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Summary of the doctoral thesis

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

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Appendix no. 1

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The doctoral thesis will be available for public viewing in printed form at least five working days before the defense at the Department for Science and International Relations of the Dean's Office of the First Faculty of Medicine.

Appendix no. 1

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

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 the 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-germ-free (GF) mice. In the intervention study, we tested the hypothesis that the success of an 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 The importance of gut microbiota and integration with other data types

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).

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 does not consider other types of microbes such as viruses and phages (Gilbert et al., 2018).

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). The human gut microbiome is currently attracting a great deal of scientific attention. The gastrointestinal tract (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.

A typical aspect of the human gut microbiota 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 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).

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 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.

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).

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).

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. 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.

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. 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. It is no exaggeration to say that multi-omics studies are the future.

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 non-communicable diseases (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

The 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 samples 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 the 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 (ribosomal ribonucleic acid) sequencing. The plasma was also analyzed for short-chain fatty acids (SCFAs) concentrations and untargeted metabolomics using liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance spectroscopy (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. 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.

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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 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 the 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 was then applied to DNA (deoxyribonucleic acid) 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.

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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 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 (OGTT) 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 (Least Absolute Shrinkage and Selection Operator regression model, LASSO) 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 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 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.

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.

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 through 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), an 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.

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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, 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

AUC	area under the curve
C. elegans	*Caenorhabditis elegans*
CRP	C-reactive protein
DNA	deoxyribonucleic acid
FMT	fecal microbial transfer
GC-MS	gas chromatography-mass spectrometry
GF	germ-free
GIT	gastrointestinal tract
LASSO	Least Absolute Shrinkage and Selection Operator
LC-MS	liquid chromatography-mass spectrometry
LDL	low-density lipoprotein
NAFLD	non-alcoholic fatty liver disease
NCDs	non-communicable diseases
NMR	nuclear magnetic resonance spectroscopy
OGTT	oral glucose tolerance test
PCR	polymerase chain reaction
PERMANOVA	Permutational Analysis of Variance
qPCR	quantitative PCR
RNA	ribonucleic acid
rRNA	ribosomal RNA
SCFAs	short-chain fatty acids
T2D	type 2 diabetes
VOCs	volatile organic compounds
WD	Western-type diet

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