestinalis L1-82	ABYJ02000099	EEV00989
Roseburia inulinivorans DSM 16841	ACFY01000152	EEG92587

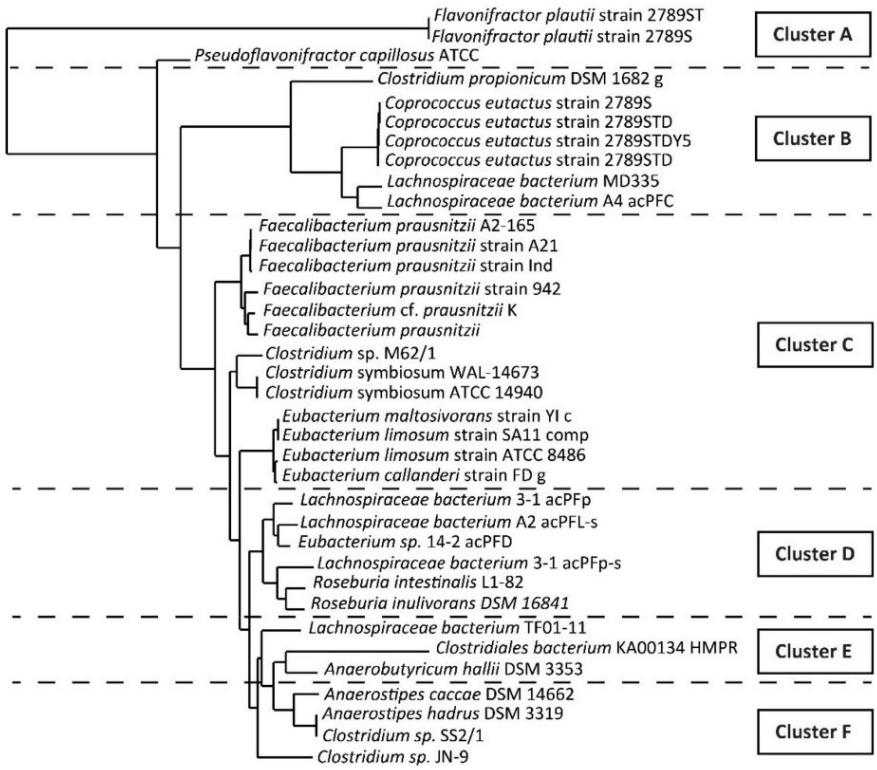


Figure 2. Results obtained from phylogenetic analysis. Six clusters were constructed (cluster A–F) based on phylogenetic distance. For each cluster, one pair of degenerate primers was designed.

Based on the phylogenetic distance, we constructed bacterial clusters and tried to design one degenerate primer for each of the suggested clusters. If we did not succeed, i.e., the variability within the group was too high, we stepped down in the phylogenetic tree to the nearest lower cluster and designed a new set of primers. Finally, we obtained six clusters (A–F) (Figure 2). For each cluster, one pair of degenerate primers was designed by using CEMASuite software (version 2.0.9) [44]. Primers were checked with Primer BLAST tool [45] to avoid any undesired cross reactivity with other gut bacteria or human DNA. The sequences and expected lengths of the degenerate primers are shown in Table 3.

3.3. Validation and Optimization of Designed Primers

Primers designed in silico were validated and optimized in DNA isolated from human stool samples. Compared to the conventional primers that perfectly match with the target sequence, the use of degenerate primers brings specific issues that must be addressed. First, a degenerate primer represents a mix of possible nucleotide combinations that do not bind to the target sequence with the same efficiency. The more degenerate the primer is, the more specific combinations may exist, and the binding efficiency to the particular target sequence is lower. Second, individual batches of the same primers are not identical, i.e., the exact combination of possible variants is unique for every single batch. Therefore, we had to experimentally define the optimal concentration of each primer for every new batch. In contrast to the usual concentration of a conventional primer (200 nM) the required concentration of degenerate primers is higher and varied in the range 200 nM–1 µM in our experiments based on the degeneracy number of each primer pair. The specificity of primer–target interaction was checked by PCR product analysis. The length of PCR

products was checked with separation resolution as good as 3 bp. For all products, the predicted and determined length agreed (Figure 3).

Table 3. Sequences and expected product lengths of each primer set.

Primer Name	Primer Sequence (5′ to 3′)	Expected Product Length (nt)
but cluster A forward	MCTGGGYATYCACACCGAG	574
but cluster A reverse	GGTGGGCGATGGAGATAA	
but cluster B forward	GGKCCBATHGARRTTGCAGA	679
but cluster B reverse	TKTCGTCMASCCABTCATAC	
but cluster C forward	GBGACTGGSTRGATTAYG	682
but cluster C reverse	TCVACRTACATYTCSGTGTG	
but cluster D forward	TGGAAYTCMTGGCATATGTC	726
but cluster D reverse	VGMRTTGTTRATGGAMATAAA	
but cluster E forward	TGHAGSABHTSWTTTTACATGGA	558
but cluster E reverse	SSCTTTGCAATGTCAACAAA	
but cluster F forward	AAATATGCCTCGHTGCYTWG	585
but cluster F reverse	ARRTARGCACCYAWAACGAAATC	

Primers designed for *but* gene are divided based on their phylogenetic distances into clusters A–F. nt = nucleotide; K = G or T; B = C or G or T; H = A or C or T; R = A or G; M = A or C; S = G or C; Y = C or T; V = A or C or G; W = A or T (as stated by IUPAC nucleotide code).

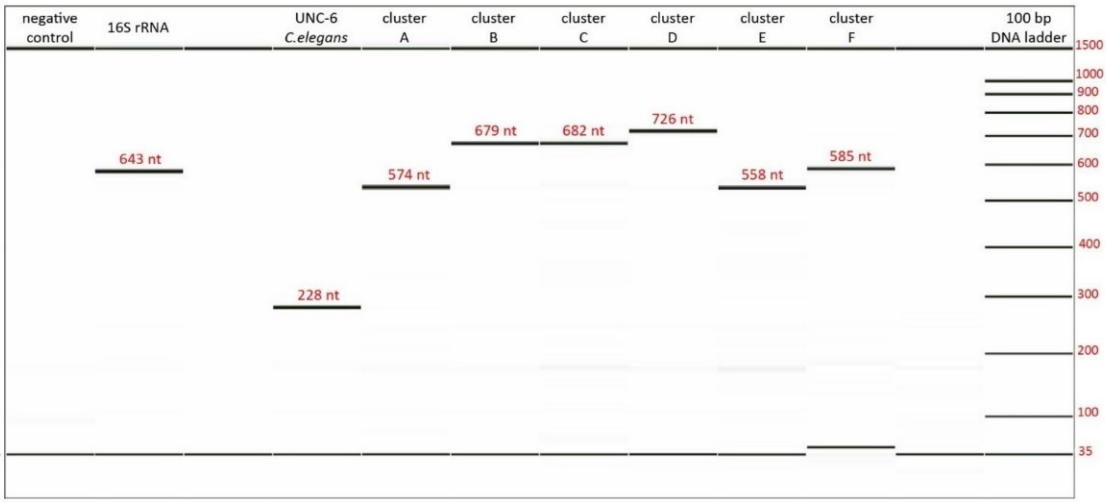


Figure 3. Results obtained from fragment analysis. Above every band, expected product length (number of nucleotides) is shown in red. The actual length can be estimated by the DNA ladder on the right.

3.4. Normalization of qPCR Results

Quantification of any target sequence by qPCR depends on the stable and robust reference (housekeeper) gene. The normalization of qPCR results in stool samples is challenging as, in this material, no housekeeper gene exists. To solve this problem, we employed two different strategies. The first is based on the spike DNA, added in a standard amount to the sample prior the isolation of DNA. To this end, we chose gene coding of the protein netrin UNC-6 from *C. elegans*, as there is minimal chance of *C. elegans* natural occurrence in the human gut. The second strategy quantifies the target gene in relation to the number of 16S rRNA gene copies, particularly the conserved sequence in the

V1–V3 region. Despite the differences between the normalization methods, their outcomes correlate, ranging from 0.4818 to 0.9331 of Spearman's R values (Figure 4), $p < 0.001$.

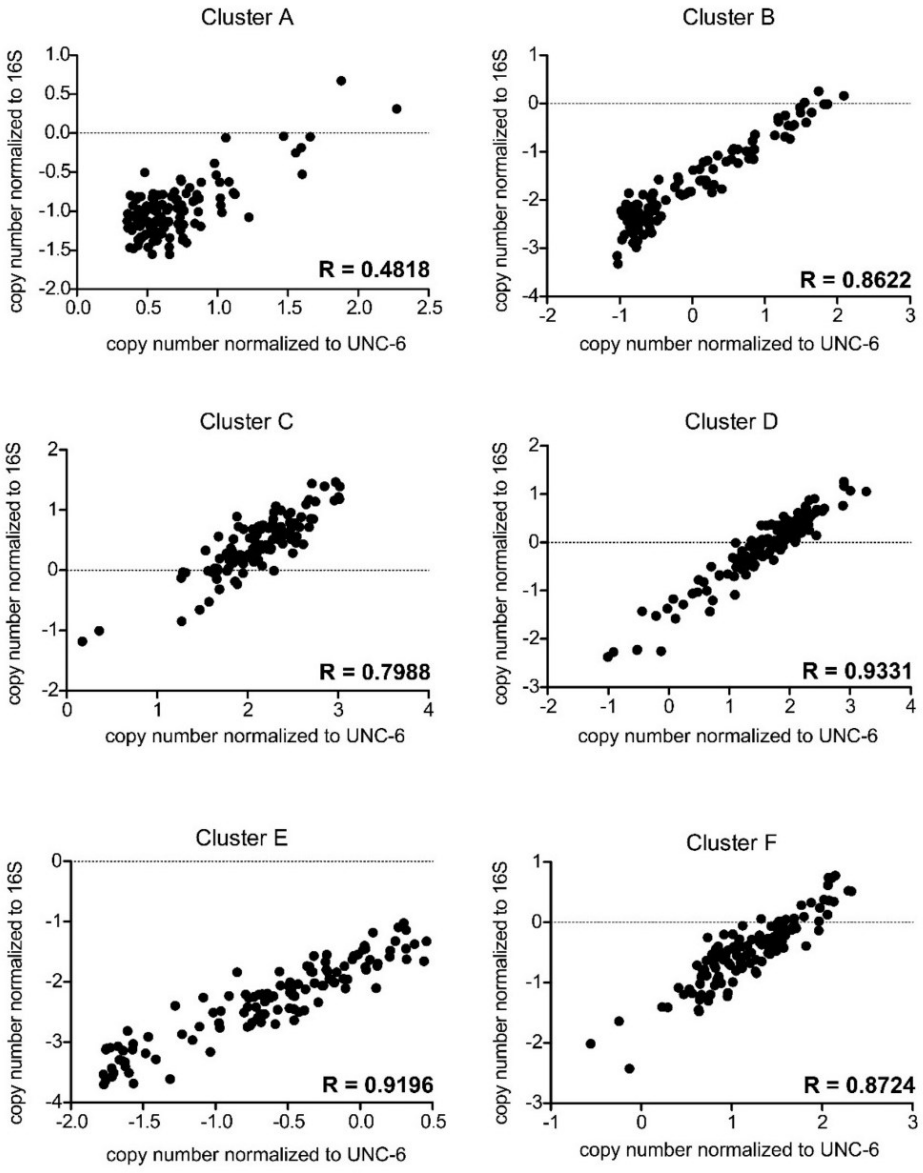


Figure 4. Correlation plots (each for cluster **A**–**F**) showing two different normalization methods for each cluster. *x*-axis: log transformed *but* copy number normalized to UNC-6 gene from *C. elegans*. *y*-axis: log transformed *but* copy number normalized to 16S rRNA gene. Spearman's R correlation values are shown.

Nevertheless, significant correlation between the outcomes of two ways of *but* gene abundance normalization does not prove that these two methods are identical. Therefore, we employed the Bland–Altman method (Figure 5). The analysis revealed that the ratio between the normalization methods does not change with the magnitude of the measured values. The results of correlation and Bland–Altman analyses revealed comparable outcomes from both normalization methods.

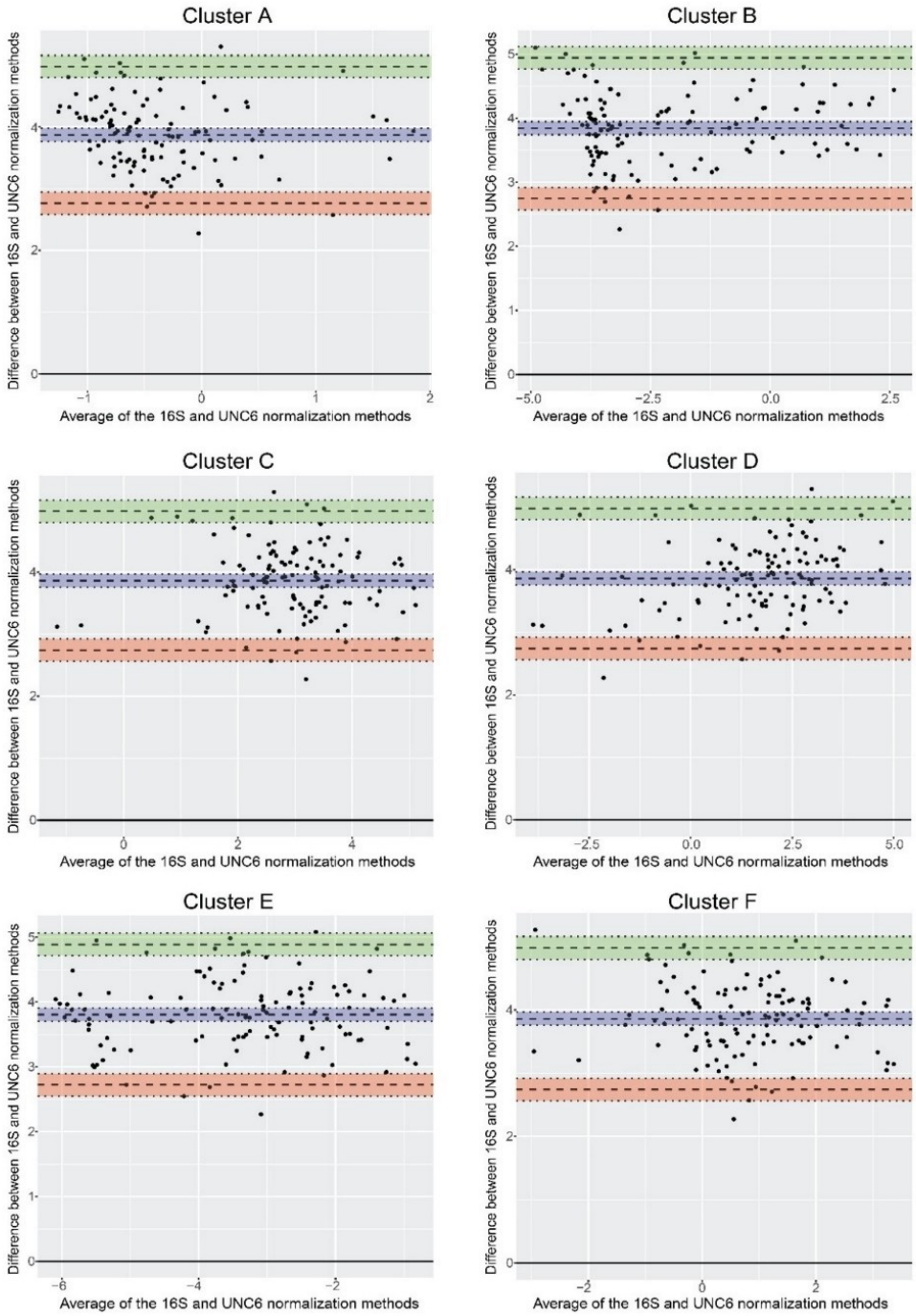


Figure 5. The Bland–Altman plots for each cluster that compare normalization methods based on the 16S rRNA or UNC-6 genes (after log-transform). The *x*-axis is the average copy number obtained by both normalization methods and the *y*-axis represents the difference between the outcomes of normalization according to 16S rRNA and UNC-6 genes. The blue region is the bias with its 95% confidence interval, green region is the upper limit of agreement with its 95% confidence interval, and the red region is the lower limit of agreement with its 95% confidence interval.

3.5. Quantification of but Gene in Populations with Contrast Phenotypes

We determined the abundance of the *but* gene in two experimental groups with different dietary habits and metabolic phenotypes, i.e., lean vegans and obese omnivores. In both vegans and omnivores, we found the highest abundance of the *but* gene was determined by primers specific for cluster C and lowest using those specific for cluster B (B < E < A < F < D < C). As shown in Table 4, vegans and obese omnivores significantly differed in the *but* gene abundance in cluster A (higher in OB) and cluster C (higher in VG). *But* gene copy number in cluster D tended to be higher in VG as well, but it did not reach statistical significance. Cluster A comprises *Flavonifractor plautii* and *Pseudoflavonifractor capillosus*. Cluster C encompasses *Faecalibacterium prausnitzii*, *Clostridium symbiosum*, *Clostridium* sp. M62/1, and three species belonging to genus *Eubacterium*. Cluster D is comprised of three species belonging to genus *Lachnospiraceae*, *Roseburia intestinalis* and *Roseburia inulivorans*, and one *Eubacterium* species. For quantification, we used both normalization methods. The results were comparable in terms of abundance of individual clusters and VG to OB ratio. In absolute values, the obtained numbers were lower using 16S rRNA gene for normalization due to the high abundance of this gene compared with the gene of interest (*but*). The difference between both normalization methods lies in the unstable 16S rRNA gene copy number per bacterial genome at various bacterial taxa. However, as shown by the downstream-statistical analysis (Table 4), both normalization methods were able to detect the same significant or insignificant differences between the subjects.

Table 4. *but* gene copy number normalized to either UNC-6 gene from *C. elegans* or to 16S rRNA gene in vegan (VG) and obese (OB) subjects. Data are given as median (IQR, interquartile range). In each sample, *but* gene copy number was determined using all primer pairs in separate qPCR reactions. The similarity of the distribution in VG and OB groups was tested using Mann–Whitney U test. The results were considered statistically significantly different at $p < 0.05$ (shown in bold).

	Cluster A		**Cluster B**		**Cluster C**		**Cluster D**		**Cluster E**		**Cluster F**	
	UNC	**16S**	**UNC**	**16S**	**UNC**	**16S**	**UNC**	**16S**	**UNC**	**16S**	**UNC**	**16S**
VG	3.5 (2.0)	0.08 (0.06)	0.45 (5.09)	0.01 (0.09)	211 (243)	4.9 (5.8)	63 (118)	1.1 (2.4)	0.28 (0.48)	0.01 (0.01)	12.5 (25.1)	0.34 (0.41)
OB	4.8 (3.5)	0.10 (0.10)	0.28 (1.91)	0.01 (0.05)	86 (71)	1.8 (1.6)	34 (76)	0.9 (1.6)	0.32 (0.61)	0.01 (0.01)	17.6 (30.9)	0.38 (0.70)
p-value	**0.004**	**0.019**	0.942	0.438	**<0.001**	**<0.001**	0.167	0.225	0.769	0.840	0.680	0.589

Aiming to validate these results, we correlated the abundance of *but* gene in different clusters with the microbiome composition of the same samples (Supplementary Table S1) determined by 16S rRNA gene sequencing (Figure 6). In the cases of clusters B, C, D, E, and F, the abundance of *but* gene most significantly correlated with the expected bacterial taxa (significance level was set to 0.05). Regarding cluster A, we found a trend to the correlation between the abundance of gene *but* and *Pseudoflavonifractor* and, to a lesser extent, *Flavonifractor*, but these correlations were not significant on the chosen significance level. Importantly, our findings, i.e., higher copy number of *but* gene in vegan group, were in line with the significantly higher amount of butyrate in vegan fecal samples, $p = 0.002$ (Figure 7).

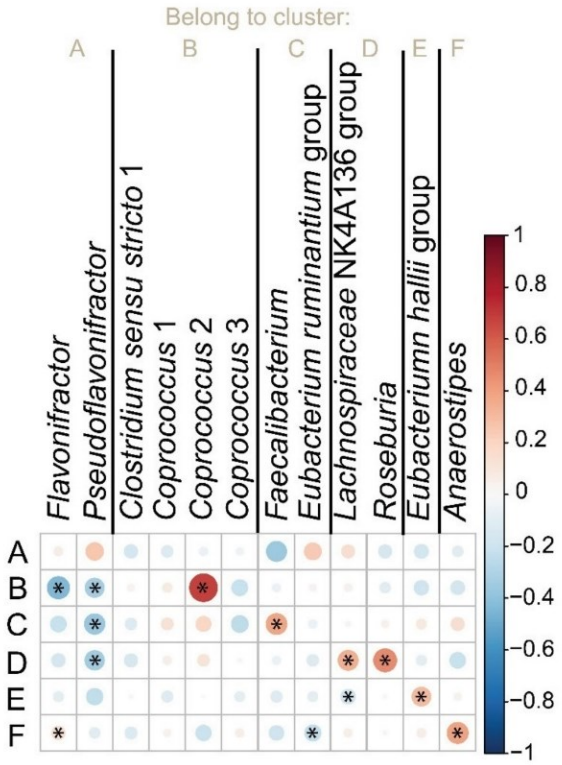


Figure 6. Graphical representation of Spearman's correlation matrix between *but* copy number identified by primers A–F and relative abundance of representative bacteria for every cluster determined by NGS. The *but* copy number was normalized to UNC-6 gene. The circle size and color intensity are proportional to Spearman's coefficient value. The red color of a circle indicates positive correlation, the blue color negative correlation. Areas with an asterisk sign inside the circle indicate that the specific correlation was significant on the significance level of 0.05.

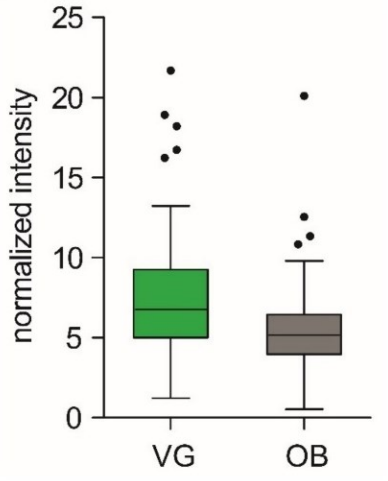


Figure 7. Butyrate content in feces of VG and OB subjects. Values represent PQN normalized intensities of butyrate-specific signal in NMR spectra. Data are presented as Tukey box plots with median and whiskers (1st, 3rd quartile).

4. Discussion

In this study, we describe the development of a widely accessible method for the assessment of functional capacity of gut microbiota for butyrate synthesis based on the qPCR quantification of bacterial butyryl-CoA:acetate CoA-transferase. The workflow includes: (i) isolation of DNA from the fecal sample; (ii) performing multiple qPCRs using degenerate primers specific for *but* gene variants; and (iii) quantification of *but* gene abundance using the selected reference gene. We further demonstrate the application of this method in the assessment of butyrate synthesis capacity in the fecal microbiome of lean vegans and healthy obese omnivores. Our results show that some *but* gene variants are far more abundant than the others; therefore, when the aim is only the approximate estimation of butyrate synthesis capacity, the method could be further simplified by using only the primers specific for the most abundant clusters of bacteria.

Quantification of a particular gene of interest (GOI) in the studied microbial population allows for deeper insight into the real functional capacity of the particular sample. On the other hand, this approach must cope with another type of limitations. Variability of GOI sequences among bacteria does not allow for designing one universal primer. This obstacle could be overcome by employing degenerate primers, but even adopting this approach, it is usually impossible to design one primer for all target sequences. We propose that the identification of phylogenetically related groups of bacteria sharing some similarity is the solution to this problem. Because of the random selection of nucleotides in the degenerate positions, individual batches of degenerate primers may significantly vary in composition. This inconvenience could be overcome by preparation of well-defined primer mixtures where each primer pair is present in the exact concentration. In practice, each primer pair may be synthesized separately and then all primers mixed in equimolar ratio. This approach may be more expensive and laborious at the beginning, but it allows for avoiding the necessity to determine the efficient primer concentration for every new batch.

The qPCR method is comparative in principle. Therefore, the selection of a stable reference gene is of utmost importance. Unfortunately, feces is quite challenging material from this point of view. It is extremely heterogeneous and varies greatly in the content of dry mass, total protein or total DNA. A standardized fecal sample processing and DNA isolation pipeline during the study is essential, as it turns out in recent years [46,47]. Furthermore, DNA isolated from feces is a mix of host, bacteria, fungi, and virus DNA at variable ratios. The final readout, i.e., the composition of the fecal microbial community, is affected by the stabilization and storage strategies used in the process of sampling. The most important factors are: (i) usage of preservation buffers; (ii) time from sample production and freezing; (iii) storage temperature, and (iv) aerobic vs. anaerobic conditions during storage [48]. A standardized fecal sample processing and DNA isolation pipeline during the study is essential, as it turns out in recent years [46,47]. In our study, we decided for sample storage without preservation buffers, as their presence makes subsequent metabolomic analysis impossible. The native samples were immediately frozen in −20 °C for maximum 7 days; long-term storage occurred at −50 °C. Because the quick-freezing stops or maximally slows down biological processes, we processed the samples under aerobic conditions, as it simplifies the sample handling for the study participants.

The most commonly known gene shared by all bacteria and, thus, the potential housekeeper gene is the gene coding for 16S rRNA. Its advantage as a reference gene is that it contains conserved sequences common to all bacteria that could serve as target sequences of primers. The quantification of the 16S rRNA gene allows for the assessment of the total bacterial "load" in the sample. The limitation of 16S rRNA gene as a reference is the unequal number of 16S rRNA copies per cell in different bacterial species.

According to Větrovský and Baldrian's in silico study [49], with a total number of 1690 bacterial genomes with 909 species identified, there was an average of 4.2 16S rRNA gene copies per genome. The copy number of 16S rRNA gene was highly taxonomically specific (for example, in the Gammaproteobacteria and Fusobacteria, the copy number varied widely between 1 and 15). In some studies [25], the 16S rRNA gene copy number

was used for normalization of the *but* gene but had to be adjusted as an average number of copies in particular bacteria; in this case, five gene copy numbers were used representing Firmicutes and Bacteriodetes. Second, the abundance of 16S rRNA gene is an order of magnitude higher than the abundance of GOI, which results in low absolute values of the GOI copy number normalized to this reference gene.

An alternative possibility of normalization in this type of material is quantification to external DNA added to the sample. This approach is independent of microbiome composition of the sample, and it may serve as a quality control of the whole process as well. The prerequisite is the choice of sequence that is not present in any organism, which could be found in the target material, in our case, in human feces. The UNC-6 gene of *C. elegans* was a good candidate as we did not find any corresponding sequence when using Nucleotide BLAST tool [30]. Despite the limitations of both approaches, we observed high correlation between the *but* gene copy number normalized to both 16S rRNA and UNC-6 sequences, and Bland–Altman analysis revealed the similarity of the outcomes of both normalization methods. Nevertheless, we should be always aware of different numbers of 16S rRNA gene copies within the bacterial population. Therefore, we prefer the normalization to external DNA added to the sample; 16S rRNA gene quantification can be used for the control of equal loading of bacterial DNA to the PCR reaction.

The rationale behind this study was to develop a widely accessible and easily implementable method that would allow for the assessment of the functional capacity of the gut microbiota. The utmost readout of metabolic performance of bacteria is the presence and quantity of the particular metabolite(s). In the case of the *but* gene, such a readout is the content of butyrate in feces. Vegans and omnivore subjects represent different phenotypes in terms of gut microbiota and metabolome composition [23,50–52]. Furthermore, a vegan diet (as well as other plant-based diets) is associated with a high production of butyrate by bacteria in the colon [50]. Based on these presumptions, we hypothesized that the *but* gene will be more expressed in vegan compared to omnivore microbiota.

Based on the phylogenetic distance, we designed six sets of degenerate primers (marked as cluster A–cluster F), every primer set targeting a different cluster of bacteria possessing the *but* gene. It was confirmed that the *but* gene was more expressed in vegans for cluster C, which encompasses abundant genera *Faecalibacterium*, *Clostridium,* and *Eubacterium*, which belong to the known butyrate producers, and *but* gene copy number detected by primers specific for this cluster was highest among all clusters tested. We also found a trend of higher *but* gene copy numbers in vegans detected for cluster D. Cluster D includes rather abundant butyrate producers *Lachnospiraceae bacterium*, *Eubacterium*, *Roseburia intestinalis,* and *Roseburia inulivorans*. Based on these results, we predicted higher butyrate production in vegans, which was confirmed by NMR analysis of butyrate content in fecal samples. Using two different approaches, the determination of *but* gene abundance in fecal DNA and direct assessment of butyrate content in feces, we confirmed the higher butyrate production capacity in vegans. This finding supports the feasibility of our method in predicting the microbial functional capacity.

As we had to employ primers specific to defined groups of bacteria, our method may provide additional information about microbiota composition. Aiming to verify this assumption, we calculated the correlation of *but* gene copy number and microbiota composition in the same sample determined by 16S rRNA sequencing. For clusters B, C, D, E and F we found strong positive correlation between the *but* gene copy number and the abundance of the target bacteria. In these clusters, the primer sequences are derived from abundant and/or highly prevalent bacteria, such as *Coprococcus* (cluster B), *Faecalibacterium* (cluster C), *Roseburia* (cluster D), *Eubacterium/Anaerobutyricum hallii* (cluster E), or *Anaerostipes* (cluster F). In cluster A, we observed a weak positive correlation (not a significant one) between *but* gene copy number and the abundance of target bacteria *Pseudoflavonifractor* and, to a lesser extent, *Flavonifractor*. Both these bacteria were identified in our sample set but with low abundance and prevalence, which might compromise the outcome of our method.

5. Conclusions

Taken together, we described a method allowing for the detection of specific bacterial genes in the gut microbiome. Our data support the presumption that the determination of *but* gene copy number on bacterial DNA reflects its taxonomic composition, particularly in the case of more abundant bacteria, as well as functional readout, in this case fecal butyrate content. In conclusion, this approach may represent an efficient tool for the estimation of microbial functional capacity. This method requires only equipment and skills routinely available in diagnostic laboratories and does not put any demands on advanced bioinformatics data analysis. Therefore, it may become a feasible tool for rapid screening of specific functional capacity of gut microbiota, i.e., allowing for personalized estimation of the usefulness of prebiotic treatment.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biom11091303/s1, Table S1: title. Microbiome composition of the fecal samples determined by 16S rRNA gene sequencing.

Author Contributions: Conceptualization, N.D. and M.C.; data curation, N.D., J.G. and M.C.; formal analysis, N.D., I.M. and M.K.; funding acquisition, J.G. and M.C.; investigation, N.D., P.V., P.S., H.P. and M.K.; methodology, N.D., M.H., P.V. and P.S.; project administration, N.D.; resources, N.D.; software, N.D. and I.M.; supervision, M.C.; validation, N.D.; visualization, N.D.; writing—original draft, N.D.; writing—review and editing, M.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The project was approved by the Ethics Committee of the Third Faculty of Medicine of the Charles University and the Ethics Committee of University Hospital Kralovske Vinohrady (EK-VP/26/0/2017) in accordance with the Declaration of Helsinki.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Sequencing data are available at European Nucleotide Archive database under the accession number PRJEB43938.

Acknowledgments: This study was supported by the Ministry of Health of the Czech Republic, grant NV-18-01-00040 and by RECETOX Research Infrastructure (ID LM2018121, MEYS CR, 2020-2022).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Tilg, H.; Moschen, A.R. Microbiota and diabetes: An evolving relationship. *Gut* **2014**, *63*, 1513–1521. [CrossRef]
2. Karlsson, F.H.; Tremaroli, V.; Nookaew, I.; Bergström, G.; Behre, C.J.; Fagerberg, B.; Nielsen, J.; Bäckhed, F. Gut metagenome in European women with normal, impaired and diabetic glucose control. *Nature* **2013**, *498*, 99–103. [CrossRef] [PubMed]
3. Qin, J.; Li, Y.; Cai, Z.; Li, S.; Zhu, J.; Zhang, F.; Liang, S.; Zhang, W.; Guan, Y.; Shen, D.; et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* **2012**, *490*, 55–60. [CrossRef] [PubMed]
4. Thorburn, A.N.; Macia, L.; Mackay, C.R. Diet, Metabolites, and "Western-Lifestyle" Inflammatory Diseases. *Immunity* **2014**, *40*, 833–842. [CrossRef] [PubMed]
5. Barko, P.C.; McMichael, M.A.; Swanson, K.S.; Williams, D.A. The Gastrointestinal Microbiome: A Review. *J. Vet. Intern. Med.* **2018**, *32*, 9–25. [CrossRef]
6. Hamer, H.M.; Jonkers, D.; Venema, K.; Vanhoutvin, S.; Troost, F.J.; Brummer, R.J. Review article: The role of butyrate on colonic function. *Aliment. Pharmacol. Ther.* **2008**, *27*, 104–119. [CrossRef] [PubMed]
7. Louis, P.; Hold, G.L.; Flint, H.J. The gut microbiota, bacterial metabolites and colorectal cancer. *Nat. Rev. Microbiol.* **2014**, *12*, 661–672. [CrossRef]
8. Verbeke, K.A.; Boobis, A.R.; Chiodini, A.; Edwards, C.A.; Franck, A.; Kleerebezem, M.; Nauta, A.; Raes, J.; Van Tol, E.A.F.; Tuohy, K.M. Towards microbial fermentation metabolites as markers for health benefits of prebiotics. *Nutr. Res. Rev.* **2015**, *28*, 42–66. [CrossRef] [PubMed]
9. Morrison, D.J.; Preston, T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes* **2016**, *7*, 189–200. [CrossRef]
10. Hernández, M.A.G.; Canfora, E.E.; Jocken, J.W.E.; Blaak, E.E. The short-chain fatty acid acetate in body weight control and insulin sensitivity. *Nutrients* **2019**, *11*, 1943. [CrossRef] [PubMed]

11. Yu, Y.; Raka, F.; Adeli, K. The Role of the Gut Microbiota in Lipid and Lipoprotein Metabolism. *J. Clin. Med.* **2019**, *8*, 2227. [CrossRef]
12. Koh, A.; De Vadder, F.; Kovatcheva-Datchary, P.; Bäckhed, F. From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. *Cell* **2016**, *165*, 1332–1345. [CrossRef]
13. Lupton, J.R. Diet Induced Changes in the Colonic Environment and Colorectal Cancer. *J. Nutr.* **2004**, *134*, 479–482. [CrossRef]
14. Guilloteau, P.; Martin, L.; Eeckhaut, V.; Ducatelle, R.; Zabielski, R.; Van Immerseel, F. From the gut to the peripheral tissues: The multiple effects of butyrate. *Nutr. Res. Rev.* **2010**, *23*, 366–384. [CrossRef]
15. Singh, N.; Gurav, A.; Sivaprakasam, S.; Brady, E.; Padia, R.; Shi, H.; Thangaraju, M.; Prasad, P.D.; Manicassamy, S.; Munn, D.H.; et al. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity* **2014**, *40*, 128–139. [CrossRef] [PubMed]
16. Richards, J.L.; Yap, Y.A.; McLeod, K.H.; MacKay, C.R.; Marinõ, E. Dietary metabolites and the gut microbiota: An alternative approach to control inflammatory and autoimmune diseases. *Clin. Transl. Immunol.* **2016**, *5*, e82. [CrossRef] [PubMed]
17. Tappenden, K.A.; McBurney, M.I. Systemic short-chain fatty acids rapidly alter gastrointestinal structure, function, and expression of early response genes. *Dig. Dis. Sci.* **1998**, *43*, 1526–1536. [CrossRef] [PubMed]
18. Burger-van Paassen, N.; Vincent, A.; Puiman, P.J.; van der Sluis, M.; Bouma, J.; Boehm, G.; van Goudoever, J.B.; Van Seuningen, I.; Renes, I.B. The regulation of intestinal mucin MUC2 expression by short-chain fatty acids: Implications for epithelial protection. *Biochem. J.* **2009**, *420*, 211–219. [CrossRef]
19. Blaak, E.E.; Canfora, E.E.; Theis, S.; Frost, G.; Groen, A.K.; Mithieux, G.; Nauta, A.; Scott, K.; Stahl, B.; van Harsselaar, J.; et al. Short chain fatty acids in human gut and metabolic health. *Benef. Microbes* **2020**, *11*, 411–455. [CrossRef] [PubMed]
20. Lampe, J.W.; Navarro, S.L.; Hullar, M.A.J.; Shojaie, A. Inter-individual differences in response to dietary intervention: Integrating omics platforms towards personalised dietary recommendations. *Proc. Nutr. Soc.* **2013**, *72*, 207–218. [CrossRef]
21. Makki, K.; Deehan, E.C.; Walter, J.; Bäckhed, F. The Impact of Dietary Fiber on Gut Microbiota in Host Health and Disease. *Cell Host Microbe* **2018**, *23*, 705–715. [CrossRef] [PubMed]
22. Laudadio, I.; Fulci, V.; Palone, F.; Stronati, L.; Cucchiara, S.; Carissimi, C. Quantitative Assessment of Shotgun Metagenomics and 16S rDNA Amplicon Sequencing in the Study of Human Gut Microbiome. *Omics J. Integr. Biol.* **2018**, *22*, 248–254. [CrossRef] [PubMed]
23. Sanschagrin, S.; Yergeau, E. Next-generation sequencing of 16S ribosomal RNA gene amplicons. *J. Vis. Exp.* **2014**, *90*, 3–8. [CrossRef] [PubMed]
24. Fiorini, N.; Lipman, D.J.; Lu, Z. Towards PubMed 2.0. *Elife* **2017**, *6*, 4–7. [CrossRef] [PubMed]
25. Vital, M.; Penton, C.R.; Wang, Q.; Young, V.B.; Antonopoulos, D.A.; Sogin, M.L.; Morrison, H.G.; Raffals, L.; Chang, E.B.; Huffnagle, G.B.; et al. A gene-targeted approach to investigate the intestinal butyrate-producing bacterial community. *Microbiome* **2013**, *1*, 1–14. [CrossRef] [PubMed]
26. Anand, S.; Kaur, H.; Mande, S.S. Comparative in silico analysis of butyrate production pathways in gut commensals and pathogens. *Front. Microbiol.* **2016**, *7*, 1–12. [CrossRef]
27. Louis, P.; Duncan, S.H.; McCrae, S.I.; Millar, J.; Jackson, M.S.; Flint, H.J. Restricted Distribution of the Butyrate Kinase Pathway among Butyrate-Producing Bacteria from the Human Colon. *J. Bacteriol.* **2004**, *186*, 2099–2106. [CrossRef]
28. Lissemore, J.L.; Lackner, L.L.; Fedoriw, G.D.; De Stasio, E.A. Isolation of Caenorhabditis elegans genomic DNA and detection of deletions in the unc-93 gene using PCR. *Biochem. Mol. Biol. Educ.* **2005**, *33*, 219–226. [CrossRef]
29. Untergasser, A.; Cutcutache, I.; Koressaar, T.; Ye, J.; Faircloth, B.C.; Remm, M.; Rozen, S.G. Primer3-new capabilities and interfaces. *Nucleic Acids Res.* **2012**, *40*, 1–12. [CrossRef]
30. McGinnis, S.; Madden, T.L. BLAST: At the core of a powerful and diverse set of sequence analysis tools. *Nucleic Acids Res.* **2004**, *32*, 20–25. [CrossRef]
31. Apprill, A.; Mcnally, S.; Parsons, R.; Weber, L. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat. Microb. Ecol.* **2015**, *75*, 129–137. [CrossRef]
32. Budinska, E.; Gojda, J.; Heczkova, M.; Bratova, M.; Dankova, H.; Wohl, P.; Bastova, H.; Lanska, V.; Kostovcik, M.; Dastych, M.; et al. Microbiome and Metabolome Profiles Associated With Different Types of Short Bowel Syndrome: Implications for Treatment. *J. Parenter. Enter. Nutr.* **2020**, *44*, 105–118. [CrossRef]
33. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef] [PubMed]
34. Caporaso, J.G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Peña, A.G.; Goodrich, J.K.; Gordon, J.I.; et al. Correspondence QIIME allows analysis of high- throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing. *Nat. Publ. Gr.* **2010**, *7*, 335–336. [CrossRef]
35. Pelantová, H.; Bugáňová, M.; Anýž, J.; Železná, B.; Maletínská, L.; Novák, D.; Haluzík, M.; Kuzma, M. Strategy for NMR metabolomic analysis of urine in mouse models of obesity- from sample collection to interpretation of acquired data. *J. Pharm. Biomed. Anal.* **2015**, *115*, 225–235. [CrossRef] [PubMed]
36. R core Team. A language and environment for statistical computing. *Found. Stat. Comput.* **2013**, *2*, 1–12.
37. Aitchison, J. The Statistical Analysis of Compositional Data. *Stat. Anal. Compos. Data* **1986**, *44*, 139–177. [CrossRef]
38. Fish, J.A.; Chai, B.; Wang, Q.; Sun, Y.; Brown, C.T.; Tiedje, J.M.; Cole, J.R. FunGene: The functional gene pipeline and repository. *Front. Microbiol.* **2013**, *4*, 1–14. [CrossRef]

39. Louis, P.; Flint, H.J. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol. Lett.* **2009**, *294*, 1–8. [CrossRef]
40. Louis, P.; Flint, H.J. Formation of propionate and butyrate by the human colonic microbiota. *Environ. Microbiol.* **2017**, *19*, 29–41. [CrossRef]
41. Louis, P.; McCrae, S.I.; Charrier, C.; Flint, H.J. Organization of butyrate synthetic genes in human colonic bacteria: Phylogenetic conservation and horizontal gene transfer. *FEMS Microbiol. Lett.* **2007**, *269*, 240–247. [CrossRef]
42. Thompson, J.D.; Gibson, T.J.; Higgins, D.G. Multiple Sequence Alignment Using ClustalW and ClustalX. *Curr. Protoc. Bioinform.* **2003**, 1–22. [CrossRef]
43. Dereeper, A.; Guignon, V.; Blanc, G.; Audic, S.; Buffet, S.; Chevenet, F.; Dufayard, J.F.; Guindon, S.; Lefort, V.; Lescot, M.; et al. Phylogeny.fr: Robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res.* **2008**, *36*, 465–469. [CrossRef]
44. Lane, C.E.; Hulgan, D.; O'Quinn, K.; Benton, M.G. CEMAsuite: Open source degenerate PCR primer design. *Bioinformatics* **2015**, *31*, 3688–3690. [CrossRef] [PubMed]
45. Ye, J.; Coulouris, G.; Zaretskaya, I.; Cutcutache, I.; Rozen, S.; Madden, T.L. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinform.* **2012**, *13*, 134. [CrossRef]
46. Costea, P.I.; Zeller, G.; Sunagawa, S.; Pelletier, E.; Alberti, A.; Levenez, F.; Tramontano, M.; Driessen, M.; Hercog, R.; Jung, F.E.; et al. Towards standards for human fecal sample processing in metagenomic studies. *Nat. Biotechnol.* **2017**, *35*, 1069–1076. [CrossRef]
47. Leigh Greathouse, K.; Sinha, R.; Vogtmann, E. DNA extraction for human microbiome studies: The issue of standardization. *BMC Microbiol.* **2018**, *18*, 212. [CrossRef] [PubMed]
48. Jenkins, S.V.; Vang, K.B.; Gies, A.; Griffin, R.J.; Jun, S.; Nookaew, I.; Dings, R.P.M. Sample storage conditions induce post- collection biases in microbiome profiles. *BMC Microbiol.* **2018**, *18*, 227. [CrossRef] [PubMed]
49. Větrovský, T.; Baldrian, P. The Variability of the 16S rRNA Gene in Bacterial Genomes and Its Consequences for Bacterial Community Analyses. *PLoS ONE* **2013**, *8*, e57923. [CrossRef] [PubMed]
50. Dinu, M.; Abbate, R.; Gensini, G.F.; Casini, A.; Sofi, F. Vegetarian, vegan diets and multiple health outcomes: A systematic review with meta-analysis of observational studies. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 3640–3649. [CrossRef]
51. Jia, W.; Zhen, J.; Liu, A.; Yuan, J.; Wu, X.; Zhao, P.; Zhao, L.; Li, X.; Liu, Q.; Huang, G.; et al. Long-Term Vegan Meditation Improved Human Gut Microbiota. *Evid. Based Complement. Altern. Med.* **2020**, *2020*, 9517897. [CrossRef]
52. Wu, G.D.; Compher, C.; Chen, E.Z.; Smith, S.A.; Shah, R.D.; Bittinger, K.; Chehoud, C.; Albenberg, L.G.; Nessel, L.; Gilroy, E.; et al. Comparative metabolomics in vegans and omnivores reveal constraints on diet-dependent gut microbiota metabolite production. *Gut* **2016**, *65*, 63–72. [CrossRef] [PubMed]

3.3 Exploration of the possibilities of the manipulation of gut microbiota by the dietary fiber inulin in the personalized treatment of T2D

Obesity and associated metabolic diseases, such as T2D, are a major global health challenge, and the gut microbiota has been suggested to play a critical role in their development. Although many studies suggest an association between T2D and gut dysbiosis, results on the composition and function of the microbiota are inconsistent and sometimes contradictory. Diet plays an important role in shaping the microbiome, and dietary interventions focused on modulating the composition and/or performance of the gut microbiota appear to be a promising therapeutic target. This study aimed to determine whether the gut microbial composition and metabolome differ in lean healthy, obese healthy, and obese diabetic drug-naive T2D patients, whether the effects of inulin on glucose tolerance and insulin sensitivity can be explained by the response of the gut microbiota to inulin intervention, and whether this response can be predicted from the initial microbiome and metabolome signature.

The observational part of the study involved screening patients with pre/diabetes (DM, n = 49), metabolically healthy overweight/obese patients (OB, n = 66) and a lean healthy cohort (LH, n = 32). All cohorts had their blood, urine and stool samples collected. An oral glucose tolerance test was performed and 3-day prospected dietary records were obtained. The prospective part of the study involved 27 DM patients and the effect of inulin supplementation (10 g/day for three months) on glucose disposal and insulin sensitivity was investigated. Various outcomes were measured during the whole study, including gut microbial composition, SCFAs in plasma, volatile organic compounds (VOCs) in feces and metabolites in serum measured by NMR.

Microbiome and metabolome composition varied across groups. The DM and LH groups represented opposite poles of the abundance spectrum, whereas OB was found to be more similar to DM. Concerning microbiome composition, multivariable statistics revealed significant differences in β-diversity between LH, OB, and DM phenotypes (PERMANOVA test). The univariable analysis identified 37 taxa that had significantly different abundance among the groups. A machine learning approach (LASSO regression model) was used to discriminate the groups based on microbial composition, but the outcome was not satisfactory. When OB and DM data sets were grouped, the accuracy of the model increased to 75%. Significant differences were also found in the β-diversity of VOCs between the groups, with pairwise analysis confirming significant differences between OB and DM groups compared to the LH group. Univariable analysis followed by effect size analysis revealed ten VOCs with significantly different abundance between groups. Nonanoic acid was more abundant, while all other compounds, including SCFAs esters, were less abundant in the OB and DM groups compared to LH controls. The machine learning model achieved only 52% accuracy for classifying subjects into three categories but increased to 81% when obese and diabetic subjects were combined into one category. The LASSO model based on serum metabolome data was able to classify unknown subjects into the categories LH, OB, or DM with an accuracy of 74%. The integrated LASSO model, which combined all variables, allowed better classification between groups, with an accuracy of 77%. Taken together, our results demonstrate that microbiome and metabolome composition differ between lean participants and subjects with obesity, but do not allow to discriminate between obese subjects with and without diabetes.

Inulin supplementation in 27 subjects with obesity and diabetes led to a significant change in their microbiota composition (PERMANOVA <0.001). Several bacterial taxa, including butyrate producers such as *Faecalibacterium*, *Anaerostipes* or *Eubacterium halii* and other bacteria considered beneficial

such as *Lactobacillus*, *Bifidobacterium* and *Akkermansia*, increased after treatment. Conversely, some bacterial taxa abundances decreased after supplementation, for example, those known to be associated with protein fermentation. After the inulin treatment, there was a significant increase in the concentration of butyric acid, propionic acid, and asparagine in the serum, while the concentration of glycerol and 2-propanol decreased. Inulin intake also affected markers of glucose tolerance and insulin sensitivity, but the individual response varied greatly. Nevertheless, significant improvement in glucose tolerance (measured as 120 min OGTT glucose) was observed in the entire group that received the intervention, along with a tendency towards a reduction in the area under the curve (AUC) for OGTT glucose and fasting glycemia. Linear regression models were fitted with all glucose metabolism parameters as outcome variables and all omics and clinical variables as predictors. We identified potential predictors of individual response to inulin treatment independently on pre-intervention glycemic parameters, such as serum BCAA derivatives, serum 3-hydroxyisobutyrate, fecal indole and various bacterial taxa (*Ruminiclostridium*, *Lachnoclostridium*, *Eubacterium halii*).

In conclusion, this study provides valuable insights into the role of gut microbiota in the development of metabolic diseases and the potential use of dietary interventions to modulate the microbiota and improve metabolic health. The findings highlight the complex nature of microbial changes underlying the development of TD2 and obesity but also suggest that inulin supplementation can lead to significant improvements in glucose tolerance and insulin sensitivity, as well as changes in microbiota composition and metabolome. These findings may help personalize treatment options and improve outcomes for patients with metabolic diseases who have struggled to achieve success through lifestyle changes alone.

This article was published on the 21st of April 2023 in Nutrition & Diabetes, IF = 4.73

ARTICLE OPEN

Check for updates

Multi-omics signatures in new-onset diabetes predict metabolic response to dietary inulin: findings from an observational study followed by an interventional trial

N. Ďásková[1,12], I. Modos[2], M. Krbcová[3], M. Kuzma[4], H. Pelantová[4], J. Hradecký[5], M. Heczková[2], M. Bratová[2], P. Videňská[6], P. Šplíchalová[7], M. Králová[8,12], M. Heniková[3], J. Potočková[3], A. Ouřadová[3], R. Landberg[9], T. Kühn[10,11,13], M. Cahová[2,13]✉ and J. Gojda[3,13]

AIM: The metabolic performance of the gut microbiota contributes to the onset of type 2 diabetes. However, targeted dietary interventions are limited by the highly variable inter-individual response. We hypothesized (1) that the composition of the complex gut microbiome and metabolome (MIME) differ across metabolic spectra (lean-obese-diabetes); (2) that specific MIME patterns could explain the differential responses to dietary inulin; and (3) that the response can be predicted based on baseline MIME signature and clinical characteristics.

METHOD: Forty-nine patients with newly diagnosed pre/diabetes (DM), 66 metabolically healthy overweight/obese (OB), and 32 healthy lean (LH) volunteers were compared in a cross-sectional case-control study integrating clinical variables, dietary intake, gut microbiome, and fecal/serum metabolomes (16 S rRNA sequencing, metabolomics profiling). Subsequently, 27 DM were recruited for a predictive study: 3 months of dietary inulin (10 g/day) intervention.

RESULTS: MIME composition was different between groups. While the DM and LH groups represented opposite poles of the abundance spectrum, OB was closer to DM. Inulin supplementation was associated with an overall improvement in glycemic indices, though the response was very variable, with a shift in microbiome composition toward a more favorable profile and increased serum butyric and propionic acid concentrations. The improved glycemic outcomes of inulin treatment were dependent on better baseline glycemic status and variables related to the gut microbiota, including the abundance of certain bacterial taxa (i.e., *Blautia, Eubacterium halii group, Lachnoclostridium, Ruminiclostridium, Dialister*, or *Phascolarctobacterium*), serum concentrations of branched-chain amino acid derivatives and asparagine, and fecal concentrations of indole and several other volatile organic compounds.

CONCLUSION: We demonstrated that obesity is a stronger determinant of different MIME patterns than impaired glucose metabolism. The large inter-individual variability in the metabolic effects of dietary inulin was explained by differences in baseline glycemic status and MIME signatures. These could be further validated to personalize nutritional interventions in patients with newly diagnosed diabetes.

Nutrition and Diabetes (2023)13:7 ; https://doi.org/10.1038/s41387-023-00235-5

Obesity and its associated metabolic diseases, including type 2 diabetes, currently represent one of the greatest challenges to global health care [1]. Recently, it has been suggested that the composition and performance of the gut microbiota contribute to individual risks. The critical role of the gut microbiota in the development of obesity was suggested by a seminal study by Turnbaugh [2], followed by others confirming differences in microbiota composition between lean and obese individuals [3, 4]. Further research showed an association between the gut microbiota and the development of type 2 diabetes [5–8], with evidence of a specific gut microbiota signature characteristic of prediabetes [9, 10]. However, while many studies suggest that type 2 diabetes is associated with gut dysbiosis [11], results on the composition and function of the microbiota are inconsistent and sometimes contradictory. For example, α-diversity has been reported to be significantly lower [6, 12, 13], not significantly

[1]First Faculty of Medicine, Charles University, Prague, Czech Republic. [2]Institute for Clinical and Experimental Medicine, Prague, Czech Republic. [3]Department of Internal Medicine, Kralovske Vinohrady University Hospital and Third Faculty of Medicine, Charles University, Prague, Czech Republic. [4]Institute of Microbiology of the CAS, Prague, Czech Republic. [5]Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Prague, Czech Republic. [6]Mendel University, Department of Chemistry and Biochemistry, Brno, Czech Republic. [7]RECETOX, Faculty of Science Masaryk University, Brno, Czech Republic. [8]Ambis University, Department of Economics and Management, Prague, Czech Republic. [9]Division of Food and Nutrition Science, Department of Biology and Biological Engineering, Chalmers University of Technology, Goteborg, Sweden. [10]Institute of Global Food Security, Queen's University Belfast, Belfast, UK. [11]Heidelberg Institute of Global Health (HIGH), Medical Faculty and University Hospital, Heidelberg University, Heidelberg, Germany. [12]Present address: Institute for Clinical and Experimental Medicine, Prague, Czech Republic. [13]These authors contributed equally: T. Kühn, M. Cahová, J. Gojda.
✉email: monika.cahova@ikem.cz

Received: 9 August 2022 Revised: 22 March 2023 Accepted: 6 April 2023
Published online: 21 April 2023

reduced [14], or comparable to nondiabetic subjects in patients with T2D [15, 16]. Most studies report significant differences in the composition of the gut microbiota between diseased and healthy subjects [17], but they differ greatly with respect to specific taxa. Some studies show that T2D is associated with an increased Firmicutes/Bacteroidetes ratio [6, 13, 14, 18, 19], whereas others report a significant increase [14, 18] or decrease [6, 13] in Proteobacteria. At the genus level, there are few dysregulated taxa that have been consistently reported, i.e., an increase in *Streptococcus* [9, 15, 20], *Escherichia* [15, 21, 22], *Veillonella* [6, 21], Lactobacillus [13, 18, 23], and *Collinsella* [12, 15]; decrease in *Akkermansia* [15, 18], *Dialister* [15, 19], *Haemophilus* [12, 15], *Roseburia* [12, 15], and *Faecalibacterium* [10, 12, 13], whereas many others show changes in both directions [17]. Diet composition is a known risk factor for the development of type 2 diabetes. In addition to direct effects on host physiology, diet plays an important role in shaping the microbiome, thereby influencing its metabolic program [24]. Therefore, dietary interventions focused on modulating the composition and/or performance of the gut microbiota appear to be a promising therapeutic target. Supplementation with prebiotic supplements, and dietary fiber in particular, is often recommended as a beneficial treatment for non-communicable diseases, but controlled clinical trials indicate pronounced differences in response to treatment, with considerable personal variability [25]. The underlying causes are not yet clear, but strong inter-individual differences in microbial response to dietary fiber likely play a key role [26, 27]. Therefore, the identification of the microbial taxa that mediate the beneficial effects of dietary fiber may open new avenues for individualized treatment approaches [28]. In the present study, we aimed to determine (i) whether the composition of the complex gut microbiome and metabolome (MIME) differ in lean healthy, obese healthy, and obese diabetic drug-naive type 2 diabetic patients; (ii) whether the effects of inulin on glucose tolerance and insulin sensitivity can be explained, at least in part, by the response of the gut microbiota to inulin intervention; and (iii) whether this response can be predicted from the initial MIME signature.

MATERIAL AND METHODS

The current study was performed within the framework of the TRIEMA project: Treatment of Insulin Resistance by Modification of Gut Microbiota (ClinicalTrials.gov Identifier: NCT03710850). The first study from the project has been already published [24].

Study design and population

Observational study. Forty-nine newly diagnosed patients with pre/diabetes (DM: BMI >25, fasting glycemia >5.6 mM, and/or 2hOGTT glycemia >7.8 mM), 66 metabolically healthy overweight/obese (OB: BMI >25) and 32 lean healthy (LH: BMI <25) subjects were screened and enrolled in the cross-sectional case-control study. A clinical visit was scheduled after enrollment. Volunteers were examined after a 12-h overnight fast; blood and urine samples were collected; a clinical examination, bioimpedance analysis, and oral glucose tolerance test (OGTT, 75 g glucose) were performed. A prospective 3-day dietary record and stool samples were collected from each participant. Dietary records and stool samples were obtained no longer than a week after the clinical visit.

Prospective study. Twenty-seven patients (DM) were then enrolled in a one-arm, non-controlled intervention study in which they were fed 10 g of inulin daily for 3 months. The sample size determination for the intervention study was calculated for the primary outcome, glucose disposal (GD). According to GD, standard deviations ranged from 1.8 to 2.5 mg/kg/min in both insulin-sensitive and insulin-resistant individuals, with high insulin levels (i.e., 80 mU/m^2) showing less variability with SD up to 0.51 [29]. We anticipate that participants will respond individually to the intervention, and we will divide them into tertiles (responders, neutral, and non-responders). If we consider a difference between changes of 20% (i.e., ~1.5 mg/kg/min) to be significant to have 90% power to detect a difference at the 0.05 alpha level, we must have 6 subjects in each group. To account for dropouts or incomplete data, we aimed to have at least 9 subjects in each group (i.e., responders vs. non-responders). Baseline and post-intervention examinations were identical to those described above. In addition, indirect calorimetry and a two-step glucose clamp (10 and 80 mIU/m^2 BSA insulin dose) were performed [30]. Insulin sensitivity (IS) of adipose tissue was expressed as the change in non-esterified fatty acids (NEFA) and plasma glycerol levels from baseline to the steady state of the first step of the clamp, whereas IS of skeletal muscle was expressed as space-corrected glucose infusion rate per kg fat-free mass (Mcor mg/kg FFM/min) and metabolic clearance of glucose divided by steady-state insulinemia (MCR/I, ml/kg FFM/min) at the steady state of the second step. Detailed calculations are described in Supplementary Material. All participants signed an informed consent before enrollment in each respective study. The research protocol was approved by the Ethics Committee of University Hospital Kralovske Vinohrady (EK-VP /26/0/2017) in accordance with the Declaration of Helsinki. The study was registered under NCT03710850.

Gut microbiome analysis

DNA from stool samples was isolated using the QIAmp PowerFecal DNA Kit (Qiagen, Hilden, Germany), and the V4 region of the bacterial 16 S rRNA gene was amplified by PCR. Sequencing was performed using the Miseq reagent kit V2 with a MiSeq instrument (Illumina, Hayward, CA, USA). The raw sequences were processed using a DADA2 Amplicon Denoiser [31].

Short-chain fatty acids (SCFA) in plasma

SCFA were analyzed in plasma by LC-MS according to a method described before [32].

Volatile compounds (VOCs) analysis in feces

Volatile fingerprinting of fecal samples was performed using an Agilent 7890B gas chromatograph (Santa Clara, California, USA) coupled to a Pegasus 4D time-of-flight mass spectrometer (LECO, Geleen, The Netherlands). Data acquisition and initial data processing were performed using instrumental SW ChromaTOF by LECO.

NMR analyses

Serum samples (after protein precipitation) were measured on a 600 MHz Bruker Avance III spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a 5 mm TCI cryogenic probe head. The concentrations of individual metabolites, identified by comparison of proton and carbon chemical shift with the HMDB database, were expressed as PQN [33] normalized intensities of corresponding signals in CPMG spectra. The list of quantified metabolites with corresponding ^{1}H and ^{13}C chemical shifts is given in Table S1. The representative ^{1}H NMR spectrum is shown in Fig. S1.

Statistics

The statistical analyses were performed using R software packages and in-house scripts [34]. The microbiome and VOCs data were treated as compositional (proportions of total read count in each sample or proportion of the total area of selected masses), and before all statistical analyses, the data were transformed by centered log-ratio (clr) transformation with a multiplicative simple replacement for handling zero values. According to their abundance and prevalence, the bacteria were classified as "core microbial taxa" when fulfilling the following conditions, i.e. abundance of >0.1% and prevalence of >75% at least in one experimental group. Other microbial taxa were classified as rare.

All methods are described in detail in Supplementary Material.

RESULTS

Observational study: clinical characteristics

The clinical characteristics of the study participants are shown in Table 1. As expected, the groups differed in terms of glycemic indices, insulin sensitivity, and beta cell function. Biomarkers of lipid metabolism were significantly elevated in both the OB and DM groups compared with LH.

Observational study: fecal microbiome composition

In all samples, we found 44,332 amplicon sequence variants (ASVs) and identified 13 phyla, 30 classes, 56 orders, 104 families, and 367 genera. Considering only the ASVs, all α-diversity indices were

Table 1. Group characteristics for lean (LH), obese (OB) and persons with pre/diabetes (DM).

	LH	DM	OB	K–W test	DMCT		
					LH vs OB	LH vs DM	OB vs DM
General characteristics							
Sex (F/M)	16/16	26/23	47/19				
Weight (kg)	74.8 [23.1]	99.5 [17.4]	87.2 [25.8]	˂0.05	˂0.001	˂0.001	˂0.01
Age (years)	30.9 [11.0]	58.3 [13.1]	51.3 [14.2]	˂0.05	˂0.001	˂0.001	n.s.
BMI (kg/m^2)	23.0 [4.0]	34.9 [9.1]	30.8 [6.6]	˂0.05	˂0.001	˂0.001	˂0.05
WHR	0.8 [0.1]	1.0 [0.1]	0.9 [0.1]	˂0.05	˂0.001	˂0.001	˂0.05
Body composition							
Fat (kg)	14.2 [4.8]	39.5[22.3]	32.9[14.7]	˂0.05	˂0.001	˂0.001	n.s.
FFM (kg)	56.5 [22.5]	61.3[14.8]	51.9[17.3]	˂0.05	n.s.	n.s.	˂0.05
TBW (kg)	41.4 [16.5]	44.9[10.8]	38.0[12.6]	˂0.05	n.s.	n.s.	˂0.05
Macronutrient intake							
Total energy (kcal/day)	2101[1583]	2017[879]	1777[555]	n.s.	N/A	N/A	N/A
Proteins (g/day)	81 [29]	82 [33]	72 [28.5]	n.s.	N/A	N/A	N/A
Lipids (g/day)	83 [49]	79 [40]	65 [35.5]	˂0.05	˂0.05	n.s.	n.s.
Carbohydrates (g/day)	232 [98]	207 [96]	197 [73.5]	n.s.	N/A	N/A	N/A
Dietary fiber (g/day)	18 [19]	16 [9]	15 [7.5]	n.s.	N/A	N/A	N/A
Glucose metabolism							
Fasting glucose (mmol/l)	4.8 [0.3]	5.9 [0.8]	5.3 [0.6]	˂0.05	˂0.001	˂0.001	˂0.001
2 h OGTT glucose (mmol/l)	5.7 [1.1]	8.9 [3.1]	6.4 [1.6]	˂0.05	n.s.	˂0.001	˂0.001
AUC for OGTT glucose (mmol/l × 120 min^{-1})	254 [114]	499 [282]	239 [150]	˂0.05	n.s.	˂0.001	˂0.001
AUC for OGTT insulin (mIU/l × 120 min^{-1})	3890[2707]	8948[6596]	6453[4122]	˂0.05	˂0.01	˂0.001	˂0.05
Insulin (mIU/l)	4.0 [2.7]	15.9 [8.6]	9.5 [5.7]	˂0.05	˂0.001	˂0.001	˂0.001
C-peptide (pmol/l)	233 [97]	769 [357]	5.3 [0.6]	˂0.05	˂0.001	˂0.001	˂0.01
HbA1c (mmol/mol)	32 [2]	38 [7]	6.4 [1.6]	˂0.05	˂0.001	˂0.001	˂0.001
Matsuda index	10.2 [6.4]	2.0 [1.7]	4.0 [3.4]	˂0.05	˂0.01	˂0.001	˂0.001
Insulinogenic index	0.8 [0.7]	0.8 [1.0]	1.1 [1.0]	˂0.05	n.s.	n.s.	n.s.
Oral disposition index	6.7 [4.9]	1.9 [1.2]	4.9 [5.7]	˂0.001	n.s.	˂0.001	˂0.001
Beta cell index	163 [134]	45 [25]	108 [145]	˂0.001	n.s.	˂0.001	˂0.001
TyG index	0.51 [0.67]	1.54[0.59]	1.01[0.60]	˂0.05	˂0.001	˂0.001	˂0.01
Lipid metabolism							
Total cholesterol (mmol/l)	4.30 [1.09]	5.01 [1.23]	5.15 [1.24]	˂0.05	˂0.01	˂0.05	n.s.
HDL-C (mmol/l)	1.67 [0.47]	1.26 [0.30]	1.39 [0.56]	˂0.05	˂0.05	˂0.001	n.s.
LDL-C (mmol/l)	2.37 [1.15]	3.05 [1.40]	3.06 [1.16]	˂0.05	˂0.001	˂0.05	n.s.
Triacylglycerols (mmol/l)	0.69 [0.52]	1.53 [0.93]	1.10 [0.71]	˂0.05	˂0.001	˂0.001	˂0.05
Inflammatory markers							
CRP (mg/l)	0.7 [0.9]	3.3 [4.5]	2.3 [4.0]	˂0.05	˂0.001	˂0.001	n.s.
Stool characteristics							
pH in feces	7.26 [0.67]	7.04 [0.52]	7.27 [0.50]	n.s.	N/A	N/A	N/A
dry mass (%)	25.1 [8.9]	24.5 [9.9]	23.0 [6.9]	n.s.	N/A	N/A	N/A

Data were given as median [71].
AUC area under the curve during oral glucose tolerance test, *BMI* body mass index, *CRP* C-reactive protein, *DMCT* Dunn's multiple comparison test, *FFM* fat-free mass, *HDL-C* high-density lipoprotein–cholesterol, *HbA1* glycated hemoglobin, *K–W* Kruskal–Wallis test, *LDL-C* low-density lipoprotein–cholesterol, *N/A* not applicable, *ns* not significant, *TyG index* ln (fasting triglyceride × fasting glucose)/2; *TBW* total body water, *WHR* waist-hip ratio. Insulinogenic index (ΔINS 0-30/ΔGLU 0-30), *ISI-M* Matsuda–deFronzo index; oral disposition index (IGI*ISI); beta cell index (iAUC$_{insulin}$/iAUC$_{glucose}$)*ISI.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

significantly lower in OB and DM compared with LH, whereas no differences were found between the DM and OB groups (Fig. S2). When ASVs were aggregated and classified at the genus level, only the Shannon index remained significantly lower in OB and DM compared with LH (Fig. S3).

At the phylum level, the microbiota composition was dominated by Firmicutes and Bacteroidetes, followed by much less abundant Actinobacteria, Proteobacteria, and Verrucomicrobia. The median abundance of all other phyla was less than 0.01%. There were no significant differences in the

representation of individual phyla (Table S2). The separation of individual samples at the genus level is visualized in Fig. 1A. Multivariable statistics revealed significant differences in β-diversity ($p \leq 0.001$), and pairwise analysis confirmed significant differences between OB vs. LH ($p < 0.001$) and DM vs. LH ($p < 0.001$), but not between DM and OB. Using univariable analysis, we identified 37 taxa that had significantly different abundance among groups; 15 of them met the criteria of "core" microbiota, i.e., an abundance of >0.05% and a prevalence of >75% in at least one group (Fig. 1B and Table S3), accounting for 45% of all core genera. Thirteen core genera were more abundant in LH compared to the other two groups, while *Pseudobutyrivibrio* and *Lachnoclostridium* were enriched only in DM. Confirmed butyrate producers, i.e., *Anaerostipes, Eubacterium halii, Faecalibacterium, Christensenellaceae R-7 group*, were more abundant in the core microbiota LH than in the core microbiota OB or DM. Most of the taxa enriched in DM and/or OB belong to the "non-core" taxa. Among them, potentially harmful genera were identified (*Fusobacterium, Megasphera,* and *Desulfovibrio*). Significant positive correlations were found between *Fusobacterium* abundance and C-peptide concentration in all groups. The common or unique taxa specific to the groups are shown in Fig. S4.

The discrimination of the groups as a function of microbiome composition was investigated using a machine learning approach (LASSO regression model). This model, which has an accuracy of 51% and a sensitivity of 66% (LH), 50% (OB), and 43% (DM), does not reliably classify LH, OB, and DM (Fig. S5). When we grouped OB and DM, the accuracy of the model increased to 75% and the sensitivity to 65% (LH) (Fig. S6).

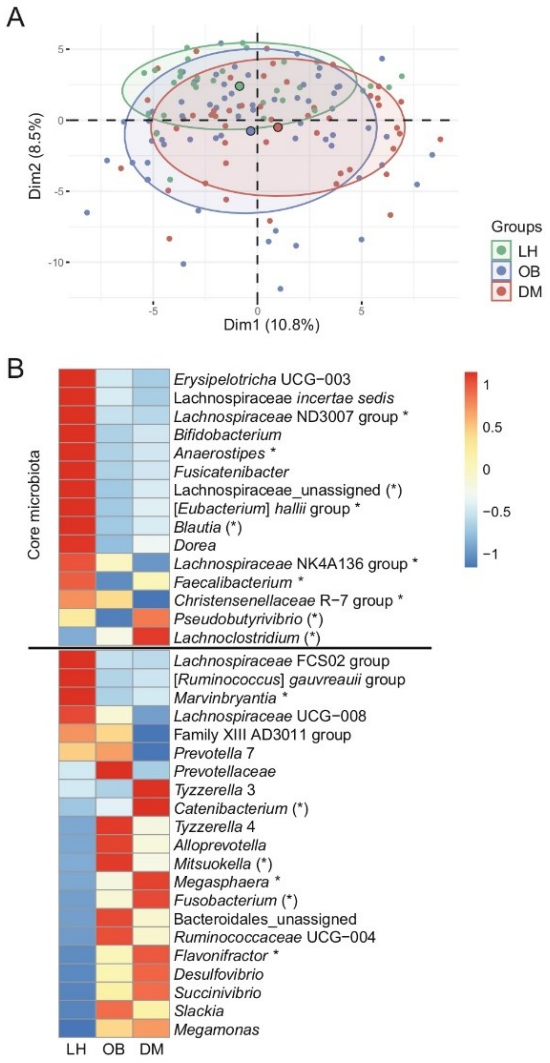


Fig. 1 Fecal microbiome composition. A 2D PCA scores plot on genera level after clr transformation. The explained variance of each component is included in the axis labels. The large points represent the centroids of each group. **B** Abundances of all significant genera (FDR <0.1). Proportional data were used. Each cell then represents the mean in each group for the corresponding genera. Rows were z-scaled. Core genera are defined by the condition abundance >0.05% and prevalence >75% at least in one group. Genera marked by * are confirmed butyrate producers, and genera marked by (*) are potential butyrate producers.

Observational study: fecal metabolome

In the fecal metabolome, we identified 185 different VOCs. Within this subset, 113 VOCs were of very low abundance (˂0.1%), 54 VOCs each accounted for 0.1–1% of the total, 12 VOCs accounted for 1–5% of the total, and six were very abundant (>5%). The separation of individual samples is visualized in Fig. 2. Multivariable statistics revealed significant differences in β-diversity ($p = 0.0017$). The pairwise analysis confirmed significant differences between the DM vs. LH groups ($p < 0.01$) and OB vs. LH ($p < 0.05$), but not between DM and OB.

Univariable analysis followed by effect size analysis revealed ten VOCs with significantly different abundance between groups (FDR

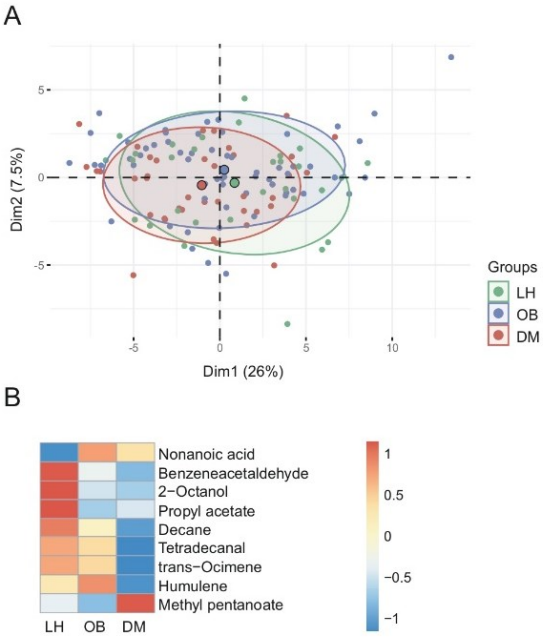


Fig. 2 Fecal metabolome composition. A 2D PCA scores plot on VOCs abundances after clr transformation. Only VOCs meeting condition $AUC_{x,p} \geq 0.1\%$ $AUC_{total,p}$ are shown. The explained variance of each component is included in the axis labels. The large points represent the centroids of each group. **B** Abundances of significant metabolites. Proportional data were used. Each cell then represents the median in each group for the corresponding metabolite. Rows were z-scaled.

$p \leq 0.1$) (Fig. 2B and Table S4). Nonanoic acid was more abundant, while all other compounds, including SCFA esters, were less abundant in the OB and DM groups compared to LH. Only methyl pentanoate showed an opposite pattern in the DM and OB groups (DM>LH = OB) (Fig. S7). Nonanoic acid correlated positively with the TyG index in all groups.

A LASSO model created for the classification of tested subjects into three categories (LH vs OB vs DM) achieved only 52% accuracy and only 48% (LH), 54% (OB), and 53% (DM) sensitivity (Fig. S8). When we combined subjects from OB and DM into one category, classification accuracy increased to 80.5%, but sensitivity remained low at 52% (Fig. S9).

Observational study: serum/plasma metabolome

To determine the composition of the serum metabolome, we used an untargeted NMR approach and LC-MS analyzes that allows accurate determination of SCFA concentration in plasma. In total, we identified 35 quantified analytes by NMR and nine SCFAs by LC-MS, only acetate/acetic acid was identified by both methods. PERMANOVA analysis suggested the separation of the groups, and subsequent pairwise tests revealed significant differences ($p \leq 0.001$) in serum metabolome composition between all compared pairs.

The univariable analysis identified 21 metabolites that were significantly different in abundance between groups (Fig. 3B and Table S5). Based on the univariable analysis, we identified LH, OB, and DM-specific groups of serum metabolites. For most metabolites, the DM and LH groups represented the opposite poles of the abundance spectra, with OB closer to the DM group. All three groups differed in serum concentrations of intermediates of saccharide metabolism (glucose, lactate, and mannose) and two amino acids (AA) (glutamine, alanine). The concentration of seven compounds, including three SCFA (propionic acid, succinic acid, valeric acid), two AA (tyrosine, histidine), and glycerol was comparable at OB and DM, but differed from LH. Six compounds, including two branched-chain amino acid (BCAA) derivatives (2-oxoisovalerate, 3-methyl-2-oxovalerate), 2-hydroxybutyrate, acetone, 2-propanol, and formic acid, presented a specific DM-associated signature (Fig. S10).

A LASSO -model based on serum metabolome data was able to classify unknown subjects into the categories LH, OB, or DM with an accuracy of 74% and a sensitivity of 90% (LH), 72% (OB), or 65% (DM) (Fig. S11). When we grouped subjects from OB and DM groups together, model accuracy increased to 89% and sensitivity (LH) increased to 88% (Fig. S12). None of the models selected glucose as a key discriminant.

Observational study: integrative analysis

We further investigated whether a combination of all variables would allow better classification between groups. With this integrated LASSO model, an unknown subject could be assigned to one of the three groups (LH, OB, and DM) with an accuracy of 77% and a sensitivity of 88% (LH), 79% (OB), and 66% (DM), respectively. LASSO coefficients included five variables from the microbiome dataset, one variable from the fecal metabolome dataset, and nine variables from the serum metabolome dataset (Fig. S13). When we constructed the LASSO model only for two groups (LH vs. OB + DM), we were able to classify an unknown subject with 91% accuracy and 89% sensitivity. Ten microbes, five fecal VOCs, and 11 serum metabolites contributed to the discrimination between groups (Fig. S14).

Finally, we looked for a possible complex interaction between different MIME components in individual groups. Figure 4 depicts the positive and negative Spearman correlations among datasets filtered by $|\rho| > 0.5$; these correlations unravel differences in interaction networks within each group. In the LH group, we observed a rich network among variables both within and outside the datasets, whereas the complexity in OB and DM was much lower.

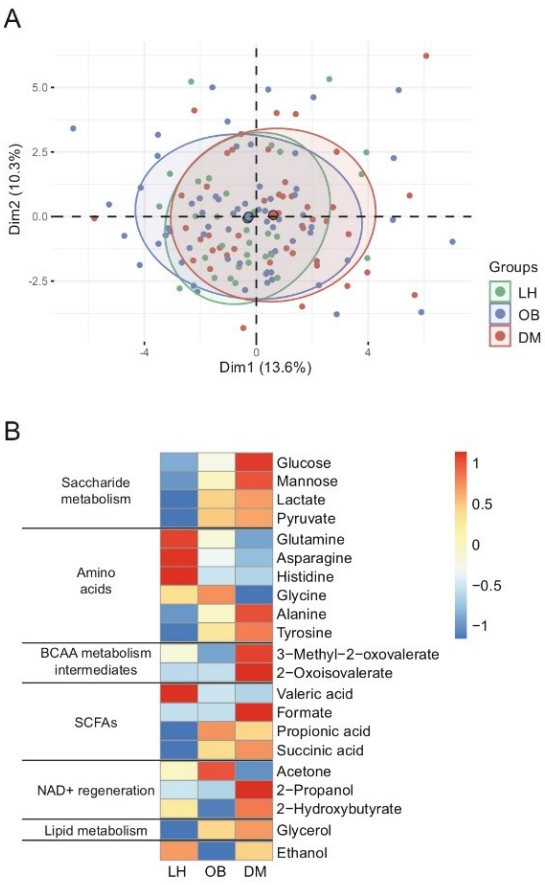


Fig. 3 Serum metabolome composition. A 2D PCA scores plot. The explained variance of each component is included in the axis labels. The large points represent the centroids of each group. **B** Abundances of significant metabolites. Each cell then represents the median in each group for the corresponding metabolite. Rows were z-scaled.

Prospective study: effect of inulin on omics signature

Twenty-seven newly diagnosed DM subjects participated in a three-month, single-arm, non-controlled intervention study in which they were administered inulin (10 g/day) without other antidiabetic medications and/or lifestyle interventions. No clinically significant adverse events occurred, and all subjects completed the study. The inulin intervention was associated with a significant change in microbiota composition (PERMANOVA $p < 0.001$) and a significant decrease in α-diversity (Fig. 5A, B). At the phylum level, the abundance of Bacteroidetes and Proteobacteria significantly decreased, whereas the proportion of Actinobacteria and Verrucomicrobia significantly increased (Table S6). Univariable analysis revealed 28 taxa with significantly different abundance before and after inulin treatment (Fig. 5C and Table S7). The abundance of 16 bacterial taxa (genera or higher taxonomic units), including confirmed butyrate producers such as *Faecalibacterium*, *Anaerostipes*, and *Eubacterium halii* or bacteria considered beneficial such as *Lactobacillus, Bifidobacterium*, and *Akkermansia*, increased after treatment. The abundance of 12 taxa, including *Alistipes, Odoribacter*, or *Bacteroides*, decreased.

In serum and feces, inulin intake was not associated with a shift in total metabolome composition, but using univariable analysis, we identified several metabolites that were significantly different

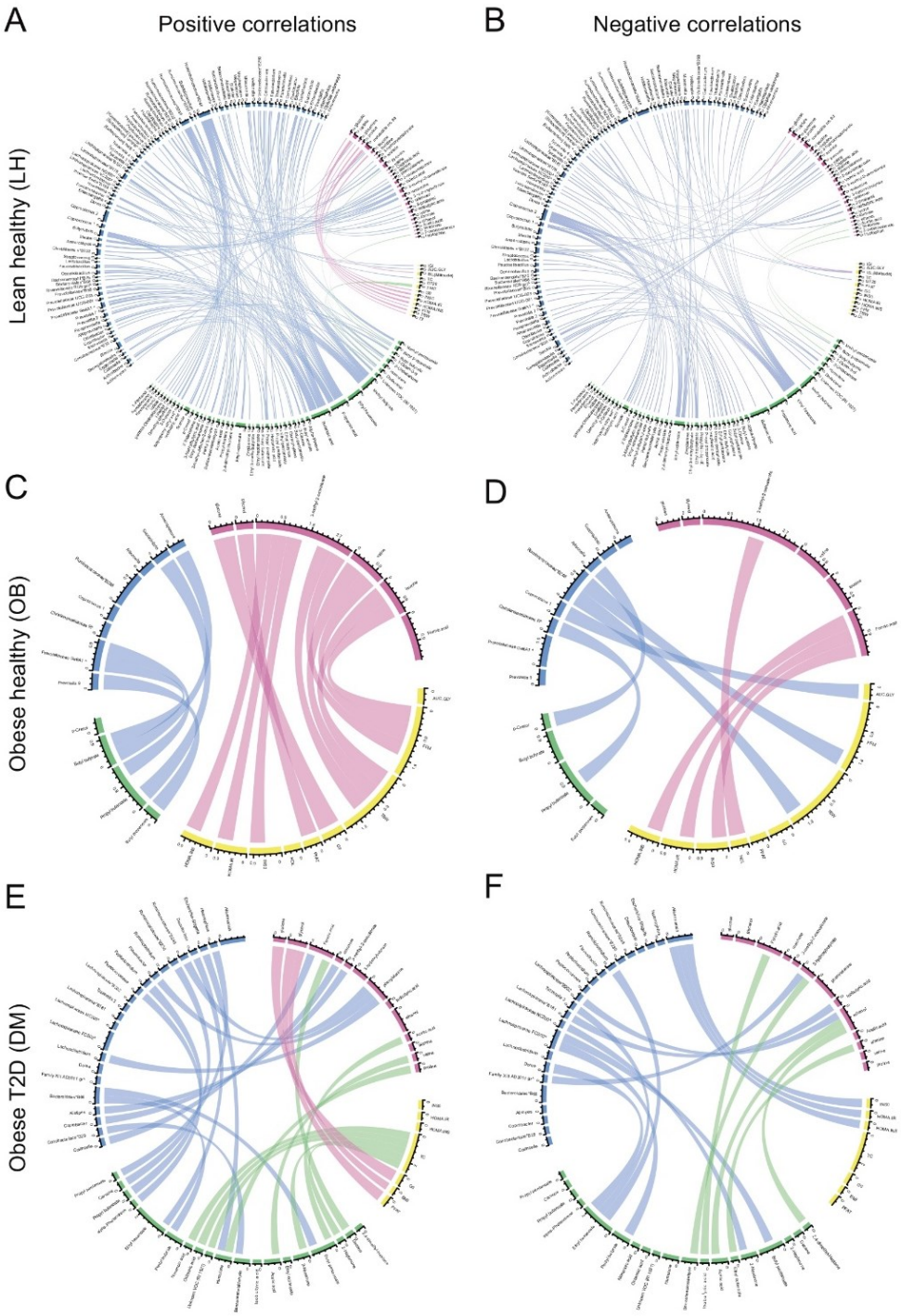


Fig. 4 Correlation chord diagrams between variables of different datasets. Spearman correlations were calculated for each group (LH, OB, DM) separately. Only correlations among variables from different datasets (clinical variables, microbiome, serum, and fecal metabolome) and characterized by $|\rho| > 0.5$ are presented. Positive (**A**, **C**, **E**) and negative (**B**, **D**, **F**) correlations are shown separately. The colors on the circuit code individual datasets, the color of the edges corresponds to one of the datasets that are linked by the edge. Blue: microbiome; green: fecal metabolome; yellow: clinical variables; violet: serum metabolome.

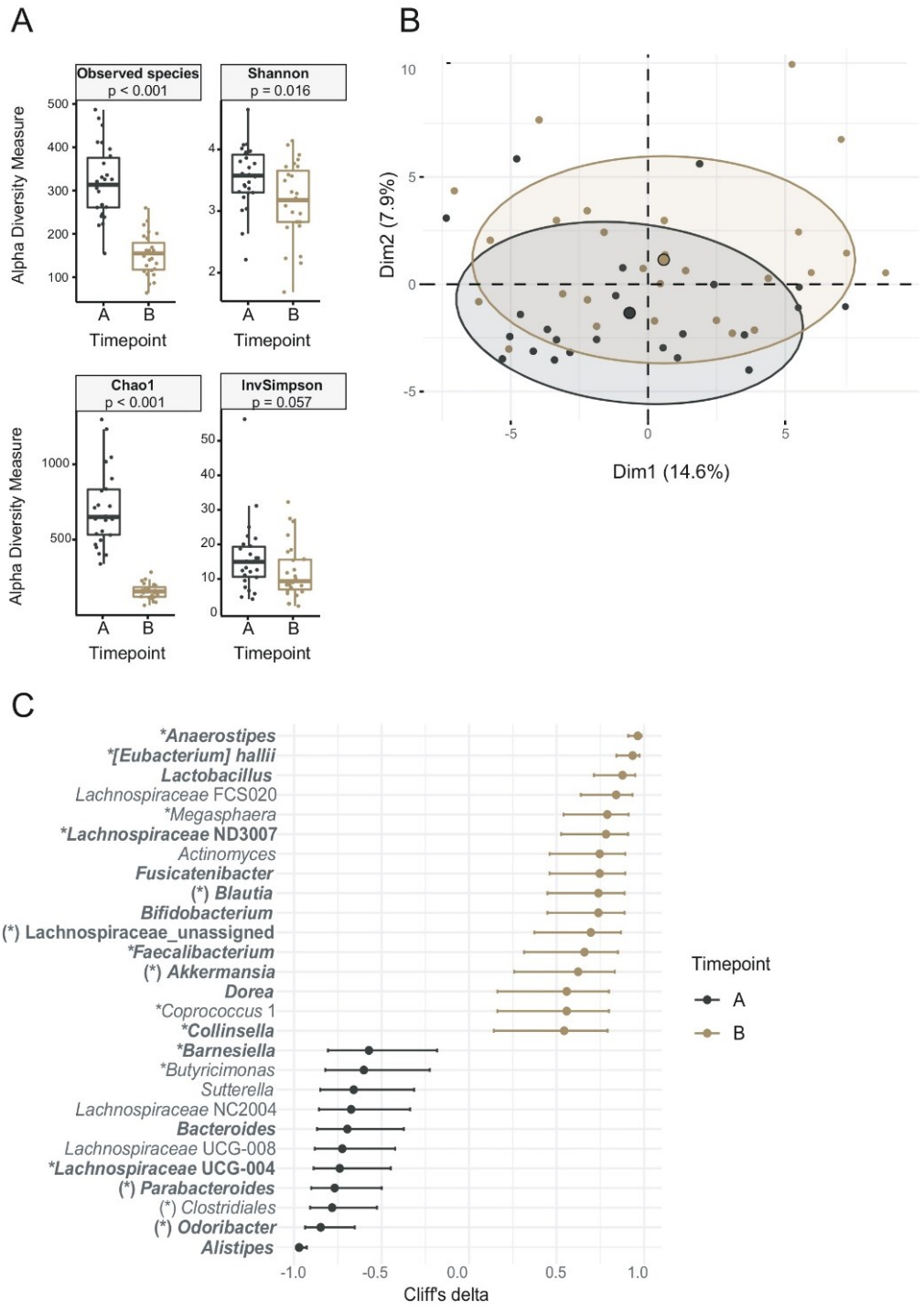


Fig. 5 Effect of inulin on fecal microbiome composition. **A** Alpha diversity calculated on rarefied ASV data, *p* value represents the result of Wilcoxon test; **B** 2D PCA scores plot on genera level. The explained variance of each component is included in the axis labels. The large points represent the centroids of each group. **C** Biomarker bacterial genera. Prior to all calculations, data were clr transformed. Biomarkers were generated from univariable discriminant analysis (FDR ≥0.1), with effect size estimated by Cliff's delta with a 95% confidence interval. A, time point prior to intervention; B, time point post-intervention. Core genera (bold) are defined by the condition abundance >0.05% and prevalence >75% at least in one group. Genera marked by * are confirmed butyrate producers, and genera marked by (*) are potential butyrate producers. ASV, amplicon sequence variant.

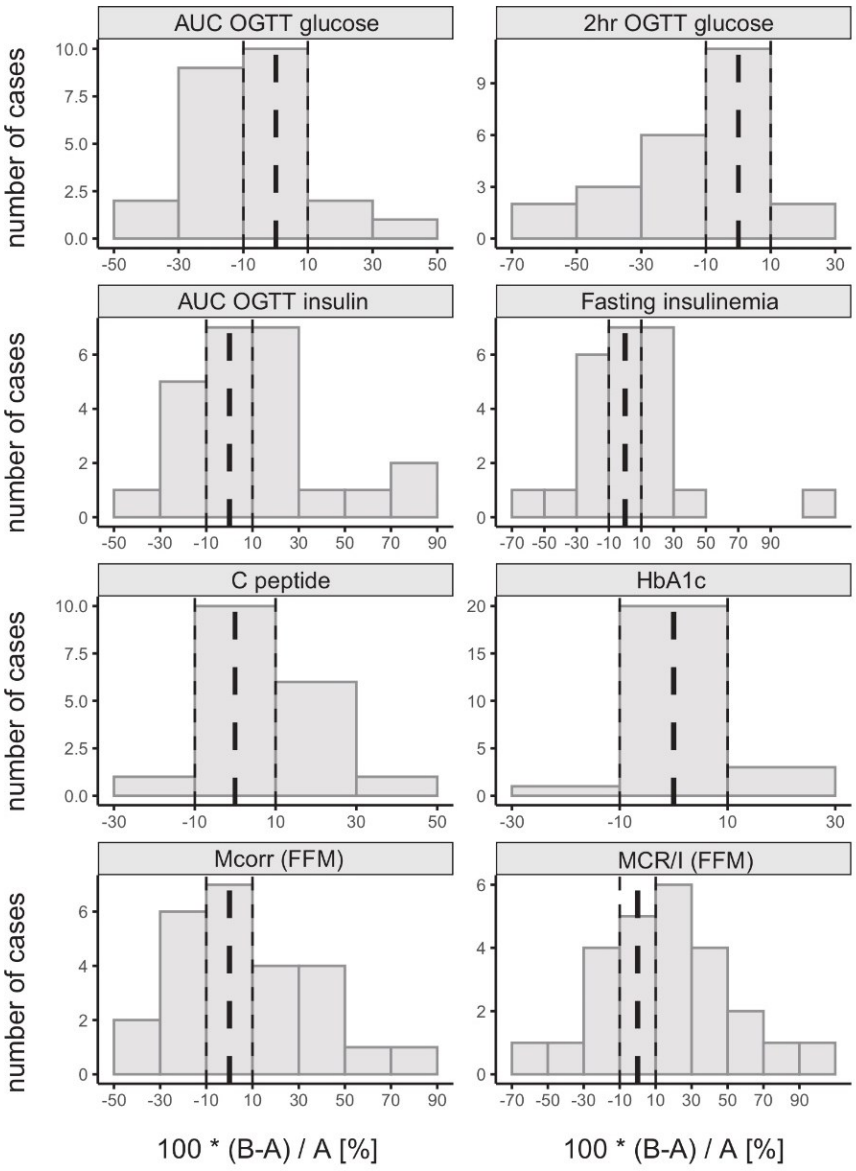


Fig. 6 Effect of inulin supplementation on selected markers of glucose metabolism. Data are expressed as the percentual change baseline to post-intervention. A, time point prior to intervention; B, time point post-intervention. Dashed line, 0%; dotted lines, ±10% range.

before and after the intervention. In serum, the concentration of butyric acid, propionic acid, and asparagine increased significantly, whereas the concentration of glycerol and 2-propanol decreased after inulin treatment (Fig. S15 and Table S8). In feces, three VOCs were significantly different in abundance ($p < 0.05$) before and after inulin treatment, including two propionic acid esters (increased) and 1-hexanol (decreased) (Table S9). However, the significance disappeared after multiple comparisons.

Prospective study: effect of inulin on glucose metabolism

Inulin intake affected markers of glucose tolerance and insulin sensitivity, but the individual response was highly variable; we observed positive, no, or negative changes for each of the variables (Fig. 6 and Table S10). In the entire intervention group, we observed a significant improvement in glucose tolerance (120 min OGTT glucose) and a trend toward a reduction in AUC for OGTT glucose and fasting glycemia. Skeletal muscle insulin sensitivity, measured by glucose clamp and expressed as MCR/I value, increased by more than 10% after the intervention compared with baseline in 14 subjects (from +11.4 to +62.4%), whereas it did not change or decrease in 13 subjects (from +4.8 to −48.7%). A similar distribution was observed for other indices of insulin sensitivity (Mcorr corrected for FFM, AUC OGTT insulin, and fasting insulinemia).

Prospective study: predictors of the metabolic effect of inulin

Because we replicated previous findings of large inter-individual differences in metabolic responses to inulin, we sought to identify predictors of these differences. To this end, we built linear regression models for all glucose metabolism parameters studied

Table 2. Predictors of the inulin treatment effect on glucose homeostasis parameters.

outcome	predictor	$\hat{\beta}_x$	*p* val βx	$\hat{\beta}_y$	*p* val βy	R^2
AUC OGTT glucose	*Ruminiclostridium*	−41.60	0.015	−0.11	0.236	0.393
	Lachnospiraceae_incertae sedis	40.37	0.015	−0.18	0.044	0.249
	Lachnoclostridium	35.83	0.033	−0.16	0.083	0.317
	3-methyl-2-oxovalerate	37.78	0.018	−0.18	0.044	0.326
	alanine	36.28	0.024	−0.19	0.040	0.287
	ethanol	−51.31	0.001	−0.21	0.008	0.501
2 h OGTT glucose	AUC OGTT insulin	1.00	0.027	−0.03	0.876	0.286
	fasting insulinemia	0.91	0.037	−0.10	0.536	0.181
	HOMA INS	0.88	0.046	−0.12	0.487	0.215
	Eubacterium halii group	0.99	0.032	−0.17	0.321	0.280
	3-methyl-2-oxovalerate	1.08	0.012	−0.09	0.585	0.287
	3-hydroxyisobutyrate	0.98	0.024	−0.14	0.392	0.278
	2-oxoisocaproate	0.93	0.033	−0.12	0.462	0.229
	pyruvate	0.93	0.034	−0.09	0.580	0.245
	indole	1.33	0.002	−0.11	0.465	0.350
	tridecanol	1.17	0.008	−0.13	0.426	0.300
	ç-Dodecalactone	1.13	0.012	−0.18	0.276	0.269
	methyl heptenone	1.05	0.020	−0.16	0.360	0.217
	2-undecanone	1.05	0.021	−0.16	0.341	0.240
	methyl butanal	1.02	0.024	−0.11	0.527	0.214
MCR/I (FFM)	ISI (Matsuda)	0.01	0.005	−0.67	0.008	0.313
	AUC OGTT insulin	−0.01	0.005	−0.53	0.017	0.291
	2 hr OGTT insulinemia	−0.01	0.024	−0.45	0.051	0.251
	HOMA INS	−0.01	0.027	−0.57	0.029	0.208
	fasting insulinemia	−0.01	0.030	−0.56	0.032	0.269
	HOMA IR	−0.01	0.036	−0.55	0.035	0.205
	IGI	−0.01	0.045	−0.40	0.080	0.189
	Blautia	−0.01	0.027	−0.22	0.329	0.222
	[Eubacterium] hallii group	−0.01	0.030	−0.16	0.451	0.180
	asparagine	0.01	0.011	−0.31	0.121	0.230
Δ Mcorr (FFM)	ISI (Matsuda)	1.02	0.001	−0.53	0.025	0.474
	AUC OGTT insulin	−0.90	0.002	−0.31	0.132	0.335
	HOMA INS	−0.90	0.003	−0.38	0.080	0.318
	Fasting insulinemia	−0.88	0.003	−0.37	0.090	0.326
	HOMA IR	−0.86	0.006	−0.38	0.091	0.243
	IGI	−0.75	0.009	−0.20	0.367	0.243
	2 h OGTT insulinemia	−0.66	0.029	−0.24	0.293	0.201
	Dialister	−0.58	0.038	−0.01	0.979	0.172
	Phascolarctobacterium	0.55	0.048	0.08	0.705	0.203
	asparagine	0.75	0.011	−0.26	0.239	0.210

The data shown in the table are derived from the linear regression model described by the equation $Y^{(B)} - Y^{(A)} = \beta_0 + \beta_Y Y^{(A)} + \beta_X X^{(A)} + \varepsilon$, where $Y^{(A)}$ stands for outcome variable at time A; $Y^{(B)}$ stands for outcome variable at time B, B > A; $X^{(A)}$ stands for a standardized variable at time A representing in each model any single clinical, metabolome or microbiome variable; β_x, β_y are model coefficients; ε stands for random error. Fecal metabolites were filtered by the condition Σ $AUC_x \geq 0.1\%$ Σ AUC_{total} across all samples; bacteria were filtered by the condition median abundance ≥0.1% of the total Σ of bacteria across all samples. *HbA1C* glycosylated hemoglobin, *Mcorr* glucose disposal space corrected and adjusted to fat-free mass, *MCR/I* metabolic clearance rate of glucose space corrected and adjusted to fat-free mass divided by steady-state insulinemia, *OGTT* oral glucose tolerance test, R^2 proportion of variation in y explained by the predictors obtained using bootstrapping (50 iterations).

as outcome variables, with all clinical or omics variables as predictors; we omitted variables with significant coefficients that had high leverage (Figs. S16–S19). Despite the limitations of our model, it showed several potentially interesting findings (summarized in Table 2). For example, the effect of inulin on skeletal muscle insulin sensitivity (Mcorr and MCR/I) could be predicted from pre-intervention glycemic measures. In contrast, the MIME predictors of the inulin effect were mostly not associated with pre-intervention outcome variables. Change in AUC OGTT glucose was negatively associated with an initial abundance of

Ruminiclostridium, whereas increases in Mcorr and MCR/I were associated with higher initial serum asparagine (both parameters) and lower *Dialister* (Mcorr) or *Blautia* and *Eubacterium halii* (MCR/I). Initial serum concentrations of BCAA derivatives were positively associated with increases in AUC and 2-hour OGTT glucose. All results are summarized in Table 2.

DISCUSSION

Our main findings are: (i) obesity is the dominant factor determining the MIME signature, whereas glycemic status has a lesser additional influence; (ii) the metabolic response to inulin supplementation in individuals with newly diagnosed prediabetes/diabetes is highly variable but can be predicted, at least in part, from baseline clinical characteristics and MIME signatures. Indeed, more insulin-resistant individuals with poorer glycemic indices and elevated circulating BCAA derivatives and fecal indole and p-cresol are less likely to respond to inulin supplementation.

Observational study: gut microbiome and metabolome

Obesity is a prominent risk factor for the development of type 2 diabetes. Numerous studies have identified groups of bacterial taxa that are enriched or depleted in obesity and type 2 diabetes, and despite considerable heterogeneity in the results, some common observations have been noted. First, type 2 diabetes is associated with the depletion of potentially beneficial bacteria rather than the presence of some dominant potentially harmful bacteria. Second, the abundance of butyrate producers and the functional potential for butyrate production is reduced in type 2 diabetes [10, 20, 35]. Third, the diversity of the microbiota is lower in diseased individuals compared with healthy controls [6, 36].

Some of our results are consistent with the above, whereas others are contradictory. In contrast to the results of Wu [10], the change in the composition of the gut microbiota in our study was not related to glycemic status but mainly to obesity. The dominant butyrate producers, such as *Faecalibacterium*, *Anaerostipes*, *Eubacterium halii*, or *Blautia* were significantly less abundant in the microbiota of DM and OB, but we did not detect lower SCFA concentrations in either feces or serum. In contrast, MCFA, nonanoic and decanoic acids were elevated in OB and DM. MCFA can originate from dietary sources [35], but also from microbial or yeast fermentation [37]. SCFA and MCFA have different immunomodulatory properties; whereas SCFA attenuate inflammation, MCFA have the opposite effect [35, 38, 39]. In addition, MCFA may enhance intestinal permeation because of their physicochemical properties as anionic surfactants [40]. Based on these findings, we might suggest that it is not the lower level of SCFA but the increased level of MCFA in the lumen that contributes to the complications associated with obesity, such as impaired intestinal barrier function or chronic low-grade inflammation.

Observational study: serum metabolome

The serum metabolome signature of obesity and diabetes overlapped greatly in the study. Compared to lean subjects, both the OB and DM signatures follow the same direction and differ only in magnitude. The "adiposity signature," which is similar in both OB and DM, includes SCFA (succinic and propionic acid increased, while valeric acid decreased), aromatic AA tyrosine (increased), and two other AA (histidine and asparagine, decreased). The concentration shift of five other metabolites, i.e., intermediates of saccharide metabolism (glucose, lactate, and mannose, increased) and AA glutamine and alanine, follows the concordant direction to LH, but there is a significant difference among all three groups. Six metabolites are specific for DM. This signature consists of three BCAA derivatives, formic acid, 2-hydroxybutyrate, acetone, and 2-propanol.

Our findings are consistent with previously published observations [41, 42]. Some signature metabolites could be attributed to altered saccharide metabolism in obesity and diabetes, such as glucose, mannose, and lactate. 2-propanol, acetone, and 2-hydroxybutyrate might be related to NADH/NAD+ redox imbalance, which has been proposed as one of the features of T2D [43].

Some other signature metabolites, i.e., SCFA and BCAA, are located at the interface between the host and microbiota. SCFA in serum have not previously been described as components of an obesity-related serum signature, probably because of the analytical difficulties associated with their determination in serum. They are exclusively microbial products, some of which (circulating butyric acid and propionic acid) have been associated with beneficial effects [44]. Elevated circulating BCAAs have been associated with insulin-resistance conditions such as obesity, diabetes [45], and even cancer [46]. For mammals, BCAAs are essential and must be supplied from external sources. Recent research has deciphered the importance of the gut microbiota in modulating the availability of many necessary compounds, including BCAA, to the host [47].

Inulin intervention and the effects on microbiota composition and performance

Three months of regular consumption of 10 g inulin/day was associated with a significant shift in the composition of the microbiota, characterized by a marked increase in potentially beneficial bacteria, many of which are capable of butyrate production [48]. At the same time, several bacterial taxa were depleted, such as those associated with the fermentation of proteins [49, 50]. This observation is largely consistent with previously published reports [51, 52].

We did not detect a significant shift in the composition of the fecal metabolome, although there was a non-significant trend toward an increase in SCFA esters content. Participants were asked not to change their dietary habits, and the only difference before and after the intervention was the amount of inulin consumed. This change could primarily increase the production of SCFA, but these compounds are readily utilized by other microbes or colonocytes at the site of their production, and only about 5% of SCFA are excreted in the feces [53]. A small fraction of SCFA from the intestine may enter the bloodstream, and indeed we observed a significant increase in serum butyric and propionic acid concentrations at the end of the intervention. Muller et al. [54] have previously reported that it is not fecal but circulating SCFA, particularly butyrate, that can provide a link between the gut microbiota and whole-body insulin sensitivity. SCFA are ligands of the G protein-coupled receptors GPR41 and GPR43, which are expressed in many tissues, including adipose tissue and skeletal muscle [55, 56]. Animal studies have shown that oral administration of SCFA or intravenous infusion improves insulin sensitivity [54].

Predicting the individual effect of an inulin intervention

The increasing understanding of the role of the microbiome in host physiology opened new avenues for research focused on the possibility of predicting the outcome of a given intervention based on the individual MIME setting. Clinically relevant results have been obtained in cancer research, e.g., the success of therapy with Anti-programmed Cell Death Protein-1 (PD−1) has been shown to depend significantly on the baseline composition of the patient's gut microbiota [57–60]. MIME has also been successfully used to predict the response of IBD patients to a low FODMAP diet [61] or anti-TNF treatment [62], the efficacy of synbiotic treatment of gastrointestinal disease in children [63], or the prediction of the clinical outcome of bariatric surgery [64]. The gut microbiota may serve as a biomarker for selecting the most effective drugs for the treatment of rheumatoid arthritis [65], and gut bacterial signatures have even been described to characterize the diagnosis and predict treatment outcomes in bipolar depression [66].

Inulin-type dietary fiber is thought to alleviate several features of metabolic syndrome; however, results from human studies are inconsistent. A recent systematic review [67], which included 33 RCTs, showed that inulin intake (average 11 g/day) significantly reduced blood glucose, total cholesterol, and TAG in individuals with prediabetes and diabetes. However, a common feature of all included studies was the wide heterogeneity of individual responses to treatment, making clear dietary recommendations difficult. Therefore, we sought to identify factors that would allow a personalized assessment of the efficacy of inulin treatment. We found that patients with a profile suggestive of less impaired glucose homeostasis were likely to improve metabolically. In addition, we identified several other potential predictors that were not dependent on pre-intervention glycemic indices, including lower serum BCAA derivatives (3-methy-2-oxovalerate, 2-oxoisocaproate), serum 3-hydroxyisobutyrate (product of NADH oxidation), fecal indole, and/or various bacteria (*Ruminiclostridium, Lachnoclostridium, Eubacterium halii*, etc.), which could allow a more accurate prediction of inulin intervention outcomes. In the prediabetes phase, patients are often advised to change their lifestyle and diet. Despite initial adherence to advice, outcomes may be highly variable, and patients who have failed despite their best efforts may be demotivated to adhere to further recommendations. The tool of predicting the individual appropriateness of a particular intervention, in this case, the administration of inulin, would help personalize treatment so that it has a higher chance of success in potential responders and does not expose potential non-responders to repeated failures.

Strengths and limitations of the study

There are several strengths of the study. First, the DM group included only participants with newly diagnosed type 2 diabetes prior and/or concomitant treatment, thus excluding confounding effects of antidiabetic drugs on the effects of inulin. Second, we did not rely solely on the measurement of fecal SCFA as the only indicator of SCFA production in the colon, but used a highly sensitive LC-MS method that allows its quantification in serum. Third, we evaluated the complex effects of the inulin intervention using a multi-omics approach. Nevertheless, the study is limited by several factors. First, we were able to include only a limited number of subjects, and the results were not validated in an independent cohort. For this, the results were internally validated by permutation tests. Second, the lean healthy subjects differed from the OB or DM groups by age, because obesity and associated comorbidities are more common in older populations. Age is one of the external factors affecting microbiota composition, but this is especially true for very young children or the elderly (over 70 years of age). In adolescence and adulthood, the composition of the microbiome is remarkably stable in terms of diversity indices, PCA metrics, or representation of selected taxa [68–70]. Therefore, we believe that the age difference in our population did not result in a significant bias. Third, we did not control dietary intake during the prospective intervention study with inulin because we did not want to further burden participants and increase the risk of dropping out of the study, but all participants were explicitly asked to maintain their usual dietary habits. An indirect measure of adherence to the habitual diet may be the BMI of participants, which did not change significantly during the intervention period. Finally, the prospective study design was a single-arm, non-controlled intervention study, so the causality of the effect of inulin on metabolic outcomes cannot be inferred. The small number of participants in the prospective study did not allow us to build more complex models to account for possible synergies among predictors. Because the study aimed to explore predictors, and we found several novel biomarkers that predict response to inulin treatment, these will need to be validated on a larger scale in future studies.

In summary, we showed that the gut microbiota and metabolome profiles in OB and DM differed from those of lean healthy individuals, whereas the differences between OB and DM were less pronounced. We identified several omics-derived biomarkers that may play a central role in the development of obesity-associated metabolic changes. In patients with newly diagnosed pre/diabetes, we observed substantial inter-individual variability in the effects of inulin on glucose homeostasis and identified several predictors of treatment response. If replicated in further studies with other populations, the identified predictors could facilitate the estimation of inulin intervention outcomes, paving the way for the concept of personalized dietary management of early diabetes.

DATA AVAILABILITY

The sequencing data were available at the SRA database under the accession number PRJNA823864. The other datasets generated and analyzed during the current study are available from the corresponding author on request.

REFERENCES

1. Cheng HL, Medlow S, Steinbeck K. The health consequences of obesity in young adulthood. Curr Obes Rep. 2016;5:30–7.
2. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006;444:1027–31.
3. Cornejo-Pareja I, Munoz-Garach A, Clemente-Postigo M, Tinahones FJ. Importance of gut microbiota in obesity. Eur J Clin Nutr. 2019;72:26–37.
4. Lim YY, Lee YS, Ooi DSQ. Engineering the gut microbiome for treatment of obesity: a review of current understanding and progress. Biotechnol J. 2020;15:e2000013.
5. Qin J, Li Y, Cai Z, Li S, Zhu J, Zhang F, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature. 2012;490:55–60.
6. Nuli R, Cai J, Kadeer A, Zhang Y, Mohemaiti P. Integrative analysis toward different glucose tolerance-related gut microbiota and diet. Front Endocrinol. 2019;10:295.
7. Zhou W, Sailani MR, Contrepois K, Zhou Y, Ahadi S, Leopold SR, et al. Longitudinal multi-omics of host-microbe dynamics in prediabetes. Nature. 2019;569:663–71.
8. Forslund K, Hildebrand F, Nielsen T, Falony G, Le Chatelier E, Sunagawa S, et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. Nature. 2015;528:262–6.
9. Allin KH, Tremaroli V, Caesar R, Jensen BAH, Damgaard MTF, Bahl MI, et al. Aberrant intestinal microbiota in individuals with prediabetes. Diabetologia. 2018;61:810–20.
10. Wu H, Tremaroli V, Schmidt C, Lundqvist A, Olsson LM, Kramer M, et al. The gut microbiota in prediabetes and diabetes: a population-based cross-sectional study. Cell Metab. 2020;32:379–90.e3.
11. Tilg H, Moschen AR. Microbiota and diabetes: an evolving relationship. Gut. 2014;63:1513–21.
12. Zhang X, Shen D, Fang Z, Jie Z, Qiu X, Zhang C, et al. Human gut microbiota changes reveal the progression of glucose intolerance. PLoS ONE. 2013;8:e71108.
13. Bhute SS, Suryavanshi MV, Joshi SM, Yajnik CS, Shouche YS, Ghaskadbi SS. Gut microbial diversity assessment of Indian type-2-diabetics reveals alterations in Eubacteria, Archaea, and Eukaryotes. Front Microbiol. 2017;8:214.
14. Zhao L, Lou H, Peng Y, Chen S, Zhang Y, Li X. Comprehensive relationships between gut microbiome and faecal metabolome in individuals with type 2 diabetes and its complications. Endocrine. 2019;66:526–37.
15. Zhong H, Ren H, Lu Y, Fang C, Hou G, Yang Z, et al. Distinct gut metagenomics and metaproteomics signatures in prediabetics and treatment-naive type 2 diabetics. EBioMedicine. 2019;47:373–83.
16. Wang L, Yu X, Xu X, Ming J, Wang Z, Gao B, et al. The fecal microbiota is already altered in normoglycemic individuals who go on to have type 2 diabetes. Front Cell Infect Microbiol. 2021;11:598672.
17. Letchumanan G, Abdullah N, Marlini M, Baharom N, Lawley B, Omar MR, et al. Gut microbiota composition in prediabetes and newly diagnosed type 2 diabetes: a systematic review of observational studies. Front Cell Infect Microbiol. 2022;12:943427.
18. Gaike AH, Paul D, Bhute S, Dhotre DP, Pande P, Upadhyaya S, et al. The gut microbial diversity of newly diagnosed diabetics but not of prediabetics is significantly different from that of healthy nondiabetics. mSystems. 2020;5:e00578-19.

19. Li L, Li C, Lv M, Hu Q, Guo L, Xiong D. Correlation between alterations of gut microbiota and miR-122-5p expression in patients with type 2 diabetes mellitus. Ann Transl Med. 2020;8:1481.
20. Karlsson FH, Tremaroli V, Nookaew I, Bergstrom G, Behre CJ, Fagerberg B, et al. Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature. 2013;498:99–103.
21. Diener C, Reyes-Escogido ML, Jimenez-Ceja LM, Matus M, Gomez-Navarro CM, Chu ND, et al. Progressive shifts in the gut microbiome reflect prediabetes and diabetes development in a treatment-naive Mexican cohort. Front Endocrinol. 2020;11:602326.
22. Ghaemi F, Fateh A, Sepahy AA, Zangeneh M, Ghanei M, Siadat SD. Intestinal microbiota composition in Iranian diabetic, pre-diabetic and healthy individuals. J Diabetes Metab Disord. 2020;19:1199–203.
23. Chen PC, Chien YW, Yang SC. The alteration of gut microbiota in newly diagnosed type 2 diabetic patients. Nutrition. 2019;63-64:51–6.
24. Prochazkova M, Budinska E, Kuzma M, Pelantova H, Hradecky J, Heczkova M, et al. Vegan diet is associated with favorable effects on the metabolic performance of intestinal microbiota: a cross-sectional multi-omics study. Front Nutr. 2021;8:783302.
25. Colantonio AG, Werner SL, Brown M. The effects of prebiotics and substances with prebiotic properties on metabolic and inflammatory biomarkers in individuals with type 2 diabetes mellitus: a systematic review. J Acad Nutr Diet. 2020;120:587–607.e2.
26. Davis LM, Martinez I, Walter J, Goin C, Hutkins RW. Barcoded pyrosequencing reveals that consumption of galactooligosaccharides results in a highly specific bifidogenic response in humans. PLoS ONE. 2011;6:e25200.
27. Zhao L, Zhang F, Ding X, Wu G, Lam YY, Wang X, et al. Gut bacteria selectively promoted by dietary fibers alleviate type 2 diabetes. Science. 2018;359:1151–6.
28. Wareham NJ. Personalised prevention of type 2 diabetes. Diabetologia. 2022;65:1796–1803.
29. Le DS, Brookshire T, Krakoff J, Bunt JC. Repeatability and reproducibility of the hyperinsulinemic-euglycemic clamp and the tracer dilution technique in a controlled inpatient setting. Metabolism. 2009;58:304–10.
30. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol. 1979;237:E214–23.
31. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13:581–3.
32. Han J, Lin K, Sequeira C, Borchers CH. An isotope-labeled chemical derivatization method for the quantitation of short-chain fatty acids in human feces by liquid chromatography-tandem mass spectrometry. Anal Chim Acta. 2015;854:86–94.
33. Dieterle F, Ross A, Schlotterbeck G, Senn H. Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in 1H NMR metabonomics. Anal Chem. 2006;78:4281–90.
34. R Core Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. 2017.
35. Sam QH, Ling H, Yew WS, Tan Z, Ravikumar S, Chang MW, et al. The divergent immunomodulatory effects of short chain fatty acids and medium chain fatty acids. Int J Mol Sci. 2021;22:6453.
36. Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, et al. Richness of human gut microbiome correlates with metabolic markers. Nature. 2013;500:541–6.
37. Nissen L, Samaei SP, Babini E, Gianotti A. Gluten free sourdough bread enriched with cricket flour for protein fortification: Antioxidant improvement and Volatilome characterization. Food Chem. 2020;333:127410.
38. Luscombe VB, Lucy D, Bataille CJR, Russell AJ, Greaves DR. 20 years an orphan: is GPR84 a plausible medium-chain fatty acid-sensing receptor? DNA Cell Biol. 2020;39:1926–37.
39. Saresella M, Marventano I, Barone M, La Rosa F, Piancone F, Mendozzi L, et al. Alterations in circulating fatty acid are associated with gut microbiota dysbiosis and inflammation in multiple sclerosis. Front Immunol. 2020;11:1390.
40. Brayden DJ, Maher S, Bahar B, Walsh E. Sodium caprate-induced increases in intestinal permeability and epithelial damage are prevented by misoprostol. Eur J Pharm Biopharm. 2015;94:194–206.
41. Halama A, Suleiman NN, Kulinski M, Bettahi I, Hassoun S, Alkasem M, et al. The metabolic footprint of compromised insulin sensitivity under fasting and hyperinsulinemic-euglycemic clamp conditions in an Arab population. Sci Rep. 2020;10:17164.
42. Gall WE, Beebe K, Lawton KA, Adam KP, Mitchell MW, Nakhle PJ, et al. alpha-hydroxybutyrate is an early biomarker of insulin resistance and glucose intolerance in a nondiabetic population. PLoS ONE. 2010;5:e10883.
43. Song J, Yang X, Yan LJ. Role of pseudohypoxia in the pathogenesis of type 2 diabetes. Hypoxia. 2019;7:33–40.
44. Kumar J, Rani K, Datt C. Molecular link between dietary fibre, gut microbiota and health. Mol Biol Rep. 2020;47:6229–37.
45. Newgard CB. Interplay between lipids and branched-chain amino acids in development of insulin resistance. Cell Metab. 2012;15:606–14.
46. Rossmeislova L, Gojda J, Smolkova K. Pancreatic cancer: branched-chain amino acids as putative key metabolic regulators? Cancer Metastasis Rev. 2021;40:1115–39.
47. Gojda J, Cahova M. Gut microbiota as the link between elevated BCAA serum levels and insulin resistance. Biomolecules. 2021;11:1414.
48. Le Bastard Q, Chapelet G, Javaudin F, Lepelletier D, Batard E, Montassier E. The effects of inulin on gut microbial composition: a systematic review of evidence from human studies. Eur J Clin Microbiol Infect Dis. 2020;39:403–13.
49. Diether NE, Willing BP. Microbial fermentation of dietary protein: an important factor in diet(-)microbe(-)host interaction. Microorganisms. 2019;7:19.
50. Yao CK, Muir JG, Gibson PR. Review article: insights into colonic protein fermentation, its modulation and potential health implications. Aliment Pharm Ther. 2016;43:181–96.
51. Vandeputte D, Tito RY, Vanleeuwen R, Falony G, Raes J. Practical considerations for large-scale gut microbiome studies. FEMS Microbiol Rev. 2017;41:S154–S67.
52. Healey G, Murphy R, Butts C, Brough L, Whelan K, Coad J. Habitual dietary fibre intake influences gut microbiota response to an inulin-type fructan prebiotic: a randomised, double-blind, placebo-controlled, cross-over, human intervention study. Br J Nutr. 2018;119:176–89.
53. Rechkemmer G, Ronnau K, von Engelhardt W. Fermentation of polysaccharides and absorption of short chain fatty acids in the mammalian hindgut. Comp Biochem Physiol A Comp Physiol. 1988;90:563–8.
54. Muller M, Hernandez MAG, Goossens GH, Reijnders D, Holst JJ, Jocken JWE, et al. Circulating but not faecal short-chain fatty acids are related to insulin sensitivity, lipolysis and GLP-1 concentrations in humans. Sci Rep. 2019;9:12515.
55. Tang C, Ahmed K, Gille A, Lu S, Grone HJ, Tunaru S, et al. Loss of FFA2 and FFA3 increases insulin secretion and improves glucose tolerance in type 2 diabetes. Nat Med. 2015;21:173–7.
56. Koh A, De Vadder F, Kovatcheva-Datchary P, Backhed F. From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites. Cell. 2016;165:1332–45.
57. Mao J, Wang D, Long J, Yang X, Lin J, Song Y, et al. Gut microbiome is associated with the clinical response to anti-PD-1 based immunotherapy in hepatobiliary cancers. J Immunother Cancer. 2021;9:e003334.
58. Fahrmann JF, Saini NY, Chia-Chi C, Irajizad E, Strati P, Nair R, et al. A polyamine-centric, blood-based metabolite panel predictive of poor response to CAR-T cell therapy in large B cell lymphoma. Cell Rep. Med. 2022;3:100720.
59. Sannicolo S, Giaj Levra M, Le Gouellec A, Aspord C, Boccard J, Chaperot L, et al. Identification of a predictive metabolic signature of response to immune checkpoint inhibitors in non-small cell lung cancer: METABO-ICI clinical study protocol. Respir Med Res. 2021;80:100845.
60. McCulloch JA, Davar D, Rodrigues RR, Badger JH, Fang JR, Cole AM, et al. Intestinal microbiota signatures of clinical response and immune-related adverse events in melanoma patients treated with anti-PD-1. Nat Med. 2022;28:545–56.
61. Vervier K, Moss S, Kumar N, Adoum A, Barne M, Browne H, et al. Two microbiota subtypes identified in irritable bowel syndrome with distinct responses to the low FODMAP diet. Gut. 2022;71:1821–30.
62. Busquets D, Oliver L, Amoedo J, Ramio-Pujol S, Malagon M, Serrano M, et al. RAID prediction: pilot study of fecal microbial signature with capacity to predict response to anti-TNF treatment. Inflamm Bowel Dis. 2021;27:S63–S6.
63. Tierney BT, Versalovic J, Fasano A, Petrosino JF, Chumpitazi BP, Mayer EA, et al. Functional response to a microbial synbiotic in the gastrointestinal system of children: a randomized clinical trial. Pediatr Res. 2022. https://doi.org/10.1038/s41390-022-02289-0.
64. Vaz M, Pereira SS, Monteiro MP. Metabolomic signatures after bariatric surgery - a systematic review. Rev Endocr Metab Disord. 2022;23:503–19.
65. Wei M, Chu CQ. Prediction of treatment response: personalized medicine in the management of rheumatoid arthritis. Best Pr Res Clin Rheumatol. 2022;36:101741.
66. Lai J, Li A, Jiang J, Yuan X, Zhang P, Xi C, et al. Metagenomic analysis reveals gut bacterial signatures for diagnosis and treatment outcome prediction in bipolar depression. Psychiatry Res. 2022;307:114326.
67. Li L, Li P, Xu L. Assessing the effects of inulin-type fructan intake on body weight, blood glucose, and lipid profile: a systematic review and meta-analysis of randomized controlled trials. Food Sci Nutr. 2021;9:4598–616.
68. Odamaki T, Kato K, Sugahara H, Hashikura N, Takahashi S, Xiao JZ, et al. Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. BMC Microbiol. 2016;16:90.
69. Rodriguez JM, Murphy K, Stanton C, Ross RP, Kober OI, Juge N, et al. The composition of the gut microbiota throughout life, with an emphasis on early life. Micro Ecol Health Dis. 2015;26:26050.
70. Salazar N, Gonzalez S, Nogacka AM, Rios-Covian D, Arboleya S, Gueimonde M, et al. Microbiome: effects of ageing and diet. Curr Issues Mol Biol. 2020;36:33–62.

71. Feins EN, Ireland C, Gauvreau K, Chavez M, Callahan R, Jenkins KJ, et al. Pulmonary vein stenosis: anatomic considerations, surgical management, and outcomes. J Thorac Cardiovasc Surg. 2022;163:2198–207.e3.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Health of the Czech Republic, grant NV-18-01-00040 and by the project National Institute for Research of Metabolic and Cardiovascular Diseases (Program EXCELES, Project No. LX22NPO5104)—Funded by the European Union—Next Generation EU. Jan Gojda is supported by EFSD mentorship program supported by AstraZeneca.

AUTHOR CONTRIBUTIONS

ND, TK, MC, and JG were involved in the study concept and design. ND, MP, MK, HP, JH, MHec, MB, PV, PS, MHen, AO, JP, and RL participated in the acquisition of data. ND, IM, and MK were involved in statistical analysis. ND, IM, MK, HP, RL, TK, MK, MC, and JG participated in interpretation of data. ND, MC, TK, and JG drafted the manuscript. All authors participated in the critical revision of the manuscript and approved the final version of the manuscript. The senior and corresponding authors (JG, TK, and MC) take full responsibility for the work and/or the conduct of the study, they had access to the data and controlled the decision to publish.

CONFLICT OF INTEREST

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41387-023-00235-5.

Correspondence and requests for materials should be addressed to M. Cahová.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

3.4 Assessment of the protective effect of vegan microbiota against the influence of the obesogenic diet

As described in the introductory section, gut microbiota plays an essential role in energy homeostasis, weight control and inflammation, which are all related to NCDs like obesity, T2D or non-alcoholic fatty liver disease (NAFLD). Targeted modulation of gut microbiota and its metabolic programming is considered a potentially promising therapeutic approach in the NCDs treatment but more research on this topic is definitely needed. Fecal microbial transfer (FMT) is gaining more attention due to its potential therapeutic properties by altering the entire microbial community. As noted above, vegan or plant-based diets are associated with beneficial effects on overall health, suggesting that vegan microbiota might be desirable, and subjects adhering to plant-based diets should be explored as suitable candidates for FMT donors. However, it remains unclear how the transferred microbiota is affected by the host diet and the substrates provided.

In this study, stool from four vegan donors was used to prepare a mixed VG inoculum used for FMT transfer to GF animals. Female GF mice were colonized with VG inoculum and were paired with male GF mice. Their offspring were further used for the experiment when ex-GF humanized mice (VG) were fed either a Western-type diet (WD) or a standard diet (SD) with or without the addition of inulin (I). The same experimental design was used in conventional mice (CV). The objectives of this study were to determine whether and how the vegan microbiota may have a protective effect against an obesogenic diet, to describe the mechanistic relationships of microbiome and metabolome in these mice, and to explore the effect of fiber in enhancing the additional therapeutic potential of the vegan microbiota.

After an eight-week experimental period on specific diets, glucose and lipid homeostasis parameters, fecal microbiota composition and serum and fecal metabolome were determined. Western diet caused a significant increase in total body weight and liver triacylglycerol content in both mice models (Kruskal–Wallis test and Dunn's post hoc test with the Benjamin–Hochberg correction, $p < 0.05$). Impaired glucose homeostasis caused by the Western diet was observed only in the VG group. Inulin supplementation reversed the liver steatosis and improved glucose homeostasis in the VG group, but not in the CV group, so further analyses focused on the VG group only. Regarding microbiota in the VG group, pairwise PERMANOVA analysis on the taxonomic level species showed significant differences between all dietary groups (SD vs SD + I $p = 0.0011$, SD vs WD $p = 0.0011$, SD vs WD + I $p = 0.0011$, WD vs WD + I $p = 0.0042$). The LASSO machine learning regression model was able to classify bacteria at the species level between all pairs of groups with at least 90% accuracy, sensitivity and specificity.

Untargeted metabolome analysis identified 61 VOCs in cecum content. Inulin supplementation did not lead to an alteration of cecum metabolome in the SD diet group (paired PERMANOVA, $p > 0.1$), but resulted in a significant change in the WD group (paired PERMANOVA, $p = 0.005$). Interestingly, after inulin supplementation, we observed a shift from amino acid fermentative metabolism to saccharolytic fermentation described by a decrease of the product of tryptophan fermentation indole (only in VG_SD+I group), a decrease of methionine/cysteine fermentation product dimethyl trisulfide (in both VG_SD+I and VG_WD+I groups), increase of butyrate (only in VG_SD+I group) and increase of acetic acid (in both VG_SD+I and VG_WD+I groups). The serum metabolome assessed by NMR spectroscopy was not significantly affected by any of the treatments. Paired PERMANOVA analysis revealed no difference between groups (all paired PERMANOVA tests > 0.15).

In this animal model, we demonstrated that vegan microbiota alone may not be sufficient to counteract the negative metabolic effects of a Western-type diet. However, further supplementation by dietary fiber (in this case inulin) can protect from steatosis and impairment of glucose metabolism. Notably, this effect was only observed in humanized mice and not in conventional mice models. Furthermore, inulin supplementation in humanized mice model led to a shift in the cecal microbial community and its metabolic performance. These results suggest that the treatment of metabolic disorders by FMT should be also supported by subsequent dietary precautions in order for the treatment to be more successful.

This article was published on the 15th of January 2023 in Nutrients, IF = 6.71

nutrients

MDPI

Article

Protective Effect of Vegan Microbiota on Liver Steatosis Is Conveyed by Dietary Fiber: Implications for Fecal Microbiota Transfer Therapy

Nikola Daskova [1,2], Marie Heczkova [1], Istvan Modos [1], Jaromir Hradecky [3], Tomas Hudcovic [4], Marek Kuzma [4], Helena Pelantova [4], Irena Buskova [1], Eva Sticova [1], David Funda [4], Jaroslav Golias [4], Barbora Drabonova [5], Jarmila Jarkovska [4], Maria Kralova [6], Ivana Cibulkova [7], Jan Gojda [7] and Monika Cahova [1,*]

1 Institute for Clinical and Experimental Medicine, 14021 Prague, Czech Republic
2 First Faculty of Medicine, Charles University, Katerinska 1660/32, 12108 Prague, Czech Republic
3 Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, 16500 Prague, Czech Republic
4 Institute of Microbiology of the CAS, 14220 Prague, Czech Republic
5 Faculty of Agrobiology, Food, and Natural Resources, Czech University of Life Sciences, 16500 Prague, Czech Republic
6 Department of Applied Mathematics and Computer Science, Masaryk University, 60177 Brno, Czech Republic
7 Department of Internal Medicine, Kralovske Vinohrady University Hospital and Third Faculty of Medicine, Charles University, 10000 Prague, Czech Republic
* Correspondence: monika.cahova@ikem.cz

Abstract: Fecal microbiota transfer may serve as a therapeutic tool for treating obesity and related disorders but currently, there is no consensus regarding the optimal donor characteristics. We studied how microbiota from vegan donors, who exhibit a low incidence of non-communicable diseases, impact on metabolic effects of an obesogenic diet and the potential role of dietary inulin in mediating these effects. Ex-germ-free animals were colonized with human vegan microbiota and fed a standard or Western-type diet (WD) with or without inulin supplementation. Despite the colonization with vegan microbiota, WD induced excessive weight gain, impaired glucose metabolism, insulin resistance, and liver steatosis. However, supplementation with inulin reversed steatosis and improved glucose homeostasis. In contrast, inulin did not affect WD-induced metabolic changes in non-humanized conventional mice. In vegan microbiota-colonized mice, inulin supplementation resulted in a significant change in gut microbiota composition and its metabolic performance, inducing the shift from proteolytic towards saccharolytic fermentation (decrease of sulfur-containing compounds, increase of SCFA). We found that (i) vegan microbiota alone does not protect against adverse effects of WD; and (ii) supplementation with inulin reversed steatosis and normalized glucose metabolism. This phenomenon is associated with the shift in microbiota composition and accentuation of saccharolytic fermentation at the expense of proteolytic fermentation.

Keywords: fecal microbiota transfer; vegan microbiota; liver steatosis; inulin; proteolytic fermentation

Citation: Daskova, N.; Heczkova, M.; Modos, I.; Hradecky, J.; Hudcovic, T.; Kuzma, M.; Pelantova, H.; Buskova, I.; Sticova, E.; Funda, D.; et al. Protective Effect of Vegan Microbiota on Liver Steatosis Is Conveyed by Dietary Fiber: Implications for Fecal Microbiota Transfer Therapy. *Nutrients* **2023**, *15*, 454. https://doi.org/10.3390/nu15020454

Academic Editors: Montserrat Esteve and Maria del Mar Romero

Received: 17 November 2022
Revised: 12 January 2023
Accepted: 13 January 2023
Published: 15 January 2023

1. Introduction

A Western-type diet characterized by a high intake of refined sugars, animal fats, and processed food, is associated with a sharp increase in the prevalence of obesity and non-alcoholic fatty liver disease (NAFLD), one of the most common liver diseases worldwide [1,2]. Gut microbiota have repeatedly been shown to be among the most important mediators between diet and obesity risk [3,4]. Several mechanisms were proposed: enhanced energy harvest, central effects on satiety perception, impairment of intestinal barrier function, and promotion of chronic inflammation [5]. Certain metagenomic patterns associated with obesity have been described in the literature [6,7], but they are significantly influenced by factors such as age, ethnicity, and geography [8].

Up to now, there is no proven pharmacological treatment for NAFLD and the therapeutic strategies are based mostly on lifestyle interventions, namely, diet [9]. There are numerous dietary regimes aimed at weight loss and the improvement of metabolic health [10,11]. Modulation of nutrient intake has several direct effects on the host physiology, such as via nutrient load. In addition, rapidly emerging research into the essential role of the human microbiome in host physiology opened up the question of whether the beneficial effects of various diets could be mediated, in some aspects, by shaping the intestinal microbiota and changing its metabolic programming. Besides delivery of prebiotic substrates or probiotic intervention, fecal microbiota transfer (FMT), a currently approved therapeutic approach in the treatment of *Clostridioides difficile* infection [12], has gained growing attention in the context of therapy of other non-communicable diseases, including obesity or metabolic syndrome [13]. In contrast to probiotic treatment, which does not induce an alteration in microbiota composition [14], FMT causes a structural change in the whole gut microbial community [12], and thus may convey complex beneficial effects. Up to now, six randomized clinical trials assessing the use of FMT from lean omnivore donors in obese and metabolic syndrome patients have been reported [13,15–18]. Meta-analysis of the data showed only a partial effect of FMT on obesity-related disorders. While there was a significant reduction in HbA1c, HDL, and LDL cholesterol levels in FMT recipients, there was no modification of weight, serum TAG content, or reduction in glycemia [19]. However, none of these studies included any dietary recommendations after FMT.

The effective use of FMT is limited by the insufficient definition of the optimal donor. According to the current literature, a vegan diet is considered a metabolic health-promoting approach [20–24], and diet is one of the main environmental factors modulating the composition of gut microbiota [25]. Therefore, vegans were implied as suitable donors, though with unequivocal results. It was shown that vegan FMT alone had a modestly beneficial effect in the treatment of steatohepatitis [26] but failed to elicit changes in trimethylamine-N-oxide production in patients with metabolic syndrome [17]. As the receivers did not change their eating habits, the vegan microbiota itself were probably unable to counteract the effect of the unhealthy obesogenic diet. In line with this observation, our previous study showed that strong adherence to a vegan diet in humans resulted in only a relatively mild effect on microbiota composition [27]. On the other hand, veganism was associated with significant modification of microbiota performance towards the beneficial metabolite spectrum, but this aspect could be manifested only in combination with a diet rich in plant-based food.

Despite these preliminary data, it has not been explored how the transferred microbiota are affected by the host gut environment and the substrates provided. Therefore, we aimed to seek whether the metabolic effects of vegan-derived FMT are mediated by diet, and particularly by dietary fiber. Using the model of ex-germ-free mice colonized with human vegan microbiota, we explored (i) how vegan microbiota protects against obesogenic (Western-type) diets; (ii) mechanistic relations in microbiome/metabolome composition, and (iii) the ability of dietary fiber (inulin) to enforce vegan microbiota therapeutic potential in the obesogenic milieu.

2. Materials and Methods

2.1. Gut Microbiota Donors

Four vegan donors who did not object to animal experiments for ethical reasons were recruited from the vegan cohort described in detail in our previous study [27]. Their clinical characteristics are given in Table 1 and the Supplemental File S1.

All of them strictly avoided all animal products for at least three years. The exclusion criteria were chronic diseases related to metabolism, diseases of the digestive tract, antibiotic therapy in the past three months, pregnancy, any chronic medication (excluding hormonal contraception), and regular alcohol consumption defined as any alcoholic drink on a daily basis. The participants were asked to donate a fresh stool sample, which was immediately processed [28]. The total bacteria number in each sample was assessed by

quantitative pan-bacterial real-time PCR (forward primer ACACTGACGACATGGTTC-TACAGAGTTGATCNTGGCTCAG, reverse primer TACGGTAGCAGAGACTTGGTCTGT-NTTANGCGGCKGCTG) and each inoculum was diluted to approx. the same bacterial abundance (3.9×10^6 CFU/μL). The mixed VG inoculum was prepared by mixing an equal amount of each sample. The aliquots were kept frozen with buffered glycerol at −80 °C until the transfer.

Table 1. Baseline clinical characteristics of stool donors. HDL-ch, HDL-cholesterol; LDL-ch, LDL-cholesterol; TC-ch, total cholesterol; TAG, serum triacylglycerol.

ID	Sex	Age	BMI	Glucose mM	TC mM	HDL-ch mM	LDL-ch mM	TAG mM	CRP Mg/L
1	F	24.5	21.8	4.86	2.8	0.89	1.33	1.28	2.1
2	M	29.1	20.3	4.65	3.23	1.6	1.45	0.41	0.3
3	M	31.2	21.4	5.04	3.68	1.21	1.93	1.2	0.3
4	F	40.5	22.5	4.9	4.58	2.16	2.12	0.66	0.5

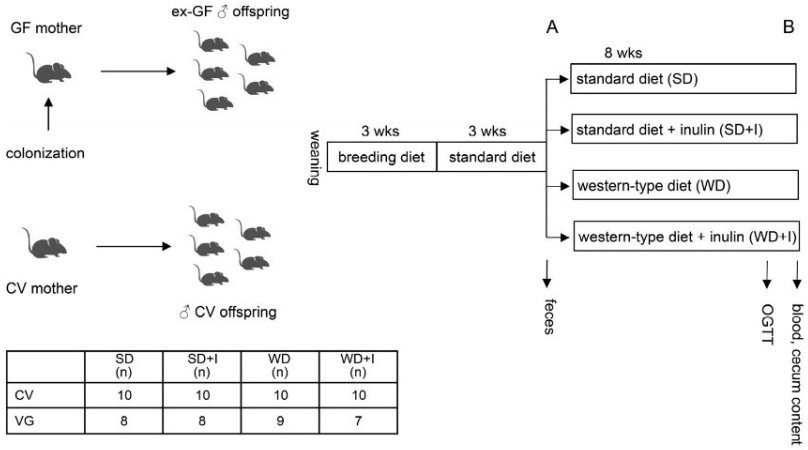
2.2. Animals

Germ-free C57Bl6 mice originated from the colony bred at the Gnotobiology laboratory Institute of Microbiology of the CAS, Novy Hradek, CR. Mice were kept under sterile conditions in Trexler-type plastic isolators, exposed to 12:12 h light–dark cycles, and supplied with autoclaved tap water and 50 kGy irradiated sterile pellet (breeding diet: Altromin 1414, Altromin, Germany) ad libitum. Axenicity was assessed every two weeks by confirming the absence of bacteria, mold, and yeast by aerobic and anaerobic cultivation of mouse feces and swabs. Female mice were colonized by mixed VG inoculum, bacterial load $\geq 1 \times 10^9$ bacteria by the means of administration on the skin, enema, and oral gavage. The colonized females were mated to germ-free males. Their male offspring, further described as VG, were kept in gnotobiotic isolators and used for the experiments. We decided to adopt this design because we aimed to create a "physiologically normal" mouse model colonized with human vegan microbiota. Recent evidence shows that maternal exposure to intestinal microbes triggers a wide range of adaptations in the offspring and the pups born to colonized mothers differ from germ-free mice colonized later during their lifetime [29,30]. Another reason is that with maternal colonization, we achieve high homogeneity of offspring colonization. After weaning, all animals were fed a breeding diet for 3 weeks. Conventional C57Bl6 mice (CV) were obtained from the breeding facility of the Institute of Microbiology of the CAS, Prague, CR. The power analysis was calculated to estimate the minimal number of animals per group according to the main outcome variable, liver TAG content (min n = 5 for $p < 0.05$ with 0.8 probability). At time point A, mice were randomly divided into four groups, each of them receiving a specific diet for another 8 weeks: SD (standard diet), SD + I (standard diet + 10% inulin), WD (western diet), WD + I (western diet + 10% inulin). The experimental design is shown in Figure 1. After 8 weeks (timepoint B), an oral glucose tolerance test was performed, animals were killed by an overdose of anesthesia, and tissue samples were collected for further analyses. Feces were collected at time points A and B. Experimental diets, the Western diet (42 kJ% fat, 43 kJ% carbohydrates, 15 kJ% protein, no. TD88137 mod.), and standard diet control of the Western (13 kJ% fat, 69 kJ% carbohydrates, 15 kJ% protein, no. CD88137) were bought from Ssniff (Soest, Germany). Inulin-supplemented diets (10% *wt*/*wt*) were custom-made by Ssniff. The diets were sterilized by irradiation. All animal experiments were conducted in concordance with the Guide for the Care and Use of Laboratory Animals (2011).

2.3. Oral Glucose Tolerance Test

An oral glucose tolerance test (OGTT) and a parallel assay of C-peptide concentration in serum were performed after the administration of a dose of glucose (1 mg. g^{-1} body weight) into overnight fasting mice. Blood was taken from the tail vein into heparinized capillaries. The blood glucose was determined with a glucometer (Roche, Basel, Switzerland)

and C-peptide concentration with an ELISA kit (Mercodia, Uppsala, Sweden). Sampling was performed at 0, 30, 60, and 120 min for each mouse.



	SD (n)	SD+I (n)	WD (n)	WD+I (n)
CV	10	10	10	10
VG	8	8	9	7

Figure 1. Experimental design. CV, conventional mice; GF, germ-free; OGTT, oral glucose tolerance test. (**A**), timepoint A (prior diet intervention); (**B**), timepoint B (after diet intervention).

2.4. Stool and Cecum Content Bacteriome Analysis

Stool samples were collected from each mouse individually at timepoint A, and the cecum content was collected at timepoint B. Samples were kept at −80 °C until the DNA was extracted using a QIAmp PowerFecal DNA Kit (Qiagen, Hilden, Germany), and the V4 region of the bacterial 16S rRNA gene was amplified by PCR. Sequencing was performed with the MiSeq reagent kit v2 using a MiSeq instrument (Illumina, Hayward, CA, USA). Raw sequences were processed using a DADA2 amplicon denoiser [31]. Subsequent taxonomic assignment was conducted by the assignTaxonomy function from the DADA2 R package using the Silva 138.1 reference database [32] at the levels L_1 (Phylum), L_2 (Class), L_3 (Order), L_4 (Family), L_5 (Genus) and L_6 (Species).

2.5. Volatile Compounds (VOCs) Analysis in Feces

Cecum content was homogenized and diluted to the equivalent of 1% (*wt*/*wt*) dry mass. Volatile fingerprinting of fecal samples was performed using an Agilent 7890B gas chromatograph (Agilent Technologies, Canta Clara, CA, USA) coupled to a Pegasus 4D time of flight mass spectrometer (LECO, USA). Volatiles were collected using solid-phase microextraction (SPME) fiber with a divinylbenzene/carboxen/polydimethylsiloxane coating from Supelco (USA). Data acquisition and initial data processing were carried out using instrumental SW ChromaTOF by LECO.

2.6. NMR Analyses

Serum samples (after protein precipitation) were measured on a 600 MHz Bruker Avance III spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a 5 mm TCI cryogenic probe head. 1D-NOESY, CPMG, and *J*-resolved experiments were performed using standard manufacturers' software Topspin 3.5. The concentrations of individual metabolites, identified by comparison of proton and carbon chemical shift with the HMDB database, were expressed as PQN [33] normalized intensities of corresponding signals in CPMG spectra. The list of quantified metabolites with corresponding ^{1}H and ^{13}C chemical shifts is given in the Supplemental Methods.

2.7. Triglyceride (TAG) Content in the Liver

Lipids were extracted from 100 mg of fresh liver tissue homogenized in 2 mL of 5%NP40 in deionized H_2O (95 °C, 5 min; room temperature, 10 min; 95 °C, 5 min). The

mixture was vortexed between the steps. After the extraction, 50 µL was used for the determination of protein concentration, and the rest of the homogenate was centrifuged for 3 min at 14,000× *g*. The clear supernatant was diluted 1:9 in deionized H_2O. The triglyceride concentration was determined using a commercially available kit (ERBA-Lachema Diagnostics, Czech Republic) and expressed as µmol TAG mg prot^{-1}.

2.8. Statistics

The statistical analyses were performed using R software packages and in-house scripts [34]. Prior to all statistical analysis, stool and cecum taxa were filtered. We kept only taxa that appeared in at least 5% of the samples. Stool and cecum microbiota univariable analysis was performed with the DESeq2 R package [35] on raw read counts. When more than one group is compared, we report both the *p*-values from the likelihood ratio test and the pairwise Wald tests. The *p*-values were adjusted using the Benjamin–Hochberg correction. The reported effect size for stool and cecum microbiota is a log 2-fold change obtained by DESeq2. The significance in univariable analysis for NMR and VOC data was obtained by the Kruskal–Wallis test and Dunn's post hoc test with the Benjamin–Hochberg correction. The reported effect size for these datasets is Cliff's delta. Alpha diversity in stool and cecum microbiota was analyzed with a vegan R package (https://CRAN.R-project.org/package=vegan, accessed on 16 November 2022). The raw counts were rarefied to 10,000 reads and then the Shannon index was computed. Statistical significance was computed by the Kruskal–Wallis test and Dunn's post hoc test with Benjamin–Hochberg correction. Principal component analysis (PCA) and Permutational MANOVA (PERMANOVA, *adonis* function from R package vegan) was used for multivariable analysis. All the datasets were centered and scaled prior to analysis. Additionally, prior to further analysis stool and cecum datasets were transformed by Variance Stabilizing Transformation. For assessing statistical significance, PERMANOVA was run with 10,000 iterations. We used Euclidean distance in both PCA and PERMANOVA. For pairwise PERMANOVA comparison, R package pairwiseAdonis was employed. To determine the discriminating ability of each dataset, we created classification models using Lasso. The classification metrics were obtained using 3-fold cross-validation with 10 repeats. To select the smallest number of variables in the models, we reported lambda = 1 se, that is, a lambda that results in the most regularized model so that the cross-validated error is within one standard error of the minimum error. Correlation networks of liver TAG content with microbiome were created as follows. First, the significantly changed taxa from the univariable analysis were identified. The Spearman correlation coefficient was computed on these taxa against the liver TAG content. The *p*-values corresponding to the correlation coefficients were adjusted using the Benjamin–Hochberg correction and the correlation networks were created with correlations having adjusted *p*-value ≤ 0.1. For the stool dataset on L_5 and L_6 levels, we show only correlations with an absolute value larger than 0.6.

3. Results

3.1. Body Composition and Glucose Homeostasis

We assessed the effect of the experimental diets on phenotype according to body composition parameters, that is, total body weight, liver weight, and triacylglycerol (TAG) content in the liver in ex-germ-free humanized mice (VG) and conventional mice (CV) (Figure 2).

As expected, the western diet induced a significant increase in total body and liver weight and liver TAG content in both models, the latter two parameters being significantly more affected in VG compared with CV mice. Inulin supplementation had no effect on WD-induced changes in CV mice, but it was associated with the decrease of liver TAG content and liver weight reaching a normal level in VG mice. We also observed a tendency to the normalization of epididymal fat pad weight in the VG_WD + I group (not shown), despite it not reaching statistical significance at $p < 0.05$. Western diet administration was associated with the deterioration of insulin secretion assessed as the fasting and

glucose-stimulated C-peptide serum concentration only in VG mice. The effect of WD was compensated by inulin treatment, as both parameters were normalized in the VG_WD + I group. Rather surprisingly, the glycemia at 30 min OGTT tended to be higher in CV mice, and in VG_WD + I it was even significantly lower compared with the CV_WD + I group. Because of the lack of the effect of inulin on metabolic phenotype in CV mice, we further focused on the VG model aiming to identify the components of the microbiome and metabolome associated with the beneficial outcome.

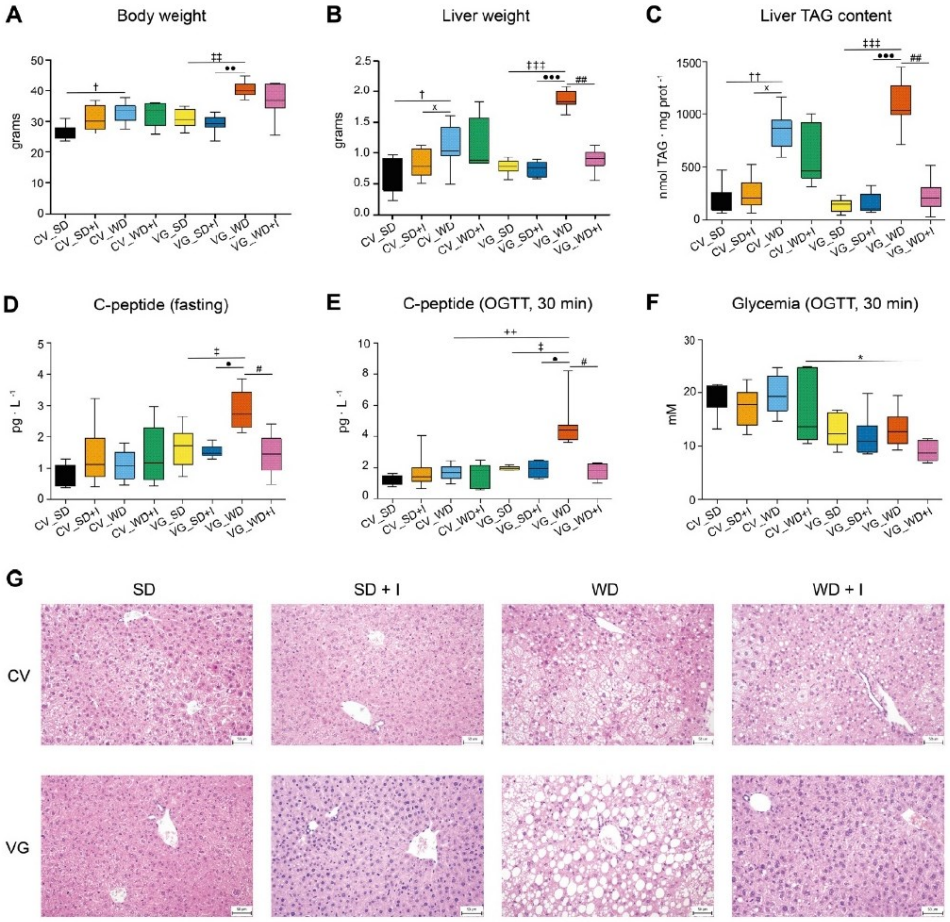


Figure 2. Effect of the diet and inulin supplementation on body composition and glucose homeostasis. (**A**): total body weight (g); (**B**): liver weight (g); (**C**): liver TAG content (nmol TAG. mg^{-1} protein); (**D**): fasting C-peptide concentration in plasma (pg. L^{-1}); (**E**): C-peptide concentration in plasma at 30 min of OGTT (pg. L^{-1}); (**F**): glycemia at 30 min of OGTT (mM); (**G**): histological assessment of liver slices. Data are shown as box plots (first and third quartile, median) with whiskers (min, max). OGTT, oral glucose tolerance test; CV, conventional mice; VG, humanized mice; SD, standard diet; SD + I, standard diet supplemented with inulin; WD, Western diet; WD + I, western diet supplemented with inulin. † $p < 0.05$, †† $p < 0.01$ CV_WD vs CV_SD; ^{x} $p < 0.05$ CV_WD vs CV_SD + I; ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ VG_WD vs VG_SD; $^{\bullet}$ $p < 0.05$, $^{\bullet\bullet}$ $p < 0.01$, $^{\bullet\bullet\bullet}$ $p < 0.001$ VG_WD vs VG_SD + I; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ VG_WD + I vs VG_WD; $^{++}$ $p < 0.01$ VG_WD vs CV_WD; $^{*}p < 0.05$ VG_WD + I vs CV_WD + I.

3.2. Cecum Microbiota Composition after Dietary and Inulin Intervention in Humanized Mice

Prior to the interventions (timepoint A), the microbiome diversity and composition in feces did not differ in mice randomly allocated to experimental groups (Figures S1 and S2). Aiming to compare the microbiota composition among the experimental groups after the intervention (timepoint B), we analyzed the cecum content because we consider it the most representative sample of microbiota in the distal intestine. The unsupervised separation of groups by variance only was visualized using PCA (Figure 3A,C). At the phylum level, the composition of the microbiota did not differ, PERMANOVA $p = 0.025$ but the analysis of dispersion test was significant which influence the result. At the species level, PERMANOVA $p < 0.001$, analysis of dispersion test was insignificant and pairwise tests proved differences between all groups (SD vs SD + I $p = 0.0011$, SD vs WD $p = 0.0011$, SD vs WD + I $p = 0.0011$, WD vs WD + I $p = 0.0042$).

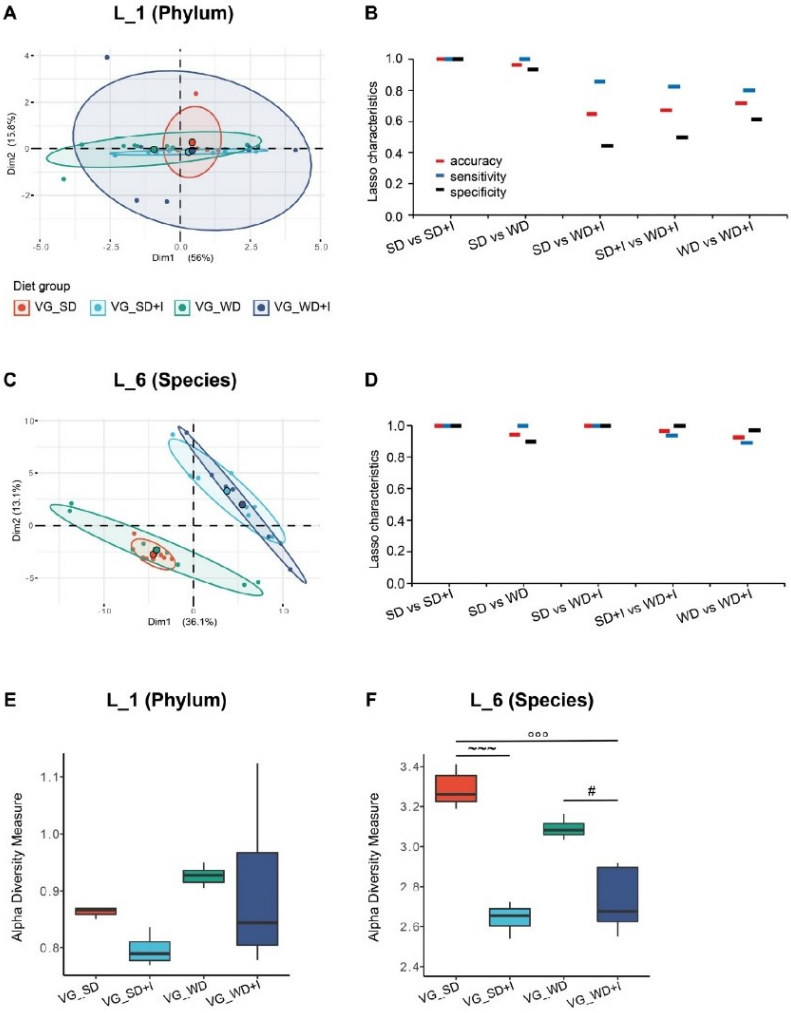


Figure 3. Cecum microbiota composition in humanized mice. (**A**,**C**): The 2D PCA scores plot. The explained variance of each component is included in the axis labels. The large points represent the centroids of each group. (**B**,**D**): Held-out characteristics of Lasso logistic regression model. (**E**,**F**): Alpha diversity of cecum microbiota assessed as Shannon index. ~~~ $p < 0.001$ VG_SD + I vs VG_SD; °°° $p < 0.001$ VG_WD + I vs VG_SD; # $p < 0.05$, p VG_WD + I vs VG_WD. Data are shown as box plots (1st and 3rd quartile, median) with whiskers (min, max).

As the next step, we adopted a machine learning approach (Lasso logistic regression) allowing for quantification of discrimination between every two pairs of groups (Figure 3B,D). At the L_1 level, we were able to reliably discriminate only VG_SD + I vs VG_SD and VG_WD vs. VG_SD groups, but the discrimination between other groups was unsatisfactory. The precision of separation increased along with reaching lower taxonomical levels. At the L_6 level, all pairs of groups could be separated with at least 90% accuracy, sensitivity, and specificity. Alpha diversity was assessed according to the Shannon index. Western diet alone increased the diversity at the L_1 (phylum) level but did not affect other taxonomical levels. Inulin treatment negatively affected the diversity at all levels independently of the background diet (VG_SD or VG_WD) (Figures 3E,F and S3).

The particular bacterial taxa discriminating between the groups were identified by univariable analysis that was performed using DESeq2. We observed an effect of all manipulations on microbiota composition at all taxonomical levels. At the L_1 level, the VG_WD group was characterized by a higher abundance of Bacteriodota, Actinobacteriota, and Verrucomicrobiota and decreased abundance of Firmicutes compared with VG_SD. Inulin treatment counteracted the WD effect on Firmicutes and potentiated the increase of Verrucomicrobia. Furthermore, inulin supplementation resulted in the decrease of Desulfobacterotota both in SD- and WD-fed mice (Table S1).

At L_6 level (species), univariable analysis unraveled 76 taxonomical units, that is, 64% of all significantly differently abundant ones among groups at FDR < 0.1 (Table S2). Considering the effect of diets and inulin, these bacteria could be divided into several groups (Figure 4). Forty bacterial taxa were affected only by inulin. In 18 bacteria, the effect of inulin was diet-dependent. Seven bacteria were significantly stimulated by inulin only in combination with SD (Figure 4A), and 11 only in combination with WD (Figure 4B). Twenty-two bacteria were affected by inulin independently of the background diet. Of those, inulin treatment stimulated the abundance of nine bacteria while the abundance of 13 bacteria was decreased (Figure 4C). Eight species were affected either positively (n = 3) or negatively (n = 5) only by WD (Figure 4D). In five taxa, the effect of WD was potentiated by inulin. The only bacteria whose abundance was positively affected by both WD and inulin was Akkermansia muciniphila, the effect of inulin being an order of magnitude stronger than the effect of diet. Four bacteria were affected negatively (Figure 4E). Finally, in 14 bacteria inulin counteracted the effect of WD. Eleven bacteria (Alistipes putredinis, Parabacteroides merdae, Sellimonas sp., Collinsella stercosis, Suterella sp., Hungatella sp., Flavonifractor sp., Lachnospiraceae NK4A136 group, Angelakisella sp., Oscillibacter sp., Bilophila sp.) were stimulated by WD while inulin supplementation negatively affected their abundance. Three bacteria (Parasutterella sp., Alistipes shahii, Lachnospiraceae_NA_NA) were suppressed by WD, while inulin partially compensated for this effect (Figure 4F). For nine taxa, we did not identify any pattern of diet or inulin effect.

3.3. Cecum VOCs Composition after the Dietary and Inulin Interventions in Humanized Mice

In total, we identified 61 VOCs in cecum content, 17 of them being significantly different among groups (Table S3). The separation of the groups is shown in Figure 5 and the PERMANOVA test confirmed that the VOCs composition of at least some of the groups differs. Inulin supplementation of SD did not result in a significant shift of cecum volatilome (pairwise PERMANNOVA $p > 0.1$), but using the Lasso logistic regression model we were able to discriminate between VG_SD and VG_SD + I groups with 89% accuracy and 86% specificity.

The univariable analysis identified five compounds significantly different between both groups; indole and 1,2-benzisothiazole were significantly decreased while tetradecanal, 1-butanol, and butanoic acid were increased in VG_SD + I compared with the VG_SD group (Figure 6A). The effect of WD alone on the VOCs spectrum in the cecum was quite modest. The PERMANOVA pairwise test was non-significant ($p = 0.374$), the accuracy of the Lasso logistic regression model was 0.78 with a specificity of 0.67 and we did not identify any differences in metabolite concentrations by UDAA. The combination of WD with inulin

supplementation led to the profound shift in cecum VOCs spectrum as revealed by PCA (Figure 5A), pairwise PERMANOVA result (p = 0.005), and the held-out characteristics of the Lasso logistic regression model (Figure 5B). Seven compounds were affected by inulin only in combination with WD (Figure 6B), all of them positive. Two compounds, unknown RI 1703 (increased) and dimethyl trisulfide (decreased), were influenced by inulin supplementation in combination with both diets (Figure 6C). Cecum content of 2-pentadecanone was increased by both WD and inulin supplementation, the effect being additive (Figure 6D). Only in the case of 2-tridecanone was the effect of inulin and WD the opposite (Figure 6E).

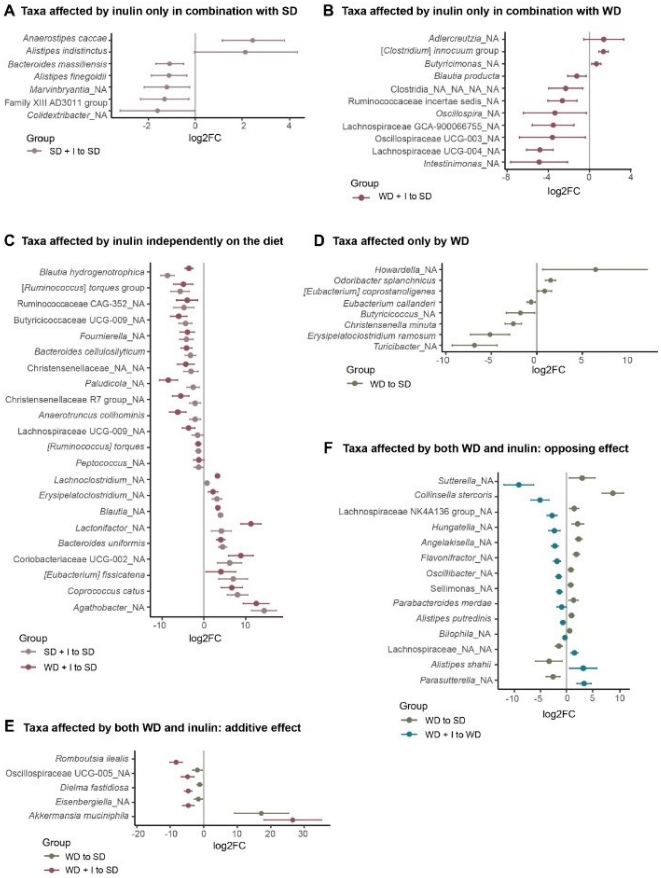


Figure 4. Bacterial taxa significantly affected by diet and/or inulin. (**A**): Taxa affected by inulin only in combination with SD (adj_pval VG_SD + I vs VG_SD < 0.1); (**B**): Taxa affected by inulin only in combination with WD (adj_pval VG_WD + I vs VG_SD < 0.1); (**C**): Taxa affected by inulin independently on the diet (adj_pval VG_SD + I vs VG_SD < 0.1 and adj_pval VG_WD + I vs VG_SD < 0.1); (**D**): Taxa affected only by WD (adj_pval VG_WD vs VG_SD < 0.1); (**E**) Taxa affected by both WD and inulin: additive effect (adj_pval VG_WD vs VG_SD < 0.1 and adj_pval VG_WD + I vs VG_WD < 0.1, effect size VG_WD vs VG_SD and VG_WD vs VG_WD + I in the same direction); (**F**) Taxa affected by both WD and inulin: opposing effect (adj_pval VG_WD vs VG_SD < 0.1 and adj_pval VG_WD + I vs VG_WD < 0.1, effect size VG_WD vs VG_SD and VG_WD vs VG_WD + I in the opposite direction). The taxa were selected according to the outcome of the univariable statistic test (Kruskal–Wallis), omnibus adj_pval < 0.1. The graph shows the effect size calculated as log2FC. adj_pval, adjusted p-value; FC, fold change.

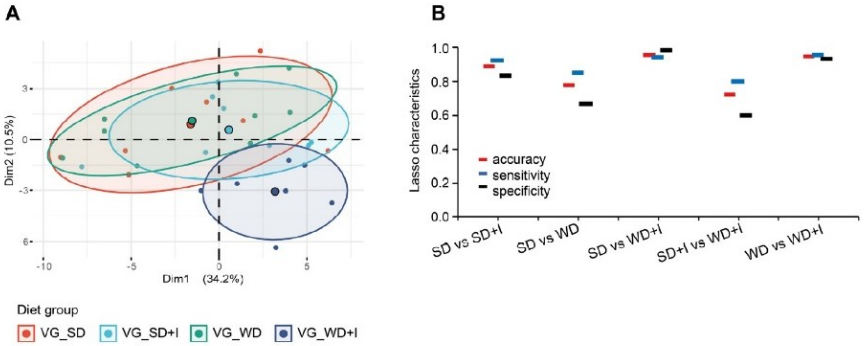


Figure 5. Cecum VOCs composition. (**A**) The 2D PCA scores plot. The explained variance of each component is included in the axis labels. The large points represent the centroids of each group. (**B**) Held-out characteristics of Lasso logistic regression model.

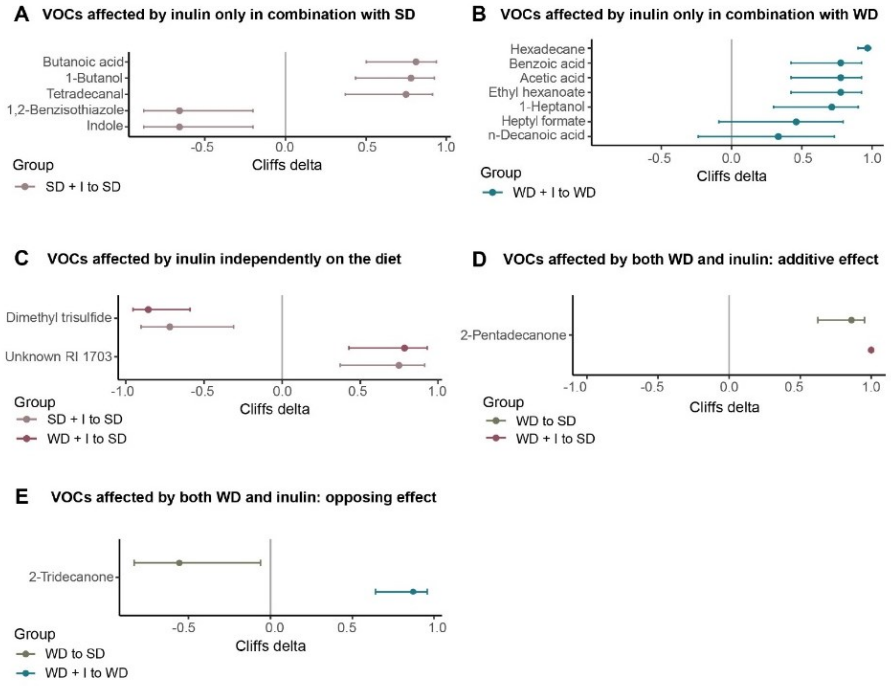


Figure 6. VOCs significantly affected by diet and/or inulin. (**A**): VOCs affected by inulin only in combination with SD (adj_pval VG_SD + I vs VG_SD < 0.1); (**B**): VOCs affected by inulin only in combination with WD (adj_pval VG_WD + I vs VG_SD < 0.1); (**C**): VOCs affected by inulin independently on the diet (adj_pval VG_SD + I vs VG_SD < 0.1 and adj_pval VG_WD + I vs VG_SD < 0.1); (**D**): VOCs affected by both WD and inulin: additive effect (adj_pval VG_WD vs VG_SD < 0.1 and adj_pval VG_WD + I vs VG_WD < 0.1, effect size VG_WD vs VG_SD and VG_WD vs VG_WD + I in the same direction); (**E**): VOCs affected by both WD and inulin: opposing effect (adj_pval VG_WD vs VG_SD < 0.1 and adj_pval VG_WD + I vs VG_WD < 0.1, effect size VG_WD vs VG_SD and VG_WD vs VG_WD + I in the opposite direction). The VOCs were selected according to the outcome of the univariable statistic test (Kruskal-Wallis), omnibus adj_pval < 0.1. The graph shows the effect size calculated as log2FC. adj_pval, adjusted *p*-value; FC, fold change; RI, retention index.

3.4. Serum Metabolome Composition after the Dietary and Inulin Interventions in Humanized Mice

The serum metabolome assessed by NMR spectroscopy was not significantly influenced by any of the treatments. PCA did not reveal any difference among the groups (all pairwise PERMANOVA tests >0.15) and the discrimination between groups based on the Lasso logistic regression model was unsatisfactory as well (Figure S4). Using univariable analysis, we did not identify any metabolite significantly different among the groups.

3.5. Integrative Analysis

Finally, we looked for possible relationships between microbiome or VOCs composition and the attenuation of WD-induced liver steatosis. To this end, we constructed the networks based on the Spearman correlations between liver TAG content and cecum bacteria or VOCs abundance in VG_WD and VG_WD + I groups (Figure **??**). Looking at the relationships between cecum microbiota and liver TAG content, we found that liver steatosis negatively correlated with the abundance of four bacteria (*Agathobacter*, *Lactonifractor*, *Bacteroides ovatus*, *Bacteroides uniformis*) that all were positively stimulated by inulin. Thirty-seven taxa correlated positively with liver TAG content including nine bacteria, whose abundance was positively affected by WD and negatively by inulin, and 12 bacteria that are negatively modulated by inulin (Figure **??**A). The correlation network between liver TAG content and VOCs compounds identified in the cecum is shown in Figure **??**B. We identified only two positive correlations, between liver TAG content and dimethyl trisulfide or propyl propanoate. Liver TAG content correlated negatively with nine compounds, including acetic acid, which is a marker of dietary fiber fermentation.

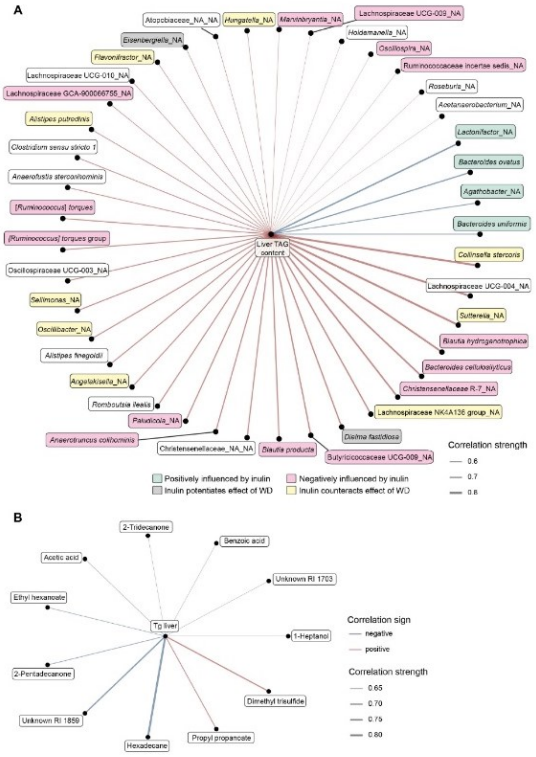


Figure 7. Correlation network between liver TAG content and cecum microbiome (**A**) and VOCs (**B**) composition. The edge width and color are proportional to the value of the correlation (red: positive; blue: negative). RI, retention index.

4. Discussion

We demonstrated in an animal model that vegan microbiota per se does not counteract the metabolically detrimental effects of a Western-type diet, but it shows the capacity to protect from NAFLD and glycemic deterioration when further supplemented with prebiotic inulin. The effect of inulin was manifested only in combination with vegan microbiota in humanized mice but not with conventional mice microbiota. Inulin supplementation in humanized mice resulted in a significant change in cecum microbiota composition with the accentuation of saccharolytic fermentation at the expense of the proteolytic one.

4.1. The Diet–Microbiota Interaction Influences the Outcomes of FMT Therapy

The landmark studies of Backhed's group [3,4] showed that in the mice model, the obese phenotype is transmissible by gut microbiota transfer. The possibility to transfer "diseased microbiota" opens the question of whether it is possible to transfer "healthy microbiota" and to use it for therapeutic purposes. The major drawback limiting the wide application of this concept is the lack of a definition of what healthy microbiota is. In the human gut, it is not possible to define the universally applicable composition of a healthy microbiome [36]. In relation to metabolic health, adherence to plant-based diets (vegetarian or vegan) was shown to be associated with potential health benefits [37], and therefore, vegan microbiota could be considered beneficial. On the other hand, in Western countries veganism has only a modest impact on gut microbiota composition [27,38,39] and the outcomes of two available vegan FMT trials were quite modest. The potential explanation may be derived from the experiment performed by Ridaura et al. who demonstrated that the invasion and colonization potential of transferred microbiota strongly depends on the diet [40]. Germ-free mice colonized with gut microbiota from a discordant twin pair, obese (Ob) and lean (Ln), repeated the donor phenotype on a standard mice diet. When co-housed and fed a low saturated fat/high fruit and vegetables diet, Ln microbiota became dominant, invaded the gut of Ob microbiota-colonized cage mates, and prevented the development of obesity. In contrast, the dominant and protective effect of Ln microbiota disappeared when mice were fed a high saturated fat/low fruit and vegetable diet. This study demonstrates how an obesogenic diet can select against human gut bacterial taxa associated with leanness [40].

Similarly, the background microbiota determines the therapeutic effect of dietary intervention. In mice models, inulin supplementation was associated with variable outcomes. Three studies were performed on C57Bl6 mice. In one study, inulin reduced the weight gain and steatosis induced by a western diet with fructose [41] while in the other inulin did not reverse the adverse effects of a high-fat diet [42]. In mice fed an n-3 fatty acid-deficient diet, inulin treatment promoted weight gain and adiposity and did not reverse the impairment of glucose homeostasis [43]. In APOE*3-Leiden.CETP mice (atherosclerosis model) inulin did not reduce hypercholesterolemia or atherosclerosis development and even resulted in manifestations of hepatic inflammation when combined with a high percentage of dietary cholesterol [44]. In our hands, inulin supplementation did not reverse the effect of the Western diet in conventionally raised C57Bl6 mice at all. Mice intestinal microbiota is profoundly different from the human and even the mice of the same strain but from different breeding facilities substantially differ in microbiota composition. Therefore, the variable outcomes of the above-mentioned studies may result from different pre-intervention gut microbiome settings. Taken together, this evidence emphasizes the strong microbiota-by-diet interactions and the implication of this relationship for therapeutic purposes.

In the previously cited vegan FMT studies, the participants were explicitly asked not to change their habitual diet. Therefore, the modest effect of vegan donor microbiota may be attributed to the diet of the FMT receivers, which did not allow for the manifestation of FMT therapeutic potential. This hypothesis is corroborated by our previous observational study comparing the gut microbiome and metabolome of adult vegan and omnivore human cohorts [27]. We found only modest differences in fecal microbiota composition but a

substantial difference in the fecal metabolome, which reflects the profoundly different diets of both groups.

4.2. The Protective Effect of Vegan Microbiota against Diet-Induced Steatosis Depends on Fiber Supplementation

We failed to show any protective potential of vegan microbiota to counteract the western diet-induced weight gain, namely the expansion of visceral fat. In contrast, inulin supplementation prevented the excessive TAG accumulation in the liver and ameliorated the impairment of glucose metabolism. We may suggest potential mechanisms based on our observations. Inulin supplementation induced a massive shift in microbiota composition and often counteracted the effect of an obesogenic diet. At the phylum level, inulin stimulated the abundance of Firmicutes, which resulted in the decreased Bacteroidota/Firmicutes ratio. In the presence of inulin, the abundance of Desulfobacterota was decreased both in VG_SD + I and VG_WD + I groups, which indicates the lower presence of sulfur compounds metabolizing bacteria and attenuation of potentially toxic sulfur-containing metabolites formation.

We further looked for the association between liver TAG content and the abundance of bacteria significantly affected by inulin supplementation. We found only four negative correlations between bacteria abundance and liver TAG content. All of them (*Lactonifactor* sp., *Agathobacter* sp., *B. ovatus*, *B. uniformis*) belonged to bacteria significantly stimulated by inulin. *Lactonifactor* ($\rho = -0.8$) converts the plant lignan secoisolariciresinol diglucoside into the bioactive enterolignans enterodiol and enterolactone [45] that have therapeutic properties, including anti-oxidant, anti-cancerous, anti-inflammatory, modulation of gene expression, anti-diabetic, estrogenic and anti-estrogenic [46]. In the cross-sectional study performed on 2294 US adults, urinary enterolactone concentration was negatively correlated with NAFLD [47]. *Agathobacter* sp. ($\rho = -0.7$), the bacteria most stimulated by inulin in our study, is a butyrate producer. It is reported to be stimulated by a different source of fiber (oatmeal, rye) and associated with lower cardiovascular disease or metabolic risk [48,49]. The depletion of *B. uniformis* was found in NAFLD [50,51] in observational studies. Treatment with *B. uniformis*, particularly when combined with fiber, ameliorated diet-induced hepatic steatosis and inflammation, restored the compromised intestinal immune defense, and improved whole-body glucose disposal [52,53]. Qiao et al. proposed the mechanism of the beneficial effect of *B. uniformis.* They proved that *B. uniformis* is able to synthesize folate and its beneficial effect may be explained, at least partly, by folate-enhanced one-carbon metabolism [54]. Most correlations between bacteria and liver TAG content were positive. Of interest, 38% of bacteria (n = 14) positively correlating with liver TAG is negatively influenced by inulin and in some bacteria (24%, n = 9), inulin even counteracts the effect of WD. Taken together, this evidence strongly suggests that the alteration of gut microbiota composition resulting from inulin supplementation may be responsible for the amelioration of steatosis.

4.3. Inulin Supplementation and Microbiota Performance

Analysis of the fecal VOCs spectrum confirmed that inulin supplementation affected the metabolic performance of cecum microbiota by accentuating saccharolytic fermentation at the expense of amino acid metabolism. This shift is documented by the (i) decrease of the product of tryptophan fermentation indole (only VG_SD + I); (ii) decrease of methionine/cysteine fermentation product dimethyl trisulfide (both VG_SD + I and VG_WD + I); (iii) increase of butanoic acid (only VG_SD + I) and (iv) increase of acetic acid (both VG_SD + I and VG_WD + I). The main fiber fermentation products are short-chain fatty acids (SCFA) whose positive effect is widely accepted [55]. Propionate and butyrate are considered unequivocally beneficial. The role of acetate is not so straightforward, as it is a lipogenic substrate and may serve as a substrate for lipid synthesis in the liver [56]. In contrast to this, Aoki et al. proposed an alternative hypothesis that acetate derived from prebiotic fermentation in the gut lumen regulates hepatic lipid metabolism and in-

sulin sensitivity via FFAR2 signaling in hepatocytes, which prevents the progression of NAFLD [56].

The health benefits of dietary fiber have been judged mainly according to the enhanced production of SCFA in the colon. Somewhat neglected is a complementary hypothesis—the beneficial effect of dietary fiber may be mediated by the inhibition of protein fermentation as well [57]. Protein fermentation yields intrinsically toxic luminal compounds that affect epithelial cell metabolism and barrier function [58]. De Preter [57] demonstrated that oligofructose-enriched inulin in a dose-dependent fashion stimulated SCFA production and in parallel inhibited the formation of sulfur-containing compounds like dimethyl tri(di)sulfide or methional. Our data support the hypothesis that inulin supplementation attenuated the proteolytic fermentation in the colon. We observed a significantly decreased content of dimethyl trisulfide in both the VG_SD + I and VG_WD + I group and this compound strongly positively correlated with liver TAG content ($\rho = 0.75$). The acetic acid concentration in the cecum was significantly increased in the VG_WD + I group and negatively correlated with steatosis ($\rho = -0.65$). Therefore, we hypothesize that attenuation of amino acid fermentation resulting from acidification of the cecal/colonic lumen by SCFA and catabolic repression imposed by the increased saccharolytic fermentation [57] may represent an additional protective mechanism of prebiotic supplementation.

5. Conclusions

Using the model of ex-germ-free mice humanized with mixed human vegan microbiota we found that it does not protect against the adverse effects of a Western-type diet like obesity, liver steatosis, and compromised glucose homeostasis. In contrast, supplementation of the Western diet with inulin reversed the steatosis and ameliorated glucose metabolism, though it did not affect the weight gain. Inulin supplementation resulted in a significant change in the gut microbiota composition and its metabolic performance, inducing the shift from proteolytic towards saccharolytic fermentation. Our results offer an explanation for the relatively modest success of FMT in treating metabolic disorders when healthy microbiota were applied into an unhealthy environment without subsequent dietary support. In the context of the potential use of FMT with vegan microbiota in the therapy of metabolic non-communicable diseases, our study points out that it is not only the particular microbiota transfer, but also the following dietary intervention with inulin or other dietary fiber and/or dietary change that is necessary for therapeutic success.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu15020454/s1, Supplemental methods Table S1 The cecum microbiota composition: level L_1 (phylum). Table S2 The cecum microbiota composition: level L_6 (species). Table S3 Volatile organics compounds spectrum in the cecum. Figure S1 Alpha diversity of fecal microbiota in timepoint A assessed as Shannon index. Figure S2 The microbiota composition in timepoint A. Figure S3 Alpha diversity of cecum microbiota in timepoint B assessed as Shannon index. Figure S4 Serum metabolome composition. File S1: Supplemental Method [59–64].

Author Contributions: Conceptualization: M.C., M.H. and J.G. (Jan Gojda); Methodology: M.C., H.P. and J.H.; Formal analysis, M.H., N.D., I.M., J.H., M.K. (Marek Kuzma) and M.K. (Maria Kralova); Funding acquisition, M.K. (Marek Kuzma) and J.G. (Jan Gojda); Investigation, M.H., J.H., T.H., M.K. (Marek Kuzma), I.B., E.S., D.F., J.G. (Jaroslav Golias), B.D., J.J., I.C. and J.G. (Jan Gojda); Visualization, N.D. and E.S.; Writing—original draft, M.H.; Writing—review & editing, M.H., N.D., I.M., J.H., M.K. (Marek Kuzma), M.K. (Maria Kralova) and J.G. (Jan Gojda). All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Health of the Czech Republic, grant NV-18-01-00040, by MH CR–DRO (Institute for Clinical and Experimental medicine–IKEM, IN 00023001) and by the project National Institute for Research of Metabolic and Cardiovascular Diseases (Programme EXCELES, Project No. LX22NPO5104)—Funded by the European Union—Next Generation EU.

Institutional Review Board Statement: The human donors of the stool were recruited as a part of the study approved by the Ethics Committee of University Hospital Kralovske Vinohrady (EK-

VP/26/0/2017) in accordance with the Declaration of Helsinki. The animal study protocol was approved by the Review Board (or Ethics Committee) of the Ministry of Health CR (protocol code 22/2018 from 12 April 2018).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Sequencing data are available at the Sequence Read Archive database under the accession number PRJNA891477 https://www.ncbi.nlm.nih.gov/sra/PRJNA891477, accessed on 16 November 2022.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Office of Communications, U.S. *Agriculture Fact Book*; Office of Communications, U.S. Department of Agriculture: Beltsville, MD, USA, 2003.
2. Younossi, Z.; Anstee, Q.M.; Marietti, M.; Hardy, T.; Henry, L.; Eslam, M.; George, J.; Bugianesi, E. Global burden of NAFLD and NASH: Trends, predictions, risk factors and prevention. *Nat. Rev. Gastroenterol. Hepatol.* **2018**, *15*, 11–20. [CrossRef]
3. Backhed, F.; Ding, H.; Wang, T.; Hooper, L.V.; Koh, G.Y.; Nagy, A.; Semenkovich, C.F.; Gordon, J.I. The gut microbiota as an environmental factor that regulates fat storage. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 15718–15723. [CrossRef]
4. Backhed, F.; Manchester, J.K.; Semenkovich, C.F.; Gordon, J.I. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 979–984. [CrossRef]
5. Tsai, F.; Coyle, W.J. The microbiome and obesity: Is obesity linked to our gut flora? *Curr. Gastroenterol. Rep.* **2009**, *11*, 307–313. [CrossRef]
6. Marotz, C.A.; Zarrinpar, A. Treating Obesity and Metabolic Syndrome with Fecal Microbiota Transplantation. *Yale J. Biol. Med.* **2016**, *89*, 383–388. [PubMed]
7. Greathouse, K.L.; Sinha, R.; Vogtmann, E. DNA extraction for human microbiome studies: The issue of standardization. *Genome Biol.* **2019**, *20*, 212. [CrossRef] [PubMed]
8. Lim, Y.Y.; Lee, Y.S.; Ooi, D.S.Q. Engineering the Gut Microbiome for Treatment of Obesity: A Review of Current Understanding and Progress. *Biotechnol. J.* **2020**, *15*, e2000013. [CrossRef]
9. European Association for the Study of the, L.; European Association for the Study of, D.; European Association for the Study of, O. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J. Hepatol.* **2016**, *64*, 1388–1402. [CrossRef]
10. Subhan, F.B.; Chan, C.B. Review of Dietary Practices of the 21st Century: Facts and Fallacies. *Can. J. Diabetes* **2016**, *40*, 348–354. [CrossRef] [PubMed]
11. Yannakoulia, M.; Poulimeneas, D.; Mamalaki, E.; Anastasiou, C.A. Dietary modifications for weight loss and weight loss maintenance. *Metabolism* **2019**, *92*, 153–162. [CrossRef] [PubMed]
12. van Nood, E.; Vrieze, A.; Nieuwdorp, M.; Fuentes, S.; Zoetendal, E.G.; de Vos, W.M.; Visser, C.E.; Kuijper, E.J.; Bartelsman, J.F.; Tijssen, J.G.; et al. Duodenal infusion of donor feces for recurrent Clostridium difficile. *N. Engl. J. Med.* **2013**, *368*, 407–415. [CrossRef]
13. Vrieze, A.; Van Nood, E.; Holleman, F.; Salojarvi, J.; Kootte, R.S.; Bartelsman, J.F.; Dallinga-Thie, G.M.; Ackermans, M.T.; Serlie, M.J.; Oozeer, R.; et al. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology* **2012**, *143*, 913–916.e7. [CrossRef] [PubMed]
14. Kristensen, N.B.; Bryrup, T.; Allin, K.H.; Nielsen, T.; Hansen, T.H.; Pedersen, O. Alterations in fecal microbiota composition by probiotic supplementation in healthy adults: A systematic review of randomized controlled trials. *Genome Med.* **2016**, *8*, 52. [CrossRef] [PubMed]
15. Allegretti, J.R.; Kassam, Z.; Mullish, B.H.; Chiang, A.; Carrellas, M.; Hurtado, J.; Marchesi, J.R.; McDonald, J.A.K.; Pechlivanis, A.; Barker, G.F.; et al. Effects of Fecal Microbiota Transplantation with Oral Capsules in Obese Patients. *Clin. Gastroenterol. Hepatol.* **2020**, *18*, 855–863.e852. [CrossRef]
16. Kootte, R.S.; Levin, E.; Salojarvi, J.; Smits, L.P.; Hartstra, A.V.; Udayappan, S.D.; Hermes, G.; Bouter, K.E.; Koopen, A.M.; Holst, J.J.; et al. Improvement of Insulin Sensitivity after Lean Donor Feces in Metabolic Syndrome Is Driven by Baseline Intestinal Microbiota Composition. *Cell Metab.* **2017**, *26*, 611–619.e6. [CrossRef] [PubMed]
17. Smits, L.P.; Kootte, R.S.; Levin, E.; Prodan, A.; Fuentes, S.; Zoetendal, E.G.; Wang, Z.; Levison, B.S.; Cleophas, M.C.P.; Kemper, E.M.; et al. Effect of Vegan Fecal Microbiota Transplantation on Carnitine- and Choline-Derived Trimethylamine-N-Oxide Production and Vascular Inflammation in Patients With Metabolic Syndrome. *J. Am. Heart Assoc.* **2018**, *7*, e008342. [CrossRef]

18. Yu, E.W.; Gao, L.; Stastka, P.; Cheney, M.C.; Mahabamunuge, J.; Torres Soto, M.; Ford, C.B.; Bryant, J.A.; Henn, M.R.; Hohmann, E.L. Fecal microbiota transplantation for the improvement of metabolism in obesity: The FMT-TRIM double-blind placebo-controlled pilot trial. *PLoS Med.* **2020**, *17*, e1003051. [CrossRef]
19. Proenca, I.M.; Allegretti, J.R.; Bernardo, W.M.; de Moura, D.T.H.; Ponte Neto, A.M.; Matsubayashi, C.O.; Flor, M.M.; Kotinda, A.; de Moura, E.G.H. Fecal microbiota transplantation improves metabolic syndrome parameters: Systematic review with meta-analysis based on randomized clinical trials. *Nutr. Res.* **2020**, *83*, 1–14. [CrossRef]
20. Huang, T.; Yang, B.; Zheng, J.; Li, G.; Wahlqvist, M.L.; Li, D. Cardiovascular disease mortality and cancer incidence in vegetarians: A meta-analysis and systematic review. *Ann. Nutr. Metab.* **2012**, *60*, 233–240. [CrossRef]
21. Barnard, N.D.; Cohen, J.; Jenkins, D.J.; Turner-McGrievy, G.; Gloede, L.; Green, A.; Ferdowsian, H. A low-fat vegan diet and a conventional diabetes diet in the treatment of type 2 diabetes: A randomized, controlled, 74-wk clinical trial. *Am. J. Clin. Nutr.* **2009**, *89*, 1588S–1596S. [CrossRef]
22. Kahleova, H.; Matoulek, M.; Malinska, H.; Oliyarnik, O.; Kazdova, L.; Neskudla, T.; Skoch, A.; Hajek, M.; Hill, M.; Kahle, M.; et al. Vegetarian diet improves insulin resistance and oxidative stress markers more than conventional diet in subjects with Type 2 diabetes. *Diabet Med.* **2011**, *28*, 549–559. [CrossRef] [PubMed]
23. Chen, Z.; Zuurmond, M.G.; van der Schaft, N.; Nano, J.; Wijnhoven, H.A.H.; Ikram, M.A.; Franco, O.H.; Voortman, T. Plant versus animal based diets and insulin resistance, prediabetes and type 2 diabetes: The Rotterdam Study. *Eur. J. Epidemiol.* **2018**, *33*, 883–893. [CrossRef]
24. Wright, N.; Wilson, L.; Smith, M.; Duncan, B.; McHugh, P. The BROAD study: A randomised controlled trial using a whole food plant-based diet in the community for obesity, ischaemic heart disease or diabetes. *Nutr. Diabetes* **2017**, *7*, e256. [CrossRef]
25. Muegge, B.D.; Kuczynski, J.; Knights, D.; Clemente, J.C.; Gonzalez, A.; Fontana, L.; Henrissat, B.; Knight, R.; Gordon, J.I. Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science* **2011**, *332*, 970–974. [CrossRef]
26. Witjes, J.J.; Smits, L.P.; Pekmez, C.T.; Prodan, A.; Meijnikman, A.S.; Troelstra, M.A.; Bouter, K.E.C.; Herrema, H.; Levin, E.; Holleboom, A.G.; et al. Donor Fecal Microbiota Transplantation Alters Gut Microbiota and Metabolites in Obese Individuals with Steatohepatitis. *Hepatol. Commun.* **2020**, *4*, 1578–1590. [CrossRef] [PubMed]
27. Prochazkova, M.; Budinska, E.; Kuzma, M.; Pelantova, H.; Hradecky, J.; Heczkova, M.; Daskova, N.; Bratova, M.; Modos, I.; Videnska, P.; et al. Vegan Diet Is Associated with Favorable Effects on the Metabolic Performance of Intestinal Microbiota: A Cross-Sectional Multi-Omics Study. *Front Nutr.* **2021**, *8*, 783302. [CrossRef] [PubMed]
28. Hamilton, M.J.; Weingarden, A.R.; Sadowsky, M.J.; Khoruts, A. Standardized frozen preparation for transplantation of fecal microbiota for recurrent Clostridium difficile infection. *Am. J. Gastroenterol.* **2012**, *107*, 761–767. [CrossRef]
29. Gomez de Aguero, M.; Ganal-Vonarburg, S.C.; Fuhrer, T.; Rupp, S.; Uchimura, Y.; Li, H.; Steinert, A.; Heikenwalder, M.; Hapfelmeier, S.; Sauer, U.; et al. The maternal microbiota drives early postnatal innate immune development. *Science* **2016**, *351*, 1296–1302. [CrossRef] [PubMed]
30. Nyangahu, D.D.; Jaspan, H.B. Influence of maternal microbiota during pregnancy on infant immunity. *Clin. Exp. Immunol.* **2019**, *198*, 47–56. [CrossRef]
31. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.; Holmes, S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef] [PubMed]
32. McLaren, M.R.C.; Benjamin, J. Silva 138.1 prokaryotic SSU taxonomic training data formatted for DADA2. *Zenodo* **2021**. [CrossRef]
33. Dieterle, F.; Ross, A.; Schlotterbeck, G.; Senn, H. Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in 1H NMR metabonomics. *Anal. Chem.* **2006**, *78*, 4281–4290. [CrossRef]
34. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2017.
35. Love, M.I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **2014**, *15*, 550. [CrossRef] [PubMed]
36. Najmanova, L.; Videnska, P.; Cahova, M. Healthy microbiome—A mere idea or a sound concept? *Physiol. Res.* **2022**, *71*, 719–738. [CrossRef] [PubMed]
37. Marrone, G.; Guerriero, C.; Palazzetti, D.; Lido, P.; Marolla, A.; Di Daniele, F.; Noce, A. Vegan Diet Health Benefits in Metabolic Syndrome. *Nutrients* **2021**, *13*, 817. [CrossRef] [PubMed]
38. Losno, E.A.; Sieferle, K.; Perez-Cueto, F.J.A.; Ritz, C. Vegan Diet and the Gut Microbiota Composition in Healthy Adults. *Nutrients* **2021**, *13*, 2402. [CrossRef]
39. Trefflich, I.; Jabakhanji, A.; Menzel, J.; Blaut, M.; Michalsen, A.; Lampen, A.; Abraham, K.; Weikert, C. Is a vegan or a vegetarian diet associated with the microbiota composition in the gut? Results of a new cross-sectional study and systematic review. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 2990–3004. [CrossRef] [PubMed]
40. Ridaura, V.K.; Faith, J.J.; Rey, F.E.; Cheng, J.; Duncan, A.E.; Kau, A.L.; Griffin, N.W.; Lombard, V.; Henrissat, B.; Bain, J.R.; et al. Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* **2013**, *341*, 1241214. [CrossRef]

41. Beisner, J.; Filipe Rosa, L.; Kaden-Volynets, V.; Stolzer, I.; Gunther, C.; Bischoff, S.C. Prebiotic Inulin and Sodium Butyrate Attenuate Obesity-Induced Intestinal Barrier Dysfunction by Induction of Antimicrobial Peptides. *Front. Immunol.* **2021**, *12*, 678360. [CrossRef]
42. Tan, S.; Caparros-Martin, J.A.; Matthews, V.B.; Koch, H.; O'Gara, F.; Croft, K.D.; Ward, N.C. Isoquercetin and inulin synergistically modulate the gut microbiome to prevent development of the metabolic syndrome in mice fed a high fat diet. *Sci. Rep.* **2018**, *8*, 10100. [CrossRef]
43. Pachikian, B.D.; Essaghir, A.; Demoulin, J.B.; Neyrinck, A.M.; Catry, E.; De Backer, F.C.; Dejeans, N.; Dewulf, E.M.; Sohet, F.M.; Portois, L.; et al. Hepatic n-3 polyunsaturated fatty acid depletion promotes steatosis and insulin resistance in mice: Genomic analysis of cellular targets. *PLoS ONE* **2011**, *6*, e23365. [CrossRef]
44. Hoving, L.R.; Katiraei, S.; Pronk, A.; Heijink, M.; Vonk, K.K.D.; Amghar-El Bouazzaoui, F.; Vermeulen, R.; Drinkwaard, L.; Giera, M.; van Harmelen, V.; et al. The prebiotic inulin modulates gut microbiota but does not ameliorate atherosclerosis in hypercholesterolemic APOE*3-Leiden.CETP mice. *Sci. Rep.* **2018**, *8*, 16515. [CrossRef]
45. Woting, A.; Clavel, T.; Loh, G.; Blaut, M. Bacterial transformation of dietary lignans in gnotobiotic rats. *FEMS Microbiol. Ecol.* **2010**, *72*, 507–514. [CrossRef]
46. Plaha, N.S.; Awasthi, S.; Sharma, A.; Kaushik, N. Distribution, biosynthesis and therapeutic potential of lignans. *3 Biotech* **2022**, *12*, 255. [CrossRef] [PubMed]
47. Xiong, G.; Huang, C.; Zou, Y.; Tao, Z.; Zou, J.; Huang, J. Associations of Urinary Phytoestrogen Concentrations with Nonalcoholic Fatty Liver Disease among Adults. *J. Healthc. Eng.* **2022**, *2022*, 4912961. [CrossRef]
48. Iversen, K.N.; Dicksved, J.; Zoki, C.; Fristedt, R.; Pelve, E.A.; Langton, M.; Landberg, R. The Effects of High Fiber Rye, Compared to Refined Wheat, on Gut Microbiota Composition, Plasma Short Chain Fatty Acids, and Implications for Weight Loss and Metabolic Risk Factors (the RyeWeight Study). *Nutrients* **2022**, *14*, 1669. [CrossRef] [PubMed]
49. Xu, D.; Pan, D.; Liu, H.; Yang, C.; Yang, X.; Wang, X.; Liu, F.; Feng, M.; Wu, Q.; Shen, Y.; et al. Improvement in cardiometabolic risk markers following an oatmeal diet is associated with gut microbiota in mildly hypercholesterolemic individuals. *Food Res. Int.* **2022**, *160*, 111701. [CrossRef] [PubMed]
50. Sheng, S.; Chen, J.; Zhang, Y.; Qin, Q.; Li, W.; Yan, S.; Wang, Y.; Li, T.; Gao, X.; Tang, L.; et al. Structural and Functional Alterations of Gut Microbiota in Males With Hyperuricemia and High Levels of Liver Enzymes. *Front Med.* **2021**, *8*, 779994. [CrossRef]
51. Zhou, Y.; Zheng, T.; Chen, H.; Li, Y.; Huang, H.; Chen, W.; Du, Y.; He, J.; Li, Y.; Cao, J.; et al. Microbial Intervention as a Novel Target in Treatment of Non-Alcoholic Fatty Liver Disease Progression. *Cell Physiol. Biochem.* **2018**, *51*, 2123–2135. [CrossRef]
52. Lopez-Almela, I.; Romani-Perez, M.; Bullich-Vilarrubias, C.; Benitez-Paez, A.; Gomez Del Pulgar, E.M.; Frances, R.; Liebisch, G.; Sanz, Y. Bacteroides uniformis combined with fiber amplifies metabolic and immune benefits in obese mice. *Gut Microbes* **2021**, *13*, 1–20. [CrossRef]
53. Lee, H.B.; Do, M.H.; Jhun, H.; Ha, S.K.; Song, H.S.; Roh, S.W.; Chung, W.H.; Nam, Y.D.; Park, H.Y. Amelioration of Hepatic Steatosis in Mice through Bacteroides uniformis CBA7346-Mediated Regulation of High-Fat Diet-Induced Insulin Resistance and Lipogenesis. *Nutrients* **2021**, *13*, 2989. [CrossRef]
54. Qiao, S.; Bao, L.; Wang, K.; Sun, S.; Liao, M.; Liu, C.; Zhou, N.; Ma, K.; Zhang, Y.; Chen, Y.; et al. Activation of a Specific Gut Bacteroides-Folate-Liver Axis Benefits for the Alleviation of Nonalcoholic Hepatic Steatosis. *Cell Rep.* **2020**, *32*, 108005. [CrossRef]
55. Gill, P.A.; van Zelm, M.C.; Muir, J.G.; Gibson, P.R. Review article: Short chain fatty acids as potential therapeutic agents in human gastrointestinal and inflammatory disorders. *Aliment. Pharmacol. Ther.* **2018**, *48*, 15–34. [CrossRef]
56. Weitkunat, K.; Schumann, S.; Petzke, K.J.; Blaut, M.; Loh, G.; Klaus, S. Effects of dietary inulin on bacterial growth, short-chain fatty acid production and hepatic lipid metabolism in gnotobiotic mice. *J. Nutr. Biochem.* **2015**, *26*, 929–937. [CrossRef]
57. De Preter, V.; Ghebretinsae, A.H.; Abrahantes, J.C.; Windey, K.; Rutgeerts, P.; Verbeke, K. Impact of the synbiotic combination of Lactobacillus casei shirota and oligofructose-enriched inulin on the fecal volatile metabolite profile in healthy subjects. *Mol. Nutr. Food Res.* **2011**, *55*, 714–722. [CrossRef]
58. Windey, K.; De Preter, V.; Verbeke, K. Relevance of protein fermentation to gut health. *Mol. Nutr. Food Res.* **2012**, *56*, 184–196. [CrossRef]
59. Aronesty, E. Comparison of sequencing utility programs. *Open Bioinform. J.* **2013**, *7*, 1–8. [CrossRef]
60. Caporaso, J.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Peña, A.G.; Goodrich, J.K.; Gordon, J.I.; et al. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **2010**, *7*, 335–336. [CrossRef] [PubMed]
61. Kleiner, D.E.; Brunt, E.M.; Van Natta, M.; Behling, C.; Contos, M.J.; Cummings, O.W.; Ferrell, L.D.; Liu, Y.C.; Torbenson, M.S.; Unalp-Arida, A.; et al. Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* **2005**, *41*, 1313–1321. [CrossRef] [PubMed]
62. Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glöckner, F.O. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* **2013**, *41*, e1. [CrossRef] [PubMed]

63. Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glöckner, F.O. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* **2013**, *41*, D590–D596. [CrossRef] [PubMed]
64. Wishart, D.S.; Feunang, Y.D.; Marcu, A.; Guo, A.C.; Liang, K.; Vázquez-Fresno, R.; Sajed, T.; Johnson, D.; Li, C.; Karu, N.; et al. HMDB 4.0: The human metabolome database for 2018. *Nucleic Acids Res.* **2018**, *46*, D608–D617. [CrossRef] [PubMed]

4 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

This dissertation thesis addresses the possible modification of the composition of the gut microbiota by diet not only from a taxonomic but also from a functional point of view, by incorporating metabolomics into the analyses and developing a new method to estimate the ability of the microbiota to produce the beneficial metabolite butyrate. The theoretical part of the thesis summarizes the importance of gut microbes for human health. In particular, it discusses the immersive functions of gut microbiota and its impact on human development and it also summarizes current knowledge on manipulating the gut microbiome through diet. It highlights the most reputable research that has been conducted in this area. In addition, some of the most commonly used multi-omics methods are introduced and their integration is briefly explained. The experimental part of the thesis focuses on four monothematic articles that have been published in connection with this work.

The first study describes the microbiome and metabolome profiles of healthy lean vegans and omnivores and explores the impact of plant-based diet on microbial function and to a lesser extent, microbial taxonomic composition. The study involved collecting dietary records and analyzing stool and plasma samples for various analyses such as metabolomics, bile acid spectrum determination, the SCFAs contents measurement, 16S rRNA sequencing, glucose and lipid homeostasis, and inflammation parameters. The study found that the vegan diet was associated with a higher intake of carbohydrates and dietary fiber, more favorable glucose and lipid metabolism, and lower inflammation levels compared to the omnivorous diet. The metabolome profiles differed significantly between the groups, with vegans having a higher occurrence of potentially beneficial metabolites from dietary fiber fermentation and a lower abundance of potentially harmful metabolites from amino acid fermentation products. The study highlights the importance of plant-based diets by demonstrating their positive impact on microbial function, metabolic health, and inflammation levels compared to omnivorous diets.

The second study presented a simple and cost-effective method for estimating the functional capacity of butyrate synthesis by the gut microbiota, an important process for maintaining overall health. The method involves the use of qPCR to quantify the bacterial gene encoding butyryl-CoA:acetate CoA-transferase, a key enzyme involved in butyrate synthesis, and was validated using six sets of degenerate primers. We compared two strategies for normalizing qPCR results and found that copy numbers normalized to the 16S rRNA gene and the *C. elegans*-derived DNA spike were comparable for all primer sets. We then tested the method on stool samples from healthy lean vegans and healthy obese omnivores and found that the amount of the *but* gene in the VG group was significantly different from the OB group, corresponding to significantly higher amounts of butyrate in the respective stool samples as determined by NMR. Thus, the method may represent a powerful tool for estimating the functional capacity of the gut microbiota and could be useful for individual assessment of the utility of prebiotic or dietary treatment.

The third study investigated whether gut microbiota composition and metabolome differ in lean healthy, obese healthy, and obese diabetic T2D patients without medication and whether the effects of inulin on glucose tolerance and insulin sensitivity can be explained by the response of the gut microbiota to inulin intervention and whether this response can be predicted from the initial microbiome and metabolome signature. The study found that the composition of microbiome and metabolome differed between lean participants and obese subjects, but did not distinguish well obese subjects with and without diabetes. Inulin supplementation resulted in a significant change in microbiota composition, with an increase in beneficial bacterial taxa and a decrease in potentially

harmful ones. Inulin intake also affected markers of glucose tolerance and insulin sensitivity, and potential predictors of individual response to inulin treatment were identified. These findings highlight the complex character of the gut microbiota and host metabolism response to inulin intervention and demonstrated the possibilities of personalized therapeutic microbiota manipulation.

In the fourth study, stool samples from four vegan donors were used to prepare a mixed inoculum for FMT to create humanized ex-GF mice. The aim was to investigate the protective effects of the vegan microbiota against the Western-type diet and the role of dietary fiber (inulin) in enhancing its therapeutic potential. The study found that the Western diet caused significant weight gain and triacylglycerol content in the liver in both humanized and conventional mouse models, but impaired glucose homeostasis was observed only in the humanized group. Inulin supplementation reversed liver steatosis and improved glucose homeostasis in the humanized mice group but not in the conventional mice group. The study suggests that a vegan microbiota alone may not be sufficient to counteract the negative metabolic effects of a Western-style diet, but follow-up dietary support may substantially enhance the treatment success.

This thesis concludes by highlighting the importance of gut microbiota for human health and the opportunity for dietary interventions that can influence microbial composition and function. The first study demonstrated that adherence to a plant-based diet high in carbohydrates and fiber can lead to a favorable microbial profile and metabolome associated with improved glucose and lipid metabolism and lower levels of inflammation. The second study presented a new method to estimate the functional capacity of butyrate synthesis by the gut microbiota. The third study focused on the identification of predictors of the therapeutic efficacy of inulin treatment in (pre)diabetes. Finally, a fourth study used FMT to investigate the protective effects of vegan microbiota against a Western-style diet and found that fiber may enhance the therapeutic potential of FMT. Overall, these studies highlight the potential of personalized dietary interventions to modify gut microbiota and improve metabolic health, but further research is needed to confirm these findings.

5 CONCLUSIONS

AIM 1:

We have shown that the composition of the gut microbiota of healthy lean long-term vegans and omnivores does not differ dramatically. In contrast, vegans and omnivores significantly differ in the composition of the fecal, serum, and urine metabolomes, probably as an effect of different availability of dietary substrates. Consequently, the vegan diet was associated with a lower abundance of the potentially harmful (protein fermentation products) and a higher occurrence of potentially beneficial (dietary fiber fermentation products) metabolites in feces.

AIM 2:

We developed a method for the assessment of the functional capacity of gut microbiota for butyrate synthesis based on the qPCR quantification of bacterial butyryl-CoA:acetate CoA-transferase. This method is based on qPCRs using degenerate primers specific for *but* gene variants and quantification of *but* gene abundance using the selected reference gene (16S rRNA gene or spike UNC-6 gene from *C. elegans*).

AIM 3:

In patients with newly diagnosed pre/diabetes treated with inulin, we observed considerable interindividual variability in the effects of inulin treatment on glucose homeostasis. We identified several omics-derived biomarkers that may play a central role in the development of obesity-associated metabolic changes and identified several predictors of treatment efficiency.

AIM 4:

Using the model of ex-GF mice humanized with mixed human vegan microbiota we found that it does not protect against the adverse effects of a Western-type diet like obesity, liver steatosis, and compromised glucose homeostasis. In contrast, supplementation of the Western diet with inulin reversed steatosis and ameliorated glucose metabolism, though it did not affect weight gain in this model. Inulin supplementation resulted in a significant change in the gut microbiota composition and its metabolic performance, inducing the shift from proteolytic towards saccharolytic fermentation. In the context of the potential use of fecal microbiota transfer with vegan microbiota in the therapy of metabolic NCDs, our study points out that it is not only the particular microbiota transfer but also the following dietary intervention with inulin or other dietary fiber and/or dietary change that is necessary for therapeutic success.

6 ABBREVIATIONS

AMPs	antimicrobial peptides
ASVs	Amplicon Sequence Variants
AUC	area under the curve
BCAAs	branched-chain amino acids
BCFAs	branched-chain fatty acids
BSH	bile salt hydrolases
C. elegans	*Caenorhabditis elegans*
CA	cholic acid
CAZymes	carbohydrate active enzymes
CCA	Canonical Correlation Analysis
CDCA	chenodeoxycholic acid
CRP	C-reactive protein
DCA	deoxycholic acid
DNA	deoxyribonucleic acid
FMT	fecal microbial transfer
FXR	farnesoid X receptor
GC-MS	gas chromatography mass spectrometry
GF	germ-free
GIT	gastrointestinal tract
HDL	high-density lipoprotein
HEI	Healthy Eating Index
HGP	Human Genome Project
HMOs	human milk oligosaccharides
HMP	Human Microbiome Project
IECs	intestinal stem cells
KEGG	Kyoto Encyclopedia of Genes and Genomes
LASSO	Least Absolute Shrinkage and Selection Operator
LCA	lithocholic acid
LC-MS	liquid chromatography mass spectrometry
LDL	low-density lipoprotein
LPS	lipopolysaccharides
ML	machine learning
MRSA	methicillin-resistant *Streptococcus aureus*
MS	mass spectrometry
NAFLD	non-alcoholic fatty liver disease

NCDs	non-communicable diseases
NGS	next-generation sequencing
NMR	nuclear magnetic resonance spectroscopy
OGTT	oral glucose tolerance test
OTUs	Operational Taxonomic Units
PCA	Principal Component Analysis
PCR	polymerase chain reaction
PERMANOVA	Permutational Analysis of Variance
qPCR	quantitative PCR
RNA	ribonucleic acid
rRNA	ribosomal RNA
SCFAs	short-chain fatty acids
sIgA	secretory immunoglobulin type A
TMAO	trimethylamine N-oxide
T2D	type 2 diabetes
UDCA	ursodeoxycholic acid
VOCs	volatile organic compounds
WD	Western-type diet

7 REFERENCES

Adak, A., & Khan, M. R. (2019). An insight into gut microbiota and its functionalities. *Cellular and Molecular Life Sciences*, *76*(3), 473–493. https://doi.org/10.1007/s00018-018-2943-4

Agus, A., Clément, K., & Sokol, H. (2021). Gut microbiota-derived metabolites as central regulators in metabolic disorders. *Gut*, *70*(6), 1174–1182. https://doi.org/10.1136/gutjnl-2020-323071

Agus, A., Denizot, J., Thévenot, J., Martinez-Medina, M., Massier, S., Sauvanet, P., Bernalier-Donadille, A., Denis, S., Hofman, P., Bonnet, R., Billard, E., & Barnich, N. (2016). Western diet induces a shift in microbiota composition enhancing susceptibility to Adherent-Invasive E. coli infection and intestinal inflammation. *Scientific Reports*, *6*(December 2015), 1–14. https://doi.org/10.1038/srep19032

Amieva, M., Alto, P., & Alto, P. (2016). Pathobiology of Helicobacter pylori-induced Gastric Cancer. *Gastroenterology*, *150*(1), 64–78. https://doi.org/10.1053/j.gastro.2015.09.004

Anand, S., & Mande, S. S. (2018). Diet, microbiota and gut-lung connection. *Frontiers in Microbiology*, *9*(SEP). https://doi.org/10.3389/fmicb.2018.02147

Arany, Z., & Neinast, M. (2018). Branched Chain Amino Acids in Metabolic Disease Do Elevations in BCAAs Cause Insulin. *Current Diabetes Reports*, *18*(76), 1–8.

Audebert, C., Even, G., Cian, A., Blastocystis Investigation Group, Loywick, A., Merlin, S., Viscogliosi, E., Chabé, M., El Safadi, D., Certad, G., Delhaes, L., Pereira, B., Nourrisson, C., Poirier, P., Wawrzyniak, I., Delbac, F., Morelle, C., Bastien, P., Lachaud, L., … Rabodonirina, M. (2016). Colonization with the enteric protozoa Blastocystis is associated with increased diversity of human gut bacterial microbiota. *Scientific Reports*, *6*(February), 1–11. https://doi.org/10.1038/srep25255

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Gou, Y. K., Nagy, A., Semenkovich, C. F., & Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(44), 15718–15723. https://doi.org/10.1073/pnas.0407076101

Baldeon, A. D., McDonald, D., Gonzalez, A., Knight, R., & Holscher, H. D. (2023). Diet Quality and the Fecal Microbiota in Adults in the American Gut Project Authors: *The Journal of Nutrition*. https://doi.org/10.1016/j.tjnut.2023.02.018

Be, C., Marteau, P., Pochart, P., & Inserm, U. (2001). *Comparative Study of Bacterial Groups within the Human Cecal and Fecal Microbiota*. *67*(10), 4939–4942. https://doi.org/10.1128/AEM.67.10.4939

Biagi, E., Franceschi, C., Rampelli, S., Severgnini, M., Ostan, R., Turroni, S., Consolandi, C., Quercia, S., Scurti, M., Monti, D., Capri, M., Brigidi, P., & Candela, M. (2016). Gut Microbiota and Extreme Longevity. *Current Biology*, *26*(11), 1480–1485. https://doi.org/10.1016/j.cub.2016.04.016

Blaser, M. J., & Falkow, S. (2009). What are the consequences of the disappearing human microbiota? *Nature Reviews Microbiology*, *7*(12), 887–894. https://doi.org/10.1038/nrmicro2245

Boland, M. (2016). Human digestion - a processing perspective. *Journal of the Science of Food and Agriculture*, *96*(7), 2275–2283. https://doi.org/10.1002/jsfa.7601

Bordenstein, S. R., & Theis, K. R. (2015). Host biology in light of the microbiome: Ten principles of holobionts and hologenomes. *PLoS Biology*, *13*(8), 1–23.

https://doi.org/10.1371/journal.pbio.1002226

Bustamante, C. D., De La Vega, F. M., & Burchard, E. G. (2011). Genomics for the world. *Nature*, *475*(7355), 163–165. https://doi.org/10.1038/475163a

Callahan, B. J., McMurdie, P. J., & Holmes, S. P. (2017). Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME Journal*, *11*(12), 2639–2643. https://doi.org/10.1038/ismej.2017.119

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., & Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(SUPPL. 1), 4516–4522. https://doi.org/10.1073/pnas.1000080107

Caruso, V., Song, X., Asquith, M., & Karstens, L. (2019). Performance of Microbiome Sequence Inference Methods in Environments with Varying Biomass. *MSystems*, *4*(1). https://doi.org/10.1128/msystems.00163-18

Celis, A. I., Aranda-Díaz, A., Culver, R., Xue, K., Relman, D., Shi, H., & Huang, K. C. (2022). Optimization of the 16S rRNA sequencing analysis pipeline for studying in vitro communities of gut commensals. *IScience*, *25*(4). https://doi.org/10.1016/j.isci.2022.103907

Chaudhari, D. S., Dhotre, D. P., Agarwal, D. M., Gaike, A. H., Bhalerao, D., Jadhav, P., Mongad, D., Lubree, H., Sinkar, V. P., Patil, U. K., Salvi, S., Bavdekar, A., Juvekar, S. K., & Shouche, Y. S. (2020). Gut, oral and skin microbiome of Indian patrilineal families reveal perceptible association with age. *Scientific Reports*, *10*(1), 1–13. https://doi.org/10.1038/s41598-020-62195-5

Chen, C., Song, X., Wei, W., Zhong, H., Dai, J., Lan, Z., Li, F., Yu, X., Feng, Q., Wang, Z., Xie, H., Chen, X., Zeng, C., Wen, B., Zeng, L., Du, H., Tang, H., Xu, C., Xia, Y., … Jia, H. (2017). The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nature Communications*, *8*(1). https://doi.org/10.1038/s41467-017-00901-0

Chen, X., Lu, Y., Chen, T., & Li, R. (2021). The Female Vaginal Microbiome in Health and Bacterial Vaginosis. *Frontiers in Cellular and Infection Microbiology*, *11*(April), 1–15. https://doi.org/10.3389/fcimb.2021.631972

Chen, Y., Wu, F. H., Wu, P. Q., Xing, H. Y., & Ma, T. (2022). The Role of The Tumor Microbiome in Tumor Development and Its Treatment. *Frontiers in Immunology*, *13*(July), 1–15. https://doi.org/10.3389/fimmu.2022.935846

Chiarello, M., McCauley, M., Villéger, S., & Jackson, C. R. (2022). Ranking the biases: The choice of OTUs vs. ASVs in 16S rRNA amplicon data analysis has stronger effects on diversity measures than rarefaction and OTU identity threshold. *PLoS ONE*, *17*(2 February), 1–19. https://doi.org/10.1371/journal.pone.0264443

Chong, J., & Xia, J. (2017). Computational approaches for integrative analysis of the metabolome and microbiome. *Metabolites*, *7*(4). https://doi.org/10.3390/metabo7040062

Clarke, S. F., Murphy, E. F., Sullivan, O. O., Lucey, A. J., Humphreys, M., Hogan, A., Hayes, P., Reilly, M. O., Jeffery, I. B., Wood-martin, R., Kerins, D. M., Quigley, E., Ross, R. P., Toole, P. W. O., Molloy, M. G., Falvey, E., Shanahan, F., & Cotter, P. D. (2014). *Exercise and associated dietary extremes impact on gut microbial diversity*. 1913–1920. https://doi.org/10.1136/gutjnl-2013-306541

Clarridge, J. E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews*, *17*(4), 840–862. https://doi.org/10.1128/CMR.17.4.840-862.2004

Clemente, J. C., Pehrsson, E. C., Blaser, M. J., Sandhu, K., Gao, Z., Wang, B., Magris, M., Hidalgo, G., Contreras, M., Noya-Alarcón, Ó., Lander, O., McDonald, J., Cox, M., Walter, J., Oh, P. L., Ruiz, J. F., Rodriguez, S., Shen, N., Song, S. J., … Dominguez-Bello, M. G. (2015). The microbiome of uncontacted Amerindians. *Science Advances*, *1*(3). https://doi.org/10.1126/sciadv.1500183

Conesa, A., & Beck, S. (2019). Making multi-omics data accessible to researchers. *Scientific Data*, *6*(1), 4–7. https://doi.org/10.1038/s41597-019-0258-4

Cover, T. L., & Blaser, M. J. (2009). Helicobacter pylori in health and disease. *Gastroenterology*, *136*(6), 1863–1873. https://doi.org/10.1053/j.gastro.2009.01.073

Craig Venter, J., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., Amanatides, P., Ballew, R. M., Huson, D. H., Wortman, J. R., Zhang, Q., Kodira, C. D., Zheng, X. H., Chen, L., … Zhu, X. (2001). The sequence of the human genome. *Science*, *291*(5507), 1304–1351. https://doi.org/10.1126/science.1058040

Cummings, J. H., Pomare, E. W., Branch, W. J., Naylor, C. P. E., & MacFarlane, G. T. (1987). Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut*, *28*, 1221–1227. https://doi.org/10.1136/gut.28.10.1221

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. V, Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S. B., Dutton, R. J., & Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, *505*(7484), 559–563. https://doi.org/10.1038/nature12820

De Aguiar Vallim, T. Q., Tarling, E. J., & Edwards, P. A. (2013). Pleiotropic roles of bile acids in metabolism. *Cell Metabolism*, *17*(5), 657–669. https://doi.org/10.1016/j.cmet.2013.03.013

De Angelis, M., Ferrocino, I., Calabrese, F. M., De Filippis, F., Cavallo, N., Siragusa, S., Rampelli, S., Di Cagno, R., Rantsiou, K., Vannini, L., Pellegrini, N., Lazzi, C., Turroni, S., Lorusso, N., Ventura, M., Chieppa, M., Neviani, E., Brigidi, P., O'Toole, P. W., … Cocolin, L. (2020). Diet influences the functions of the human intestinal microbiome. *Scientific Reports*, *10*(1), 4247. https://doi.org/10.1038/s41598-020-61192-y

De Filippo, C., Cavalieri, D., Di, M., Ramazzotti, M., & Baptiste, J. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *PNAS*, *107*(33), 14691–14696. https://doi.org/10.1073/pnas.1005963107

De Vos, W. M., Tilg, H., Van Hul, M., & Cani, P. D. (2022). Gut microbiome and health: Mechanistic insights. *Gut*, 1020–1032. https://doi.org/10.1136/gutjnl-2021-326789

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., & Knight, R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(26), 11971–11975. https://doi.org/10.1073/pnas.1002601107

Ducarmon, Q. R., Zwittink, R. D., Hornung, B. V. H., van Schaik, W., Young, V. B., & Kuijper, E. J. (2019). Gut Microbiota and Colonization Resistance against Bacterial Enteric Infection. *Microbiology and Molecular Biology Reviews*, *83*(3), 1–29. https://doi.org/10.1128/mmbr.00007-19

Fragiadakis, G. K., Smits, S. A., Sonnenburg, E. D., Van Treuren, W., Reid, G., Knight, R., Manjurano, A., Changalucha, J., Dominguez-Bello, M. G., Leach, J., & Sonnenburg, J. L. (2019). Links between environment, diet, and the hunter-gatherer microbiome. *Gut Microbes*, *10*(2), 216–227. https://doi.org/10.1080/19490976.2018.1494103

Gallo, R. L. (2017). Human Skin Is the Largest Epithelial Surface for Interaction with Microbes. *J Invest Dermatol.*, *137*(6), 1213–1214. https://doi.org/10.1016/j.jid.2016.11.045

Gilbert, J., Blaser, M. J., Caporaso, J. G., Jansson, J. K., Lynch, V. S., & Knight, R. (2018). Current understanding of the human microbiome. *Nat Med.*, *24*(4), 392–400. https://doi.org/10.1038/nm.4517

Goffau, M. C. De, Lager, S., Sovio, U., Gaccioli, F., Peacock, S. J., Parkhill, J., Charnock-jones, D. S., & Smith, C. S. (2019). Human placenta has no microbiome but can harbour potential pathogens. *Nature*, *572*(7769), 329–334. https://doi.org/10.1038/s41586-019-1451-5

Gojda, J., & Cahova, M. (2021). Gut microbiota as the link between elevated bcaa serum levels and insulin resistance. *Biomolecules*, *11*(10), 1–16. https://doi.org/10.3390/biom11101414

Gomaa, E. Z. (2020). Human gut microbiota/microbiome in health and diseases: a review. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *113*(12), 2019–2040. https://doi.org/10.1007/s10482-020-01474-7

Goodrich, J. K., Davenport, E. R., Beaumont, M., Jackson, M. A., Knight, R., Ober, C., Spector, T. D., Bell, J. T., Clark, A. G., & Ley, R. E. (2016). Genetic Determinants of the Gut Microbiome in UK Twins. *Cell Host and Microbe*, *19*(5), 731–743. https://doi.org/10.1016/j.chom.2016.04.017

Gu, Z., Eils, R., & Schlesner, M. (2016). Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics*, *32*(May), 2847–2849. https://doi.org/10.1093/bioinformatics/btw313

Guinane, M. C., Tadrous, A., Fouhy, F., Ryan, C. A., Dempsey, E. M., Murphy, B., Andrews, E., Cotter, P. D., Stanton, C., & Ross, R. P. (2013). Microbial Composition of Human Appendices from Patients following. *MBio*, *4*(1), 1–6. https://doi.org/10.1128/mBio.00366-12.Editor

Guzior, D. V., & Quinn, R. A. (2021). Review: microbial transformations of human bile acids. *Microbiome*, *9*(1), 1–13. https://doi.org/10.1186/s40168-021-01101-1

Hamada, H., Hiroi, T., Nishiyama, Y., Takahashi, H., Masunaga, Y., Hachimura, S., Kaminogawa, S., Takahashi-Iwanaga, H., Iwanaga, T., Kiyono, H., Yamamoto, H., & Ishikawa, H. (2002). Identification of Multiple Isolated Lymphoid Follicles on the Antimesenteric Wall of the Mouse Small Intestine. *The Journal of Immunology*, *168*(1), 57–64. https://doi.org/10.4049/jimmunol.168.1.57

Hanlon, D. E. O., Moench, T. R., & Cone, R. A. (2013). *Vaginal pH and Microbicidal Lactic Acid When Lactobacilli Dominate the Microbiota*. *8*(11), 1–8. https://doi.org/10.1371/journal.pone.0080074

Harmsen, H. J. M., Wildeboer-Veloo, A. C. M., Raangs, G. C., Wagendorp, A. A., Klijn, N., Bindels, J. G., & Welling, G. W. (2000). Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *Journal of Pediatric Gastroenterology and Nutrition*, *30*(1), 61–67. https://doi.org/10.1097/00005176-200001000-00019

Hasin, Y., Seldin, M., & Lusis, A. (2017). Multi-omics approaches to disease. *Genome Biology*, *18*(1), 1–15. https://doi.org/10.1186/s13059-017-1215-1

Hill, M. J. (1997). Intestinal flora and endogenous vitamin synthesis. In *European Journal of Cancer Prevention* (Vol. 6, pp. S43–S45). https://doi.org/10.1097/00008469-199703001-00009

Hunt, R. H., Camilleri, M., Crowe, S. E., El-Omar, E. M., Fox, J. G., Kuipers, E. J., Malfertheiner, P.,

McColl, K. E. L., Pritchard, D. M., Rugge, M., Sonnenberg, A., Sugano, K., & Tack, J. (2015). The stomach in health and disease. *Gut*, *64*(10), 1650–1668. https://doi.org/10.1136/gutjnl-2014-307595

James, K. R., Gomes, T., Elmentaite, R., Kumar, N., Gulliver, E. L., King, H. W., Stares, M. D., Bareham, B. R., Ferdinand, J. R., Velislava, N., Polański, K., Forster, S. C., Jarvis, L. B., Suchanek, O., Howlett, S., James, L. K., Jones, J. L., Meyer, K. B., & Menna, R. (2020). *Europe PMC Funders Group Distinct microbial and immune niches of the human colon*. *21*(3), 343–353. https://doi.org/10.1038/s41590-020-0602-z.Distinct

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. *Journal of Clinical Microbiology*, *45*(9), 2761–2764. https://doi.org/10.1128/JCM.01228-07

Jha, A. R., Davenport, E. R., Gautam, Y., Bhandari, D., Tandukar, S., Ng, K. M., Fragiadakis, G. K., Holmes, S., Gautam, G. P., Leach, J., Sherchand, J. B., Bustamante, C. D., & Sonnenburg, J. L. (2018). Gut microbiome transition across a lifestyle gradient in Himalaya. *PLoS Biology*, *16*(11), 1–30. https://doi.org/10.1371/journal.pbio.2005396

Jiang, D., Armour, C. R., Hu, C., Mei, M., & Tian, C. (2019). Microbiome Multi-Omics Network Analysis : Statistical Considerations , Limitations , and Opportunities. *Frontiers in Genetics*, *10*(November), 1–19. https://doi.org/10.3389/fgene.2019.00995

Johansson, M. E. V., Holmén Larsson, J. M., & Hansson, G. C. (2011). The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(SUPPL. 1), 4659–4665. https://doi.org/10.1073/pnas.1006451107

Johansson, M. E. V., Jakobsson, H. E., Holmén-Larsson, J., Schütte, A., Ermund, A., Rodríguez-Piñeiro, A. M., Arike, L., Wising, C., Svensson, F., Bäckhed, F., & Hansson, G. C. (2015). Normalization of host intestinal mucus layers requires long-term microbial colonization. *Cell Host Microbe*, *18*(5), 582–592. https://doi.org/10.1016/j.chom.2015.10.007

Jones, B. V., Begley, M., Hill, C., Gahan, C. G. M., & Marchesi, J. R. (2008). Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(36), 13580–13585. https://doi.org/10.1073/pnas.0804437105

Kalbermatter, C., Fernandez Trigo, N., Christensen, S., & Ganal-Vonarburg, S. C. (2021). Maternal Microbiota, Early Life Colonization and Breast Milk Drive Immune Development in the Newborn. *Frontiers in Immunology*, *12*(May), 1–22. https://doi.org/10.3389/fimmu.2021.683022

Kang, M., Ko, E., & Mersha, T. B. (2022). A roadmap for multi-omics data integration using deep learning. *Briefings in Bioinformatics*, *23*(1), 1–16. https://doi.org/10.1093/bib/bbab454

Kennedy, E. A., King, K. Y., & Baldridge, M. T. (2018). Mouse microbiota models: Comparing germ-free mice and antibiotics treatment as tools for modifying gut bacteria. *Frontiers in Physiology*, *9*(OCT), 1–16. https://doi.org/10.3389/fphys.2018.01534

Kim, Y. S., & Ho, S. B. (2010). Intestinal goblet cells and mucins in health and disease: Recent insights and progress. *Current Gastroenterology Reports*, *12*(5), 319–330. https://doi.org/10.1007/s11894-010-0131-2

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., & Bäckhed, F. (2016). From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. *Cell*, *165*(6), 1332–1345.

https://doi.org/10.1016/j.cell.2016.05.041

Kolodziejczyk, A. A., Zheng, D., & Elinav, E. (2019). Diet–microbiota interactions and personalized nutrition. *Nature Reviews Microbiology*, *17*(12), 742–753. https://doi.org/10.1038/s41579-019-0256-8

Kumar, J., Rani, K., & Datt, C. (2020). Molecular link between dietary fibre, gut microbiota and health. *Molecular Biology Reports*, *47*(8), 6229–6237. https://doi.org/10.1007/s11033-020-05611-3

Kundu, P., Blacher, E., Elinav, E., & Pettersson, S. (2017). Our Gut Microbiome: The Evolving Inner Self. *Cell*, *171*(7), 1481–1493. https://doi.org/10.1016/j.cell.2017.11.024

Land, M., Hauser, L., Jun, S., Nookaew, I., Leuze, M. R., Ahn, T., Karpinets, T., Lund, O., & Kora, G. (2015). *Insights from 20 years of bacterial genome sequencing*. 141–161. https://doi.org/10.1007/s10142-015-0433-4

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., Fitzhugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., Levine, R., McEwan, P., … Chen, Y. J. (2001). Initial sequencing and analysis of the human genome. *Nature*, *412*(6846), 565–566. https://doi.org/10.1038/35087627

LeBlanc, J. G., Milani, C., de Giori, G. S., Sesma, F., van Sinderen, D., & Ventura, M. (2013). Bacteria as vitamin suppliers to their host: A gut microbiota perspective. *Current Opinion in Biotechnology*, *24*(2), 160–168. https://doi.org/10.1016/j.copbio.2012.08.005

Lederberg, B. J., & McCray, A. T. (2001). ’ Ome Sweet ’ Omics-- A Genealogical Treasury of Words. *The Scientist*, *15*(7), 8.

Lee-Sarwar, K. A., Lasky-Su, J., Kelly, R. S., Litonjua, A. A., & Weiss, S. T. (2020). Metabolome–microbiome crosstalk and human disease. *Metabolites*, *10*(5), 1–10. https://doi.org/10.3390/metabo10050181

Lee, Y. S., Kim, T. Y., Kim, Y., Lee, S. H., Kim, S., Kang, S. W., Yang, J. Y., Baek, I. J., Sung, Y. H., Park, Y. Y., Hwang, S. W., Eunju, O., Kim, K. S., Liu, S., Kamada, N., Gao, N., & Kweon, M. N. (2018). Microbiota-Derived Lactate Accelerates Intestinal Stem-Cell-Mediated Epithelial Development. *Cell Host and Microbe*, *24*(6), 833-846.e6. https://doi.org/10.1016/j.chom.2018.11.002

Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P., Roy, R., Bircher, J. S., Schlegel, M. L., Tucker, T. A., Mark, D., Knight, R., & Gordon, J. I. (2008). Evolution of mammals and their gut microbes. *Science*, *320*(5883), 1647–1651. https://doi.org/10.1126/science.1155725

Li, T., & Chiang, J. Y. L. (2015). Bile acids as metabolic regulators. *Current Opinion in Gastroenterology*, *31*(2), 159–165. https://doi.org/10.1097/MOG.0000000000000156

Li, X., Liu, Y., Yang, X., Li, C., & Song, Z. (2022). The Oral Microbiota: Community Composition, Influencing Factors, Pathogenesis, and Interventions. *Frontiers in Microbiology*, *13*(April), 1–19. https://doi.org/10.3389/fmicb.2022.895537

Link, C. D. (2021). Is There a Brain Microbiome? *Neuroscience Insights*, *16*. https://doi.org/10.1177/26331055211018709

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., & Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature*, *489*(7415), 220–230. https://doi.org/10.1038/nature11550

Macpherson, A. J., Hunziker, L., McCoy, K., & Lamarre, A. (2001). IgA responses in the intestinal mucosa against pathogenic and non-pathogenic microorganisms. *Microbes and Infection*, *3*(12),

1021–1035. https://doi.org/10.1016/S1286-4579(01)01460-5

Maldonado-Contreras, A., Goldfarb, K. C., Godoy-vitorino, F., Karaoz, U., Blaser, M. J., Brodie, E. L., & Dominguez-bello, M. G. (2011). Structure of the human gastric bacterial community in relation to Helicobacter pylori status. *The ISME Journal*, *5*, 574–579. https://doi.org/10.1038/ismej.2010.149

Marchesi, J. R., & Ravel, J. (2015). The vocabulary of microbiome research: a proposal. *Microbiome*, *3*(1), 1–3. https://doi.org/10.1186/s40168-015-0094-5

Marcobal, A., Kashyap, P. C., Nelson, T. A., Aronov, P. A., Donia, M. S., Spormann, A., Fischbach, M. A., & Sonnenburg, J. L. (2013). A metabolomic view of how the human gut microbiota impacts the host metabolome using humanized and gnotobiotic mice. *ISME Journal*, *7*(10), 1933–1943. https://doi.org/10.1038/ismej.2013.89

Margulis, L. (1967). On the origin of mitosing cells. *Journal of Theoretical Biology*, *14*(3), 255–274. http://www.ncbi.nlm.nih.gov/pubmed/11541390

Margulis, L., & Fester, R. (1991). *Symbiosis as a Source of Evolutionary Innovation*. The MIT Press.

Martinez-Guryn, K., Hubert, N., Frazier, K., Urlass, S., Mark, W., Ojeda, P., Pierre, J. F., Miyoshi, J., Sontag, T., Cham, C., Reardon, C., Leone, V., & Chang, E. B. (2018). Small intestine microbiota regulate host digestive and absorptive adaptive responses to dietary lipids. *Cell Host and Microbe*, *23*(4), 458–469. https://doi.org/10.1016/j.chom.2018.03.011

Martins-de-Souza, D. (2014). Proteomics, metabolomics, and protein interactomics in the characterization of the molecular features of major depressive disorder. *Dialogues in Clinical Neuroscience*, *16*(1), 63–73. https://doi.org/10.31887/dcns.2014.16.1/dmartins

Mathieu, E., Escribano-Vazquez, U., Descamps, D., Cherbuy, C., Langella, P., Riffault, S., Remot, A., & Thomas, M. (2018). Paradigms of lung microbiota functions in health and disease, particularly, in asthma. *Frontiers in Physiology*, *9*(AUG), 1–11. https://doi.org/10.3389/fphys.2018.01168

Matsumoto, M., Kibe, R., Ooga, T., Aiba, Y., Kurihara, S., Sawaki, E., Koga, Y., & Benno, Y. (2012). Impact of intestinal microbiota on intestinal luminal metabolome. *Scientific Reports*, *2*, 1–10. https://doi.org/10.1038/srep00233

Maynard, C. L., Elson, C. O., Hatton, R. D., & Weaver, C. T. (2012). Reciprocal interactions of the intestinal microbiota and immune system. *Nature*, *489*(7415), 231–241. https://doi.org/10.1038/nature11551

McDonald, D., Hyde, E., Debelius, J. W., Morton, J. T., Gonzalez, A., Ackermann, G., Aksenov, A. A., Behsaz, B., Brennan, C., Chen, Y., DeRight Goldasich, L., Dorrestein, P. C., Dunn, R. R., Fahimipour, A. K., Gaffney, J., Gilbert, J. A., Gogul, G., Green, J. L., Hugenholtz, P., … Goldasich, D. L. (2018). American Gut: an Open Platform for Citizen Science Microbiome Research. *MSystems*, *3*(3), 1–28. http://humanfoodproject.com/

McHardy, I. H., Goudarzi, M., Tong, M., Ruegger, P. M., Schwager, E., Weger, J. R., Graeber, T. G., Sonnenburg, J. L., Horvath, S., Huttenhower, C., Mcgovern, D. P. B., Jr, A. J. F., Borneman, J., & Braun, J. (2013). Integrative analysis of the microbiome and metabolome of the human intestinal mucosal surface reveals exquisite inter-relationships. *Microbiome*, *1*(17), 1–19. https://doi.org/10.1186/2049-2618-1-17

Meng, C., Zeleznik, O. A., Thallinger, G. G., Kuster, B., Gholami, A. M., & Culhane, A. C. (2016). Dimension reduction techniques for the integrative analysis of multi-omics data. *Briefings in Bioinformatics*, *17*(4), 628–641. https://doi.org/10.1093/bib/bbv108

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., Belzer, C., Palacio, D., Rodriguez, J. M., Bode, L., Vos, W. De, & Gueimonde, M. (2017). The First Microbial Colonizers of the Human Gut : Composition , Activities , and Health Implications of the Infant Gut Microbiota. *Microbiology and Molecular Biology Reviews*, *81*(4), 1–67.

Mohajeri, M. H., Brummer, R. J. M., Rastall, R. A., Weersma, R. K., Harmsen, H. J. M., Faas, M., & Eggersdorfer, M. (2018). The role of the microbiome for human health: from basic science to clinical applications. *European Journal of Nutrition*, *57*(1), 1–14. https://doi.org/10.1007/s00394-018-1703-4

Morrison, D. J., & Preston, T. (2016). Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes*, *7*(3), 189–200. https://doi.org/10.1080/19490976.2015.1134082

Morton, E. R., Lynch, J., Froment, A., Lafosse, S., Heyer, E., Przeworski, M., Blekhman, R., & Ségurel, L. (2015). Variation in Rural African Gut Microbiota Is Strongly Correlated with Colonization by Entamoeba and Subsistence. *PLoS Genetics*, *11*(11), 1–28. https://doi.org/10.1371/journal.pgen.1005658

Muegge, B. D., Kuczynski, J., Knights, D., Clemente, J. C., González, A., Fontana, L., Henrissat, B., Knight, R., & Gordon, J. I. (2011). Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science*, *332*(6032), 970–974. https://doi.org/10.1126/science.1198719

Najmanová, L., Vídeňská, P., & Cahová, M. (2022). Healthy Microbiome – A Mere Idea or a Sound Concept? *Physiological Research*, *71*(6), 719–738. https://doi.org/10.33549/physiolres.934967

Neefs, J., Peer, Y. Van De, Rijk, P. De, Chapelle, S., Wachter, R. De, Biochemie, D., Uia, U. A., & Antwerp, B.-. (1993). Compilation of small ribosomal subunit RNA structures. *Nucleic Acids Research*, *21*(13), 3025–3049. https://doi.org/10.1093/nar/21.13.3025

Neugent, M. L., Hulyalkar, N. V., Nguyen, V. H., Zimmern, P. E., & De Nisco, N. J. (2020). Advances in understanding the human urinary microbiome and its potential role in urinary tract infection. *MBio*, *11*(2), 1–15. https://doi.org/10.1128/mBio.00218-20

Nikolov, M., Schmidt, C., & Urlaub, H. (2021). Quantitative mass spectrometry-based proteomics: An overview. In *Quantitative Methods in Proteomics* (Vol. 2228, pp. 85–100). https://doi.org/10.1007/978-1-61779-885-6_7

Oliphant, K., & Allen-Vercoe, E. (2019). Macronutrient metabolism by the human gut microbiome: Major fermentation by-products and their impact on host health. *Microbiome*, *7*(1), 1–15. https://doi.org/10.1186/s40168-019-0704-8

Olivier, M., Asmis, R., Hawkins, G. A., Howard, T. D., & Cox, L. A. (2019). The need for multi-omics biomarker signatures in precision medicine. *International Journal of Molecular Sciences*, *20*(19). https://doi.org/10.3390/ijms20194781

Ottman, N., Smidt, H., de Vos, W. M., & Belzer, C. (2012). The function of our microbiota: who is out there and what do they do? *Frontiers in Cellular and Infection Microbiology*, *2*(August), 104. https://doi.org/10.3389/fcimb.2012.00104

Ouwerkerk, J. P., De Vos, W. M., & Belzer, C. (2013). Glycobiome: Bacteria and mucus at the epithelial interface. *Best Practice and Research: Clinical Gastroenterology*, *27*(1), 25–38. https://doi.org/10.1016/j.bpg.2013.03.001

Paone, P., & Cani, P. D. (2020). Mucus barrier, mucins and gut microbiota: The expected slimy

partners? *Gut*, *69*(12), 2232–2243. https://doi.org/10.1136/gutjnl-2020-322260

Park, M., Kim, D., Moon, K., & Park, T. (2020). Integrative analysis of multi-omics data based on blockwise sparse principal components. *International Journal of Molecular Sciences*, *21*(21), 1–17. https://doi.org/10.3390/ijms21218202

Parks, D. H., Chuvochina, M., Waite, D. W., Rinke, C., Skarshewski, A., Chaumeil, P. A., & Hugenholtz, P. (2018). A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. *Nature Biotechnology*, *36*(10), 996. https://doi.org/10.1038/nbt.4229

Pasolli, E., Asnicar, F., Manara, S., Zolfo, M., Karcher, N., Armanini, F., Beghini, F., Manghi, P., Tett, A., Ghensi, P., Collado, M. C., Rice, B. L., DuLong, C., Morgan, X. C., Golden, C. D., Quince, C., Huttenhower, C., & Segata, N. (2019). Extensive Unexplored Human Microbiome Diversity Revealed by Over 150,000 Genomes from Metagenomes Spanning Age, Geography, and Lifestyle. *Cell*, *176*(3), 649-662.e20. https://doi.org/10.1016/j.cell.2019.01.001

Patangia, D. V, Ryan, C. A., Dempsey, E., & Stanton, C. (2022). *Impact of antibiotics on the human microbiome and consequences for host health*. *September 2021*, 1–23. https://doi.org/10.1002/mbo3.1260

Peck, B. C. E., Mah, A. T., Pitman, W. A., Ding, S., Lund, P. K., & Sethupathy, P. (2017). Functional transcriptomics in diverse intestinal epithelial cell types reveals robust MicroRNA sensitivity in intestinal stem cells to microbial status. *Journal of Biological Chemistry*, *292*(7), 2586–2600. https://doi.org/10.1074/jbc.M116.770099

Picard, M., Scott-Boyer, M. P., Bodein, A., Périn, O., & Droit, A. (2021). Integration strategies of multi-omics data for machine learning analysis. *Computational and Structural Biotechnology Journal*, *19*, 3735–3746. https://doi.org/10.1016/j.csbj.2021.06.030

Piovesan, A., Pelleri, M. C., Antonaros, F., Strippoli, P., Caracausi, M., & Vitale, L. (2019). On the length , weight and GC content of the human genome. *BMC Research Notes*, *12*(106), 1–7. https://doi.org/10.1186/s13104-019-4137-z

Potgieter, M., Bester, J., Kell, D. B., & Pretorius, E. (2015). *The dormant blood microbiome in chronic , inflammatory diseases*. *March*, 567–591. https://doi.org/10.1093/femsre/fuv013

Prochazkova, M., Budinska, E., Kuzma, M., Pelantova, H., Hradecky, J., Heczkova, M., Daskova, N., Bratova, M., Modos, I., Videnska, P., Splichalova, P., Sowah, S. A., Kralova, M., Henikova, M., Selinger, E., Klima, K., Chalupsky, K., Sedlacek, R., Landberg, R., … Cahova, M. (2022). Vegan Diet Is Associated With Favorable Effects on the Metabolic Performance of Intestinal Microbiota: A Cross-Sectional Multi-Omics Study. *Frontiers in Nutrition*, *8*(January), 1–18. https://doi.org/10.3389/fnut.2021.783302

Prodan, A., Tremaroli, V., Brolin, H., Zwinderman, A. H., Nieuwdorp, M., & Levin, E. (2020). Comparing bioinformatic pipelines for microbial 16S rRNA amplicon sequencing. *PLoS ONE*, *15*(1), 1–19. https://doi.org/10.1371/journal.pone.0227434

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D. R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., … Zoetendal, E. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature*, *464*(7285), 59–65. https://doi.org/10.1038/nature08821

Quince, C., Walker, A. W., Simpson, J. T., Loman, N. J., & Segata, N. (2017). Shotgun metagenomics, from sampling to analysis. *Nature Biotechnology*, *35*(9), 833–844. https://doi.org/10.1038/nbt.3935

Quinn, R. A., Melnik, A. V., Vrbanac, A., Fu, T., Patras, K. A., Christy, M. P., Bodai, Z., Belda-Ferre, P., Tripathi, A., Chung, L. K., Downes, M., Welch, R. D., Quinn, M., Humphrey, G., Panitchpakdi, M., Weldon, K. C., Aksenov, A., da Silva, R., Avila-Pacheco, J., … Dorrestein, P. C. (2020). Global chemical effects of the microbiome include new bile-acid conjugations. *Nature*, *579*(7797), 123–129. https://doi.org/10.1038/s41586-020-2047-9

Reel, P. S., Reel, S., Pearson, E., Trucco, E., & Jefferson, E. (2021). Using machine learning approaches for multi-omics data analysis: A review. *Biotechnology Advances*, *49*(March), 107739. https://doi.org/10.1016/j.biotechadv.2021.107739

Rew, L., Harris, M. D., & Goldie, J. (2022). The ketogenic diet : its impact on human gut microbiota and potential consequent health outcomes : a systematic literature review. *Gastroenterol Hepatol Bed*, *15*(4), 326–342. https://doi.org/10.22037/ghfbb.v15i4.2600

Reynoso-García, J., Miranda-Santiago, A. E., Meléndez-Vázquez, N. M., Acosta-Pagán, K., Sánchez-Rosado, M., Díaz-Rivera, J., Rosado-Quiñones, A. M., Acevedo-Márquez, L., Cruz-Roldán, L., Tosado-Rodríguez, E. L., Figueroa-Gispert, M. D. M., & Godoy-Vitorino, F. (2022). A complete guide to human microbiomes: Body niches, transmission, development, dysbiosis, and restoration. *Frontiers in Systems Biology*, *2*(951403), 1–22. https://doi.org/10.3389/fsysb.2022.951403

Ridlon, J. M., Harris, S. C., Bhowmik, S., Kang, D. J., & Hylemon, P. B. (2016). Consequences of bile salt biotransformations by intestinal bacteria. *Gut Microbes*, *7*(1), 22–39. https://doi.org/10.1080/19490976.2015.1127483

Ridlon, J. M., Kang, D. J., & Hylemon, P. B. (2006). Bile salt biotransformations by human intestinal bacteria. *Journal of Lipid Research*, *47*(2), 241–259. https://doi.org/10.1194/jlr.R500013-JLR200

Rodosthenous, T., Shahrezaei, V., & Evangelou, M. (2020). Integrating multi-OMICS data through sparse canonical correlation analysis for the prediction of complex traits: A comparison study. *Bioinformatics*, *36*(17), 4616–4625. https://doi.org/10.1093/bioinformatics/btaa530

Rosenbaum, M., Knight, R., & Leibel, R. L. (2015). The gut microbiota in human energy homeostasis and obesity. *Trends Endocrinol Metab.*, *26*(9), 493–501. https://doi.org/10.1016/j.tem.2015.07.002

Rothschild, D., Weissbrod, O., Barkan, E., Kurilshikov, A., Korem, T., Zeevi, D., Costea, P. I., Godneva, A., Kalka, I. N., Bar, N., Shilo, S., Lador, D., Vila, A. V., Zmora, N., Pevsner-Fischer, M., Israeli, D., Kosower, N., Malka, G., Wolf, B. C., … Segal, E. (2018). Environment dominates over host genetics in shaping human gut microbiota. *Nature*, *555*(7695), 210–215. https://doi.org/10.1038/nature25973

Salazar, N., Gonzalez, S., Nlgacka, A., Rios-covián, D., Arboleya, S., Gueimonde, M., & de Los Reyes-Gavilán, C. G. (2019). Microbiome: Effects of Ageing and Diets. In *Microbiota: current research and emerging trends* (Vol. 7). https://doi.org/10.21775/9781910190937.01

Sayin, S. I., Wahlström, A., Felin, J., Jäntti, S., Marschall, H. U., Bamberg, K., Angelin, B., Hyötyläinen, T., Orešič, M., & Bäckhed, F. (2013). Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. *Cell Metabolism*, *17*(2), 225–235. https://doi.org/10.1016/j.cmet.2013.01.003

Schnorr, S. L., Candela, M., Rampelli, S., Centanni, M., Consolandi, C., Basaglia, G., Turroni, S., Biagi, E., Peano, C., Severgnini, M., Fiori, J., Gotti, R., De Bellis, G., Luiselli, D., Brigidi, P., Mabulla, A., Marlowe, F., Henry, A. G., & Crittenden, A. N. (2014). Gut microbiome of the Hadza hunter-gatherers. *Nature Communications*, *5*. https://doi.org/10.1038/ncomms4654

Schoenborn, A. A., von Furstenberg, R. J., Valsaraj, S., Hussain, F. S., Stein, M., Shanahan, M. T., Henning, S. J., & Gulati, A. S. (2019). The enteric microbiota regulates jejunal Paneth cell number and function without impacting intestinal stem cells. *Gut Microbes*, *10*(1), 45–58. https://doi.org/10.1080/19490976.2018.1474321

Schroeder, B. O., & Bäckhed, F. (2016). Signals from the gut microbiota to distant organs in physiology and disease. *Nature Medicine*, *22*(10), 1079–1089. https://doi.org/10.1038/nm.4185

Sender, R., Fuchs, S., & Milo, R. (2016a). Are We Really Vastly Outnumbered ? Revisiting the Ratio of Bacterial to Host Cells in Humans. *Cell*, *164*(3), 337–340. https://doi.org/10.1016/j.cell.2016.01.013

Sender, R., Fuchs, S., & Milo, R. (2016b). Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biology*, *14*(8), 1–21. https://doi.org/10.1371/journal.pbio.1002533

Sharma, R., Schumacher, U., Ronaasen, V., & Coates, M. (1995). Rat intestinal mucosal responses to a microbial flora and different diets. *Gut*, *36*(2), 209–214. https://doi.org/10.1136/gut.36.2.209

Sharon, I., Mart, N., Pasolli, E., Fabbrini, M., Vitali, F., Agamennone, V., Dötsch, A., Selberherr, E., Grau, H., Meixner, M., Liere, K., Ercolini, D., Filippo, C. De, Caderni, G., Brigidi, P., & Turroni, S. (2022). *The Core Human Microbiome : Does It Exist and How Can We Find It ? A Critical Review of the Concept*. 1–25.

Shendure, J., Findlay M., G., & Snyder W., M. (2019). Genomic medicine - progress, pitfalls, and promise. *Cell*, *177*(1), 45–57. https://doi.org/10.1016/j.cell.2019.02.003

Simon, J. C., Marchesi, J. R., Mougel, C., & Selosse, M. A. (2019). Host-microbiota interactions: From holobiont theory to analysis. *Microbiome*, *7*(1), 1–5. https://doi.org/10.1186/s40168-019-0619-4

Singh, R. K., Chang, H. W., Yan, D., Lee, K. M., Ucmak, D., Wong, K., Abrouk, M., Farahnik, B., Nakamura, M., Zhu, T. H., Bhutani, T., & Liao, W. (2017). Influence of diet on the gut microbiome and implications for human health. *Journal of Translational Medicine*, *15*(1), 1–17. https://doi.org/10.1186/s12967-017-1175-y

Slatko, B., Gardner, A. F., & Ausubel, F. M. (2018). Overview of Next Generation Sequencing Technologies. *Curr Protoc Mol Biol.*, *122*(1), 1–15. https://doi.org/10.1002/cpmb.59

Smits, S. A., Leach, J., Sonnenburg, E. D., Gonzalez, C. G., Lichtman, J. S., Reid, G., Knight, R., Manjurano, A., Changalucha, J., Elias, J. E., Dominguez-Bello, M. G., & Sonnenburg, J. L. (2017). Seasonal Cycling in the Gut Microbiome of the Hadza Hunter- Gatherers of Tanzania. *Science*, *357*(6563), 802–806. https://doi.org/10.1126/science.aan4834.Seasonal

Sommer, F., & Bäckhed, F. (2013). The gut microbiota-masters of host development and physiology. *Nature Reviews Microbiology*, *11*(4), 227–238. https://doi.org/10.1038/nrmicro2974

Sonnenburg, J. L., & Bäckhed, F. (2014). Diet – microbiota interactions as moderators of human metabolism. *Nature*, *535*, 56–64. https://doi.org/10.1038/nature18846

Staley, C., Weingarden, A. R., Khoruts, A., & Sadowsky, M. J. (2017). Interaction of Gut Microbiota with Bile Acid Metabolism and its Influence on Disease States. *Appl Microbiol Biotechnol.*, *101*(1), 47–64. https://doi.org/10.1007/s00253-016-8006-6

Stewart, C. J., Ajami, N. J., O'Brien, J. L., Hutchinson, D. S., Smith, D. P., Wong, M. C., Ross, M. C., Lloyd, R. E., Doddapaneni, H. V., Metcalf, G. A., Muzny, D., Gibbs, R. A., Vatanen, T., Huttenhower, C., Xavier, R. J., Rewers, M., Hagopian, W., Toppari, J., Ziegler, A. G., … Petrosino,

J. F. (2018). Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature*, *562*(7728), 583–588. https://doi.org/10.1038/s41586-018-0617-x

Suhre, K., McCarthy, M. I., & Schwenk, J. M. (2021). Genetics meets proteomics: perspectives for large population-based studies. *Nature Reviews Genetics*, *22*(1), 19–37. https://doi.org/10.1038/s41576-020-0268-2

Swaney, M. H. (2021). *Living in Your Skin : Microbes , Molecules , and Mechanisms*. *January*.

Swann, J. R., Want, E. J., Geier, F. M., Spagou, K., Wilson, I. D., Sidaway, J. E., Nicholson, J. K., & Holmes, E. (2011). Systemic gut microbial modulation of bile acid metabolism in host tissue compartments. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(SUPPL. 1), 4523–4530. https://doi.org/10.1073/pnas.1006734107

Tebani, A., Afonso, C., Marret, S., & Bekri, S. (2016). Omics-based strategies in precision medicine: Toward a paradigm shift in inborn errors of metabolism investigations. *International Journal of Molecular Sciences*, *17*(9). https://doi.org/10.3390/ijms17091555

The Human Microbiome Project Consortium. (2012). Structure , function and diversity of the healthy human microbiome. *Nature*, *486*(7402), 207–214. https://doi.org/10.1038/nature11234

Tian, L., Wang, X. W., Wu, A. K., Fan, Y., Friedman, J., Dahlin, A., Waldor, M. K., Weinstock, G. M., Weiss, S. T., & Liu, Y. Y. (2020). Deciphering functional redundancy in the human microbiome. *Nature Communications*, *11*(1), 1–11. https://doi.org/10.1038/s41467-020-19940-1

Tibshirani, R. (1996). Regression Shrinkage and Selection via the Lasso. *Journal of the Royal Statistical Society*, *58*(1), 267–288.

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Ley, R. E., Sogin, M. L., Jones, W. J., Roe, B. a, Jason, P., Egholm, M., Henrissat, B., Heath, A. C., Knight, R., Gordon, J. I., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., … Gordon, J. I. (2009). A core gut microbiome between lean and obesity twins. *Nature*, *457*(7228), 480–484. https://doi.org/10.1038/nature07540.A

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., & Gordon, J. I. (2007). The Human Microbiome Project. *Nature*, *449*(7164), 804–810. https://doi.org/10.1038/nature06244

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., & Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*, *444*(7122), 1027–1031. https://doi.org/10.1038/nature05414

Turnbaugh, P. J., Ridaura, V. K., Faith, J. J., Rey, F. E., Knight, R., & Gordon, J. I. (2009). The Effect of Diet on the Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice. *Sci Transl Med.*, *1*(6), 1–19. https://doi.org/10.1126/scitranslmed.3000322

Tyakht, A. V, Kostryukova, E. S., Popenko, A. S., Belenikin, M. S., Pavlenko, A. V, Larin, A. K., Karpova, I. Y., Selezneva, O. V, Semashko, T. A., Ospanova, E. A., Babenko, V. V, Maev, I. V, Cheremushkin, S. V, Kucheryavyy, Y. A., Shcherbakov, P. L., Grinevich, V. B., Efimov, O. I., Sas, E. I., Abdulkhakov, R. A., … Alexeev, D. G. (2013). Human gut microbiota community structures in urban and rural populations in Russia. *Nature Communications*, *4*(2469), 1–9. https://doi.org/10.1038/ncomms3469

Ursell, L. K., Metcalf, J. L., Parfrey Wegener, L., & Knight, R. (2012). Defining the Human Microbiome. *Nutr Rev.*, *70*(August), 38–44. https://doi.org/10.1111/j.1753-4887.2012.00493.x

Vandenplas, Y., Carnielli, V. P., Ksiazyk, J., Luna, M. S., Migacheva, N., Mosselmans, J. M., Picaud, J. C.,

Possner, M., Singhal, A., & Wabitsch, M. (2020). Factors affecting early-life intestinal microbiota development. *Nutrition*, *78*, 110812. https://doi.org/10.1016/j.nut.2020.110812

Vatanen, T., Franzosa, E. A., Schwager, R., Tripathi, S., Arthur, T. D., Vehik, K., Lernmark, Å., Hagopian, W. A., Rewers, M. J., She, J. X., Toppari, J., Ziegler, A. G., Akolkar, B., Krischer, J. P., Stewart, C. J., Ajami, N. J., Petrosino, J. F., Gevers, D., Lähdesmäki, H., … Xavier, R. J. (2018). The human gut microbiome in early-onset type 1 diabetes from the TEDDY study. *Nature*, *562*(7728), 589–594. https://doi.org/10.1038/s41586-018-0620-2

Venegas, D. P., De La Fuente, M. K., Landskron, G., González, M. J., Quera, R., Dijkstra, G., Harmsen, H. J. M., Faber, K. N., & Hermoso, M. A. (2019). Short chain fatty acids (SCFAs)mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. *Frontiers in Immunology*, *10*(MAR). https://doi.org/10.3389/fimmu.2019.00277

Vujkovic-Cvijin, I., Sklar, J., Jiang, L., Natarajan, L., Knight, R., Belkaid, Y., Section, I., Diseases, I., Diseases, I., Program, M., Jolla, L., Jolla, L., Jolla, L., Jolla, L., & Jolla, L. (2020). Host variables confound gut microbiota studies of human disease. *Nature*, *587*(7834), 448–454. https://doi.org/10.1038/s41586-020-2881-9

Wahlström, A., Sayin, S. I., Marschall, H. U., & Bäckhed, F. (2016). Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism. *Cell Metabolism*, *24*(1), 41–50. https://doi.org/10.1016/j.cmet.2016.05.005

Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet.*, *10*(1), 57–63. https://doi.org/10.1038/nrg2484.RNA-Seq

Wastyk, H. C., Fragiadakis, G. K., Perelman, D., Dahan, D., Merrill, B. D., Yu, F. B., Topf, M., Gonzalez, C. G., Van Treuren, W., Han, S., Robinson, J. L., Elias, J. E., Sonnenburg, E. D., Gardner, C. D., & Sonnenburg, J. L. (2021). Gut-microbiota-targeted diets modulate human immune status. *Cell*, *184*(16), 4137-4153.e14. https://doi.org/10.1016/j.cell.2021.06.019

Whiteside, S. A., McGinniss, J. E., & Collman, R. G. (2021). The lung microbiome: Progress and promise. *Journal of Clinical Investigation*, *131*(15), 1–10. https://doi.org/10.1172/JCI150473

Wikoff, W. R., Anfora, A. T., Liu, J., Schultz, P. G., Lesley, S. A., Peters, E. C., & Siuzdak, G. (2009). Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(10), 3698–3703. https://doi.org/10.1073/pnas.0812874106

Woese, C. R., & Fox, G. E. (1977). *Phylogenetic structure of the prokaryotic domain : The primary kingdoms*. *74*(11), 5088–5090.

Woese, C. R., Kandlert, O., & Wheelis, M. L. (1990). *Towards a natural system of organisms : Proposal for the domains*. *87*(June), 4576–4579.

Wolfe, A. J., Toh, E., Shibata, N., Rong, R., Kenton, K., Fitzgerald, M., Mueller, E. R., Schreckenberger, P., Dong, Q., Nelson, D. E., & Brubaker, L. (2012). *Evidence of Uncultivated Bacteria in the Adult Female Bladder*. 1376–1383. https://doi.org/10.1128/JCM.05852-11

Wu, C., Zhou, F., Ren, J., Li, X., Jiang, Y., & Ma, S. (2019). A selective review of multi-level omics data integration using variable selection. *High-Throughput*, *8*(1), 1–25. https://doi.org/10.3390/ht8010004

Wu, S., Hugerth, L. W., & Schuppe-koistinen, I. (2022). The right bug in the right place : opportunities for bacterial vaginosis treatment. *NPJ Biofilms Microbiomes*, *8*(1), 1–11. https://doi.org/10.1038/s41522-022-00295-y

Xue, Z., Kable, M. E., & Marco, M. L. (2018). Impact of DNA Sequencing and Analysis Methods on 16S rRNA Gene Bacterial Community Analysis of Dairy Products. *MSphere*, *3*(5), 1–14. https://doi.org/10.1128/msphere.00410-18

Yagi, K., Huffnagle, G. B., Lukacs, N. W., & Asai, N. (2021). The lung microbiome during health and disease. *International Journal of Molecular Sciences*, *22*(19), 1–13. https://doi.org/10.3390/ijms221910872

Yatsunenko, T., Rey, F. E., Manary, M. J., & Trehan, I. (2012). Human gut microbiome viewed across age and geography Tanya. *Nature*, *486*(7402), 222–227. https://doi.org/10.1038/nature11053.Human

Ye, L., & Rawls, J. F. (2021). Microbial influences on gut development and gut-brain communication. *Development (Cambridge)*, *148*(21). https://doi.org/10.1242/dev.194936

Yu, Y., Raka, F., & Adeli, K. (2019). The Role of the Gut Microbiota in Lipid and Lipoprotein Metabolism. *Journal of Clinical Medicine*, *8*(12), 2227. https://doi.org/10.3390/jcm8122227

Zierer, J., Jackson, M. A., Kastenmüller, G., Mangino, M., Long, T., Telenti, A., Mohney, R. P., Small, K. S., Bell, J. T., Steves, C. J., Valdes, A. M., Spector, T. D., & Menni, C. (2018). The fecal metabolome as a functional readout of the gut microbiome. *Nature Genetics*, *50*(6), 790–795. https://doi.org/10.1038/s41588-018-0135-7

Zimmermann, M., Zimmermann-kogadeeva, M., & Wegmann, R. (2019). Mapping human microbiome drug metabolism by gut bacteria and their genes. *Nature*, *570*(7762), 462–467. https://doi.org/10.1038/s41586-019-1291-3

Zoetendal, E. G., Raes, J., Van Den Bogert, B., Arumugam, M., Booijink, C. C., Troost, F. J., Bork, P., Wels, M., De Vos, W. M., & Kleerebezem, M. (2012). The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. *ISME Journal*, *6*(7), 1415–1426. https://doi.org/10.1038/ismej.2011.212

8 LIST OF AUTHOR'S PUBLICATIONS

Publications with a direct relation with the thesis

Daskova N., Modos I., Krbcova M., Kuzma M., Pelantova H., Hradecky J., Heczkova M., *et al.* 2023. "Multi-omics signatures in new-onset diabetes predict metabolic response to dietary inulin: findings from an observational study followed by an interventional trial." Nutrition & Diabetes 13 (1): 1–13. https://doi.org/10.1038/s41387-023-00235-5. **IF (2023) = 4.73**

Daskova N., Heczkova M., Modos I., Hradecky J., Hudcovic T., Kuzma M., Pelantova H., *et al.* 2023. "Protective Effect of Vegan Microbiota on Liver Steatosis Is Conveyed by Dietary Fiber: Implications for Fecal Microbiota Transfer Therapy." Nutrients 15 (454): 1–18. https://doi.org/10.3390/nu15020454. **IF (2023) = 5.43**

Prochazkova M., Budinska E., Kuzma M., Pelantova H., Hradecky J., Heczkova M., Daskova N., *et al.* 2022. "Vegan Diet is Associated with Favorable Effects on the Metabolic Performance of Intestinal Microbiota: A Cross-Sectional Multi-Omics Study." Frontiers in Nutrition 8 (January): 1–18. https://doi.org/10.3389/fnut.2021.783302. **IF (2022) = 6.59**

Daskova N., Heczkova M., Modos I., Videnska P., Splichalova P., Pelantova H., Kuzma M., Gojda J. and Cahova M. 2021. "Determination of Butyrate Synthesis Capacity in Gut Microbiota: Quantification of but Gene Abundance by Qpcr in Fecal Samples." Biomolecules 11 (9). https://doi.org/10.3390/biom11091303. **IF (2021) = 5.88**

Publications with no relation to the thesis

Fabian O., Bajer L., Drastich P., Harant K., Sticova E., Daskova N., Modos I., *et al*. 2023. "A Current State of Proteomics in Adult and Pediatric Inflammatory Bowel Diseases: A Systematic Search and Review." Int. J. Mol. Sci. 24 (11): 1–28. https://doi.org/10.3390/ijms24119386. **IF (2023) = 6.21**

Kosek V., Heczkova M., Novak F., Meisnerova E., Novakova O., Zelenka J., Bechynska K., *et al*. 2020. "The ω-3 Polyunsaturated Fatty Acids and Oxidative Stress in Long-Term Parenteral Nutrition Dependent Adult Patients: Functional Lipidomics Approach." Nutrients 12 (8): 1–15. https://doi.org/10.3390/nu12082351. **IF (2020) = 5.72**

Bechynska K., Daskova N., Vrzackova N., Harant K., Heczkova M., Podzimkova K., Bratova M., *et al*. 2019. "The Effect of ω-3 Polyunsaturated Fatty Acids on the Liver Lipidome, Proteome and Bile Acid Profile: Parenteral versus Enteral Administration." Scientific Reports 9 (1): 1–14. https://doi.org/10.1038/s41598-019-54225-8. **IF (2019) = 4.16**

Seda O., Cahova M., Mikova I., Sedova L., Dankova H., Heczkova M., Bratova M., *et al*. 2019. "Hepatic Gene Expression Profiles Differentiate Steatotic and Non-Steatotic Grafts in Liver Transplant Recipients." Frontiers in Endocrinology 10 (April). https://doi.org/10.3389/fendo.2019.00270. **IF (2019) = 3.64**

Cahova M., Dankova H., Heczkova M., Bratova M., Daskova N., Bastova H., Gojda J. and Wohl P. 2019. "MicroRNAs as Potential Markers of Parenteral Nutrition-Associated Liver Disease in Adult Patients." Physiological Research 68 (4): 681–88. https://doi.org/10.33549/physiolres.934103. **IF (2019) = 1.66**