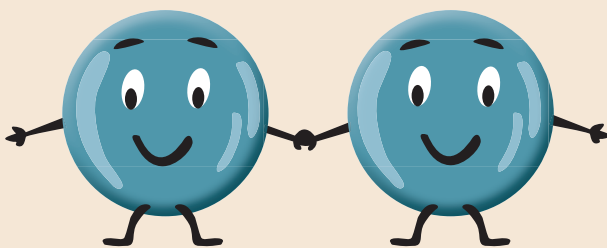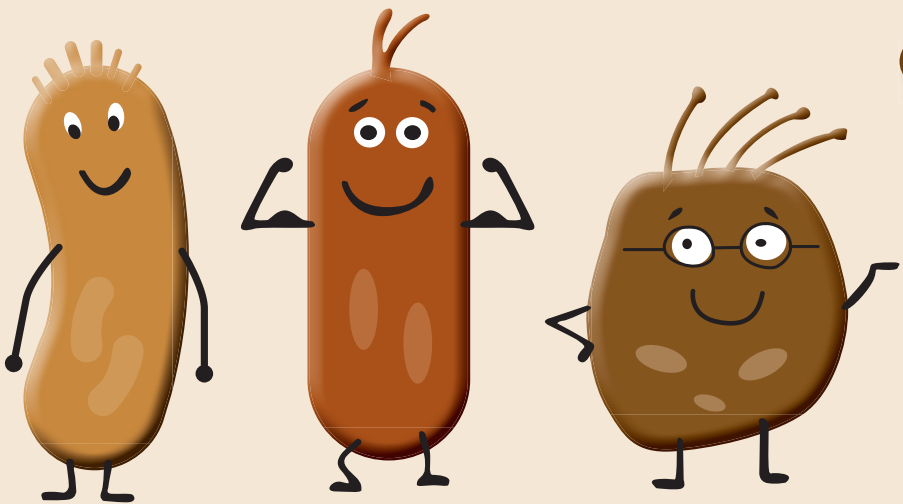# THE ROLE OF HYDROGENOTROPHIC MICROBIOTA IN THE HUMAN GUT

TAOJUN WANG

Propositions

1. Methanogen levels significantly associate with gut microbiota composition and function.
(this thesis)

2. Methanogens, sulphate-reducing bacteria and reductive acetogens coexist and do not compete for hydrogen in gut ecosystems.
(this thesis)

3. Microbes shape the daily life of human beings.

4. Embracing unexpected results is essential for scientists to think differently and explore new possibilities.

5. Supervising students is the best way to examine one's own knowledge.

6. Working efficiency speaks louder than working time.

7. Running a Marathon is not hard, but realizing that it is possible is not easy.

Propositions belonging to the thesis, entitled
The role of hydrogenotrophic microbiota in the human gut

Taojun Wang
Wageningen, 21 June 2022

The role of

hydrogenotrophic microbiota

in the human gut

Taojun Wang

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The role of
hydrogenotrophic microbiota
in the human gut

Taojun Wang

Table of contents

CHAPTER 1

General introduction and thesis outline
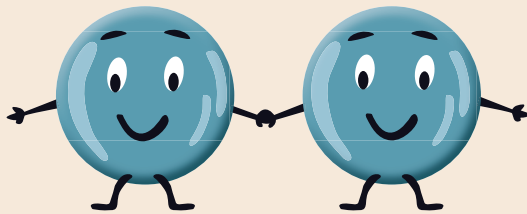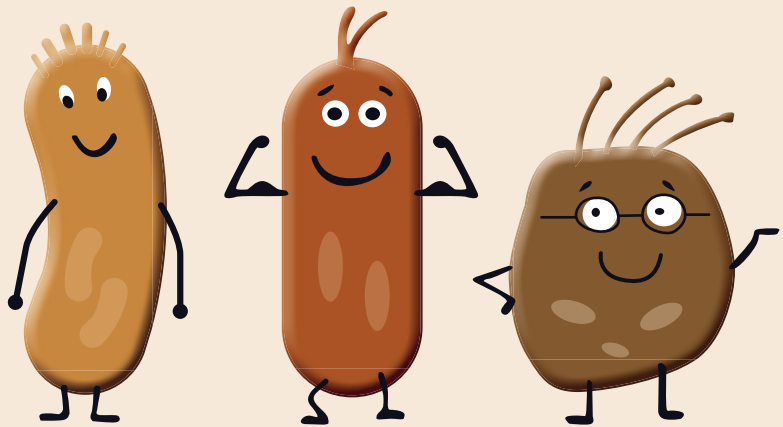
General introduction

Gut microbiota

Microorganisms are almost everywhere. These small life forms are found in water, air, soil, extreme environments, food, animals, and our human body such as skin, oral cavity, vagina and gastrointestinal tract [1]. The gastrointestinal tract, especially the colon, inhabits a dense and diverse community of microorganisms with a total number of approximately 3.9×10^{13} cells, which is similar to that of the total number of human cells in our body [2]. Collectively, these microorganisms are termed gut microbiota [3]. At the phylum level, the gut microbiota is mainly composed of *Firmicutes* and *Bacteroidetes* as well as *Actinobacteria* with lower proportions of other phyla such as *Proteobacteria* and *Verrucomicrobia* [4, 5]. At the species level, a meta-analysis including 1006 Western adults has shown that *Faecalibacterium prausnitzii*, *Oscillospira guillermondii* and *Blautia obeum* (previously called *Ruminococcus obeum*) are the top three most abundant ones [8, 9]. Although hundreds of microbial species have been isolated from the human gut [6], thousands of candidate bacterial species that were identified via analysing 11,850 human gut microbiomes have not been cultured to date and remain to be explored [7]. Furthermore, based on 1,267 metagenome datasets, approximately ten million non-redundant genes have been detected, which is 2-3 orders of magnitude more than the total number of genes in the human genome [10, 11]. Such a vast genetic functional reservoir determines the diverse metabolic capacities within the gut microbiome, such as the utilization of indigestible carbohydrates, which is complementary to human metabolism [12, 13].

The gut microbiota plays an important role in human health. Increasing evidence indicates that the composition of the human gut microbiota differs between healthy individuals and those suffering from disorders such as inflammatory bowel disease (IBD), colorectal cancer (CRC) and obesity [14-16]. In addition, it has been frequently described that compromised individuals have a lower microbial diversity compared to healthy individuals [17-19]. Moreover, the metabolites produced by the gut microbiota can have a large impact on human health. For example, butyrate is an energy source for colonocytes and protects against inflammation and CRC [20]. On the other hand, trimethylamine (TMA) can be converted to TMA *N*-oxide (TMAO) that has been associated with cardiovascular disease risk [21, 22]. Furthermore, intervention studies targeting the gut microbiota, such as those with probiotics, prebiotics or dietary components have demonstrated large differences in responsiveness between subjects, which has been suggested to be associated with the resident microbiota [23]. For example, a dietary intervention study using barley kernel-based bread described that those individuals that improved with

respect to glucose metabolism had a concomitant increase in the relative abundance of *Prevotella copri*, whereas non-responding individuals did not show this increase [24]. Thus, unravelling microbiota composition and function is fundamental to understanding the role of gut microbiota in human health and discovering potential microbial targets for interventions directed at improving health.

Many factors have been considered as drivers of gut microbiota composition, such as health status, use of antibiotics and other types of medication, genetic background, and diet [25-27]. However, several large cohort studies indicated that many of these factors collectively explain less than 20% of the observed variation in gut microbiota composition [5, 27], suggesting that individuality of microbiota composition is likely associated with specific characteristics within each microbiota. These could include the competition for substrates and specific trophic interactions between microbes, such as interspecies hydrogen transfer, which has shown to play a central role in microbial fermentation [28].

Hydrogen metabolism in gut ecosystems: a central role in microbial fermentation

Non-digestible and unabsorbed dietary components reach the colon where they are fermented by the resident microbiota resulting in the production of metabolites such as short-chain fatty acids (SCFAs), carbon dioxide and hydrogen [20, 29]. Hydrogen is produced by a range of different gut microorganisms that are collectively referred to as hydrogenogens. Primarily, hydrogen is produced via the reoxidation of reduced pyridine and flavin nucleotides. In addition, the metabolism of pyruvate is accompanied by hydrogen production. Namely, the cleavage of pyruvate results in formate, which is subsequently converted into hydrogen, and the generation of acetyl-CoA from pyruvate with the activity of pyruvate:ferredoxin oxidoreductase and hydrogenase results in hydrogen production [30]. Hydrogenases are key enzymes in hydrogen production. It has been reported that these enzymes are encoded on the genomes of more than 70% of gut microbes, and hydrogenase-bearing microorganisms mainly belong to the phyla *Bacteroidetes* and *Firmicutes* [31]. Based on the amount of the available substrate for microbial fermentation in people consuming a typical Western diet, it has been estimated that more than 13 litres of hydrogen are produced daily [32]. However, due to the difficulty of directly measuring hydrogen in the colon, flatus or exhaled breath are collected as surrogate samples to assess hydrogen metabolism in gut ecosystems. Steggerda [33] collected an average of 360 mL flatus per day from five subjects who consumed a basal diet (246 g of carbohydrates, 88 g of fat, 152 g of protein and 7 g of crude fibre), and showed that this flatus contained 19.8 % of hydrogen. Considering the fact that most of the total hydrogen is exhaled with

breath, the hydrogen concentration in the colon could be much higher than the value detected in flatus [34].

It has been suggested that fermentation processes in the gut are regulated by the production and consumption of hydrogen [30, 32]. Hydrogen accumulation leads to a high partial pressure that would inhibit the regeneration of the coenzyme NAD^+ from NADH, and thermodynamically restrict further microbial fermentation and growth [30, 35]. Therefore, the consumption of hydrogen is critical to maintain the microbial fermentation balance in gut ecosystems (Figure 1). Hydrogen-consuming microbes, collectively termed hydrogenotrophic microbiota, play an important role in using hydrogen as electron donor and maintaining a low hydrogen partial pressure in gut ecosystems [32, 36]. It has been reported that the ability of the host to harvest the energy from dietary components was enhanced by the presence of methanogens in a gnotobiotic mouse model [37]. Moreover, in another study with gnotobiotic mice, a high NAD^+/NADH ratio was maintained and the efficiency of fermentation was increased by the presence of reductive acetogens [38]. As such, the hydrogenotrophic microbiota plays an important role in microbial fermentation. However, how hydrogenotrophic microbes impact gut microbiota composition and function remains largely unknown.
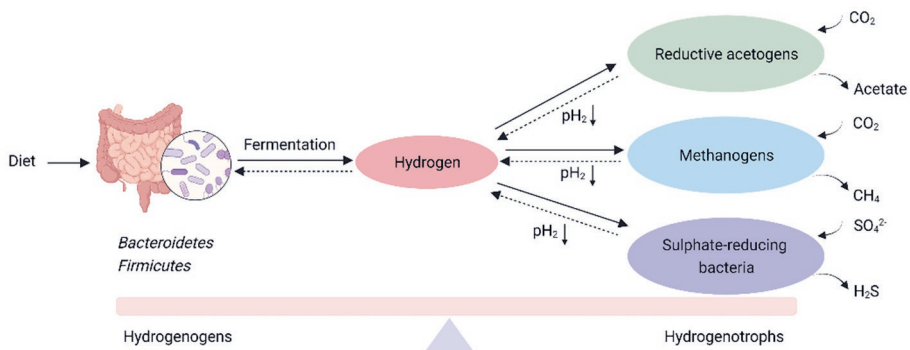
Figure 1: Schematic overview of hydrogen metabolism in gut ecosystems. Non-digestible and unabsorbed dietary components are fermented by hydrogenogens, such as members of the phyla *Bacteroidetes* and *Firmicutes*, resulting in the production of hydrogen. The accumulation of hydrogen leads to a high partial pressure that thermodynamically restricts further microbial fermentation. Hydrogenotrophs including reductive acetogens, methanogens and sulphate-reducing bacteria are able to oxidize hydrogen and thereby decrease the partial pressure of hydrogen, which maintains the balance of the overall gut microbial fermentation process. Abbreviations: pH_2, partial pressure of hydrogen. The figure is modified from [30].

Hydrogenotrophic microbiota

The hydrogenotrophic microbiota primarily consists of three functional groups in the human gut, namely methanogenic archaea, also called methanogens, that use hydrogen and carbon dioxide producing methane, sulphate-reducing bacteria (SRB) that use hydrogen and sulphate producing hydrogen sulphide, and reductive acetogens that use hydrogen and carbon dioxide producing acetate (Table 1). Thermodynamically, the most favourable reaction is sulphate reduction with a Gibbs free energy change of -152.2 kJ mol^{-1}, followed by methanogenesis with a Gibbs free energy change of -131 kJ mol^{-1}, and finally reductive acetogenesis with a Gibbs free energy change of -95 kJ mol^{-1} [39].

Table 1: Metabolic reactions of the three functional groups using hydrogen as electron donor

Functional group	Reaction	$\Delta G°´$
Methanogens	$4\ H_2 + CO_2 \longrightarrow CH_4 + 2\ H_2O$	-131 kJ mol^{-1}
Sulphate-reducing bacteria	$4\ H_2 + SO_4^{2-} + 2\ H^+ \longrightarrow H_2S + 4\ H_2O$	-152.2 kJ mol^{-1}
Reductive acetogens	$4\ H_2 + 2\ CO_2 \longrightarrow CH_3COOH + 2\ H_2O$	-95 kJ mol^{-1}

The pathways involved in hydrogen utilization are listed in Figure 2. The functional genes encoding enzymes involved in the respective hydrogenotrophic pathways have been used as molecular markers to detect and quantify the abundance of the three functional groups in gut ecosystems. Methyl coenzyme M methylreductase (MCR) plays a role in methane production (Figure 2A), and the functional gene encoding its subunit A (*mcrA*) is highly conserved and used as a marker to detect and/or quantify methanogens in anaerobic environments [40, 41]. As for SRB, genes encoding adenosine phosphosulfate (APS) reductase (APR; *aps*) and dissimilatory (bi)sulfite reductase (DSR; *dsrAB*) are highly conserved in SRB (Figure 2B) and thus are used as markers to detect and quantify SRB [42-44]. Quantification of acetogens is achieved targeting the genes encoding formyl tetrahydrofolate synthetase (FTHFS, *fhs*) and acetyl-CoA synthase (ACS, *acs*) [30].

Methanobrevibacter smithii and *Methanosphaera stadtmanae* are the two methanogenic species isolated from the human gut. *M. smithii* is the dominant methanogen in human gut ecosystems with a prevalence of up to 95.7% [45]. *M. smithii* is able to convert hydrogen and carbon dioxide, or formate to methane [46,

47]. *M. stadtmanae* uses hydrogen and methanol to produce methane [48, 49]. SRB colonize ~50% of the human guts and have a greater taxonomic diversity than methanogens with *Desulfovibro* as the dominant genus of SRB [50, 51]. The species *Desulfovibro piger*, *Desulfovibro fairfieldensis* and *Desulfovibro desulfuricans* have been isolated from human faeces with *D. piger* being the most common SRB [50, 52]. Of note is that SRB are more versatile and metabolically flexible than methanogens. Without sulphate SRB can switch to the fermentation of organic compounds, such as lactate, which in turn results in the production of hydrogen [53, 54]. Reductive acetogens are phylogenetically diverse and utilize the Wood-Ljungdahl pathway to produce acetate. In one earlier study, it has been estimated that 33% of acetate produced in the human gut is generated via this pathway [55]. *Blautia hydrogenotrophica* (previously *Ruminococcus hydrogenotrophicus*) is the most well-known and studied species [35, 56]. Besides reductive acetogenesis, *Blautia hydrogenotrophica* is also able to ferment glucose and fructose [56]. Therefore, hydrogen is only essential to methanogens.
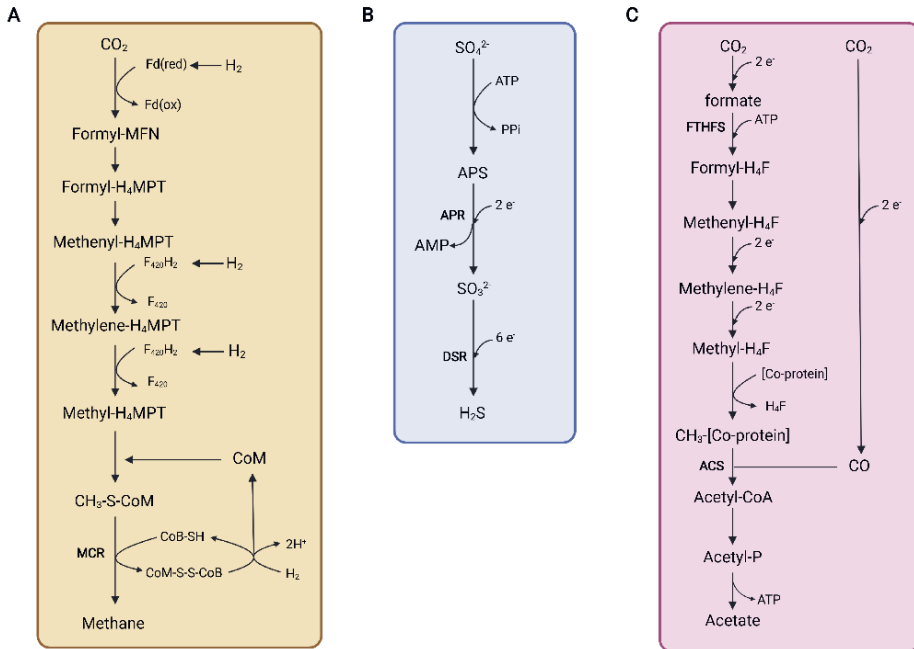
Figure 2: The three pathways involved in hydrogen utilisation in the human gut. (A) The methanogenic pathway converts hydrogen and carbon dioxide to methane. MFN, methanofuran; H_4MPT, tetrahydromethanopterin; Fd(ox), ferredoxin; Fd(red), reduced ferredoxin; F_{420}, coenzyme F_{420}; CoM/CoB, coenzyme M/B; MCR, Methyl coenzyme M methylreductase. (B) Dissimilatory sulphate-reducing pathway. APS, adenosine phosphosulfate; PPi, pyrophosphate; APR, APS reductase; DSR, dissimilatory (bi)sulfite reductase; (C) Wood-Ljungdahl pathway with two branches (the methyl branch, left branch; the carbonyl branch, right branch). H_4F, tetrahydrofolate; FTHFS, formyl- tetrahydrofolate synthetase; ACS, acetyl CoA synthase. This figure is modified from [32].

Stratification of the human population into methane excretors and non-methane excretors

The composition and abundance of hydrogenotrophic microbes in human gut ecosystems vary between individuals. The abundance of the genus *Methanobrevibacter* in the human gut shows a bimodal distribution, which means that this genus is either very abundant or nearly absent [57]. The difference in high and low numbers of methanogens is reflected in the presence of methane in exhaled breath (Figure 3). Breath tests revealed that in the Western world approximately one-third of individuals have high numbers of methanogens and show methane levels of >1 ppm (above atmospheric methane levels) in their breath [58]. Remarkably, in Africans, approximately 80% of the population emits

measurable amounts of breath methane [59]. These so-called methane excretors (MEs) have approximately 10^9 CFU/g of methanogens in the stool. In contrast, individuals who are non-methane excretors (NMEs) harbour less than 10^4 CFU/g [58, 60]. Although the methane concentration in exhaled breath shows considerable variability within person-specific range values over time [61], the methanogenic activity remained remarkably stable over 35 years despite the use of antibiotics and change of diet [58]. However, it remains speculative what mechanism drives the huge difference in amounts of methanogens between MEs and NMEs and how this impacts microbiota composition in gut ecosystems.

Methane excretors

Methane: above atmosphere level;
Methanogens: ~ 10^9/g (stool);
Ratio: ~ 33%

Breath test

Non-methane excretors

Methane: atmosphere level;
Methanogens: < 10^4/g (stool);
Ratio: ~ 67%
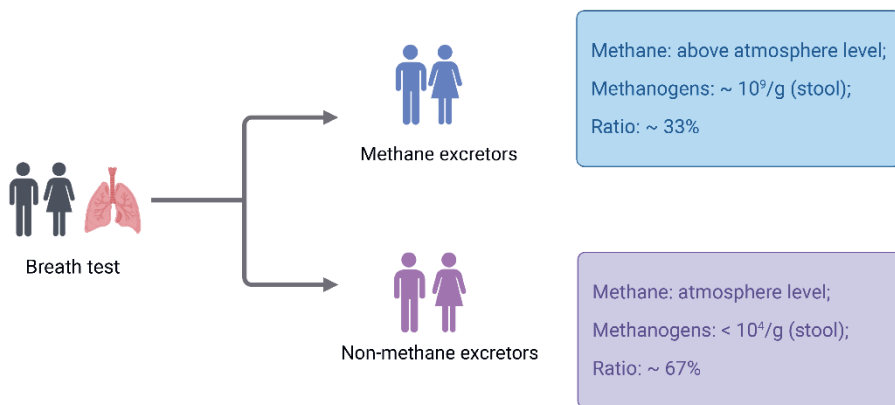
Figure 3: Schematic representation of methane excretors and non-methane excretors in the Western world

Interactions between hydrogenotrophic functional groups in gut ecosystems

Direct competition between the three hydrogenotrophic functional groups (methanogens, SRB and reductive acetogens) has been considered as all of them use hydrogen as an energy source. Thermodynamically, sulphate reduction is more favourable with the highest Gibbs free energy change compared to methanogenesis and reductive acetogenesis [39]. Moreover, SRB have the lowest threshold concentration (10-20 ppm) of hydrogen utilisation, followed by methanogens (30-100 ppm) and reductive acetogens (400–950 ppm) [62]. It has been reported that sulphate reduction completely inhibits methanogenesis when sulphate is available in sufficient amounts [63]. However, SRB switch to utilisation of other compounds, like lactate, and then play an opposite role of hydrogenogen

for syntrophic metabolism with methanogens [53, 54], suggesting that the occurrence of cross-feeding or competition depends on the nutrients provided by the environment/diet of the host.

Based on the analysis of faecal samples, it has been observed that the presence of SRB and methanogens is mutually exclusive, i.e. SRB were rarely detected in the faeces of MEs, whereas they were abundant in faeces of NMEs [64]. This was further supported by their detection in the colon of two sudden-death victims [65]. However, this mutual exclusion has not consistently been reported. Suarez et al. [66] found that methane and hydrogen sulphide were not negatively correlated when measuring the flatus. Moreover, Doré et al [67] did not observe significant differences in SRB counts between MEs and NMEs. Similarly, Hansen et al. [51] did not find a significant relationship between methanogens and SRB after analysing 87 faecal samples from monozygotic and dizygotic twin pairs. These contradictory findings suggested that the complexity of the interactions between methanogens and SRB in human gut ecosystems remains to be further studied. In contrast, consistent negative correlations were reported for reductive acetogens and methanogens in the human gut [67, 68]. In addition, inhibition of methanogens concomitantly led to higher acetate production in batch cultures inoculated with faecal samples [69]. However, competition for hydrogen between reductive acetogenesis and sulphate reduction was not demonstrated due to the fact that only a slight reduction of reductive acetogenesis was observed when very high sulphate reduction occurred [68]. Overall, it remains largely unknown what mechanisms underly the varying associations between the three hydrogenotrophic functional groups in gut ecosystems. Therefore, insight into the interactions between these three functional groups in the human gut is needed.

The human health impact of hydrogen and the activity of hydrogenotrophic microbiota

Hydrogen and metabolites of hydrogenotrophic microbiota are associated with human health. Hydrogen as the electron sink in gut ecosystems serves as antioxidant [70]. Strong oxidants such as hydroxyl radicals and peroxynitrite react with lipids, proteins or nucleic acids, which is toxic to cells, tissues and organs. Hydrogen selectively reduces hydroxyl radicals and peroxynitrite to exert protection [71, 72]. Therefore hydrogen is considered a potential therapeutic gas. However, hydrogen is not always beneficial. Higher hydrogen concentrations were detected in exhaled breath of individuals suffering from irritable bowel syndrome (IBS) compared to healthy controls, and the accumulated hydrogen is considered to be associated with the IBS symptoms abdominal pain and bloating [30, 73, 74]. As for metabolites of hydrogenotrophic microorganisms, hydrogen sulphide is highly toxic

to colonocytes and impairs their metabolic function [75]. Hydrogen sulphide has been implicated in the development of colorectal cancer (CRC) and ulcerative colitis [76], and higher SRB abundance or hydrogen sulphide concentrations have frequently been reported in ulcerative colitis patients compared to healthy controls [30, 77-79]. Moreover, hydrogen sulphide has been linked to pain-related signals in IBS patients [35, 80]. However, beneficial effects of hydrogen sulphide were also reported, including improved maintenance of mucus layer integrity [81]. Methane has been linked to increased gut transit time [82]. Higher methane production or higher abundance of methanogens have been described in individuals with functional constipation or IBS patients with constipation compared with healthy controls [83-85]. However, these associations have not been consistently observed as some studies reported no association between methane and constipation or slow colonic transit [86].

Collectively, hydrogenotrophic microbiota has been suggested to play an important role in human health, although observations vary between studies. Moreover, hydrogenotrophic microbiota is highly associated with gut microbiota composition and function. However, the underlying mechanisms as to how hydrogenotrophic functional groups determine microbiota composition and function, and the interactions between hydrogenotrophic functional groups, remain largely unknown.

Research aims and thesis outline

Unravelling the factors that determine gut microbiota composition and function is fundamental to understanding how the gut microbiota is organised, and providing leads towards strategies to modulate the gut microbiota. Hydrogenotrophic microbiota plays an essential role in overall microbial fermentation, and thus, the hydrogenotrophic microbiota could be one of the targets to alter the energy metabolism in gut ecosystems, and modulate gut microbiota composition and function. Therefore, the research described in this thesis aims to investigate how hydrogenotrophic microbiota drives overall microbiota composition, and to reveal how hydrogenotrophic functional groups interact with each other *in vitro* and *in vivo*.

This thesis comprises seven chapters. **Chapter 1** introduces the gut microbiota and its subgroup of hydrogenotrophic microbiota, and highlights the aims and motivations for conducting the studies described in this thesis. **Chapter 2** provides an overview of how diet impacts microbiota composition in adults, highlighting the current knowledge on the ecology of the microbiota and summarizing previous observations that focus on the impact of diet on microbiota composition and

metabolite production. **Chapter 3** describes the longitudinal dynamics of faecal microbiota signatures and production of SCFAs in IBS, and their association with IBS symptom severity, highlighting the importance of repeated sampling in IBS research. Since a distorted hydrogen metabolism has been suggested to be associated with IBS, in **Chapter 4**, a sub-group of subjects included in Chapter 3 is used to assess the distribution and stability of the three hydrogenotrophic functional groups in both healthy adults and IBS patients over time. To further investigate the relationships between hydrogenotrophic microorganisms, **Chapter 5** describes an *in vitro* study to investigate the interactions between the hydrogenotrophic methanogen *Methanobrevibacter smithii*, the sulphate-reducer *Desulfovibro piger* and the reductive acetogen *Blautia hydrogenotrophica*. The different organisms are incubated singly, or in binary cocultures and tricultures, providing insights into how these three hydrogenotrophic functional groups affect the growth and metabolite production of each other. **Chapter 6** focuses on the impact of methanogenesis and sulphate reduction on total microbiota composition and metabolite production. Faecal slurries from MEs or NMEs are used as inoculum for *in vitro* batch incubations*.* Methanogenesis is inhibited by adding 2-bromoethanesulfonate (BES) in MEs or stimulated by adding *M. smithii* in NMEs, and sulphate is added in both MEs and NMEs to stimulate sulphate reduction. This chapter highlights the modulation of gut microbiota composition and metabolite production by altering hydrogenotrophic species or activities. **Chapter 7** summarizes and discusses all of the research findings in this thesis and gives recommendations for future studies.

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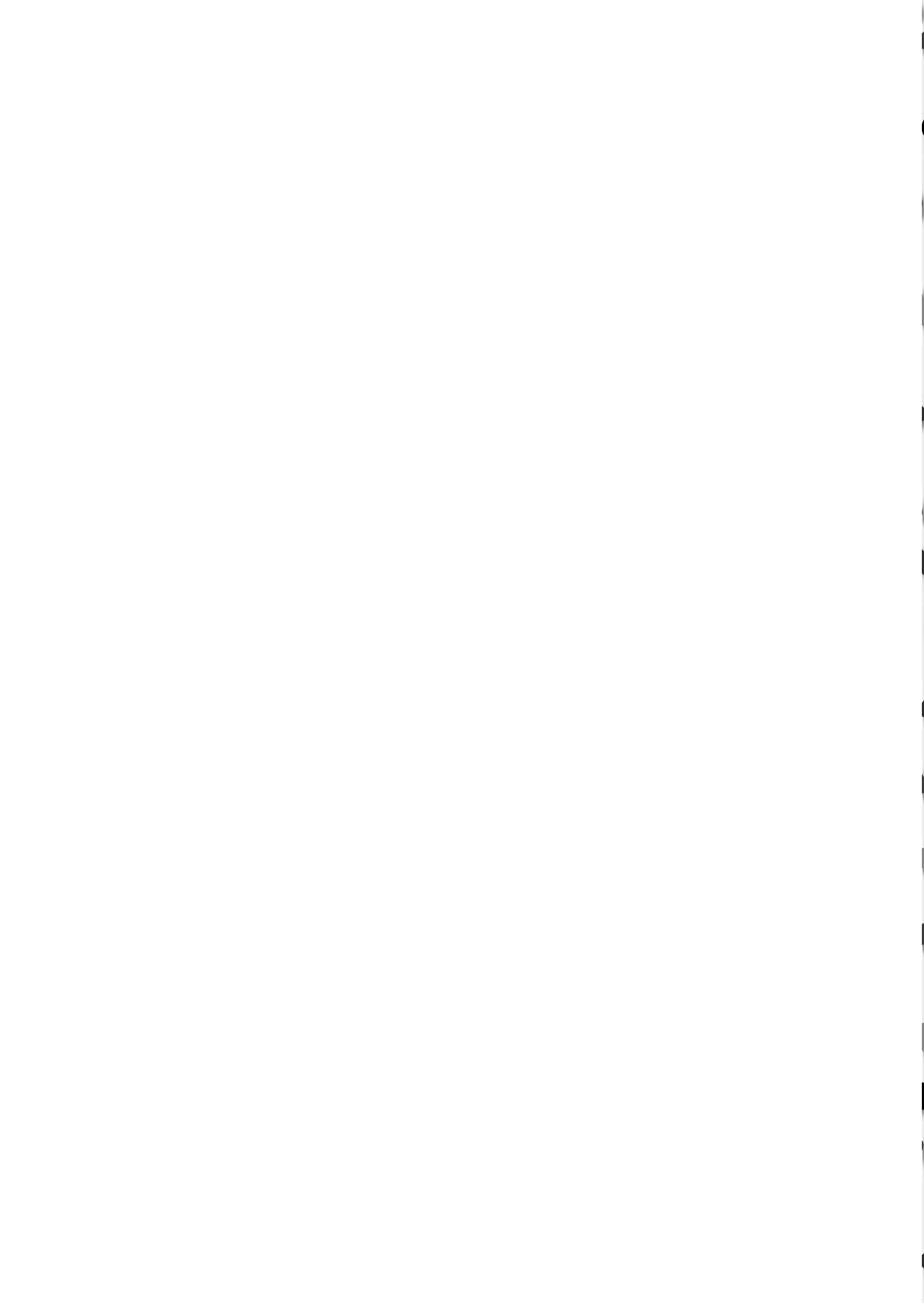
CHAPTER 2

"We are what we eat":

How diet impacts the gut microbiota in adulthood

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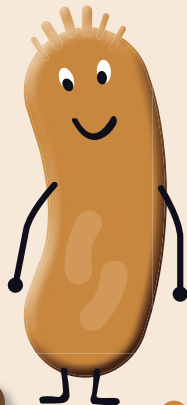
Abstract

The important role of the microbes residing in our gut, collectively called the microbiota, in human health is widely acknowledged. There are numerous factors that have an impact on the microbiota in the gut of which diet is considered a crucial one. In this chapter we highlight our current knowledge on the ecology of the microbiota in adults and how it is affected by diet. We summarize observations from different cross-sectional and intervention studies that focused on the impact of diet on microbiota composition and activity. Special attention is paid to which microbial metabolites can be produced in the gut; how these are affected by different dietary components such as carbohydrates, fat, and proteins; and how these are associated to human health. Finally, we provide recommendations for future intervention studies in order to improve our understanding of the complex interplay between microbes, diet, and ourselves.

Keywords: adult microbiome; Western diet; traditional diet; microbial stability; resilience of the gut microbiome; intestinal microbial metabolites

Introduction

The gut microbiota evolves with age from infant to adult [1, 2]. In adulthood, the gut microbiota reaches its highest diversity, compared with infants and the elderly [3-5]. Furthermore, composition of the gut microbiota is considered stable over time, although a long term study showed that significant alterations may also happen during adulthood [6]. *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* are in general the predominant phyla within the gut microbiota; however, the composition is host-specific, and each individual has a unique gut microbiota composition [7]. In contrast, the functional capacity encoded by the gut metagenome is more similar between subjects [8, 9]. Given the fact that we are all unique as human beings but our bodies function in a highly similar fashion, this is not very surprising.

A number of factors have been associated with the composition of the gut microbiota. These include the genetic background, environment, health status, use of antibiotics, and diet [10-12]. The famous expression "you are what you eat" emphasizes that diet is essential. However, we have become more aware of the significance of the fact that our daily diet not only feeds us but also provides substrates for the gut microbiota. Long-term diet is the dietary habit kept for several years or decades with relatively stable dietary components, and it has been shown to contribute to shaping gut microbial composition [13]. By contrast, short-term dietary changes have in general a minor impact on microbial composition but may change overall activity patterns [14].

Typical long-term dietary patterns, such as consumption of Western or traditional diets, have been associated with specific gut microbial profiles. For example, Western diets are rich in protein and fat, a diet that is markedly different from African diets, which are traditionally rich in dietary fibres, and these differences are reflected in the microbiota composition [1]. It was shown that even short-term consumption of a Western diet resulted in a microbiota-mediated increase in colorectal cancer (CRC) associated risk factors, while the opposite was observed with consumption of a traditional South African diet [15]. Other examples of the importance of diet with respect to human health are the association between irritable bowel syndrome (IBS) symptoms and fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAP) diets [16] as well as the associations of, among others, obesity, inflammatory bowel diseases (IBD), malnutrition, and type 2 diabetes with the gut microbiota and diets [17-20].

Overall, diet is one of the most important factors associated with human health and diseases, which can exert its effects either directly or indirectly via the gut microbiota. In this chapter, we provide an overview of our insight into the adult gut

microbiota and its association with diet.

The adult gut microbiota

The gut microbiota is individual and niche-specific

Human beings are recognised as "superorganisms" composed both of human and microbial cells. It is estimated that the total number of bacterial cells (approximately 3.9×10^{13}) is similar to that of human cells [21]. Collectively, the gut microbiota comprises more than 1,000 microbial species, harbouring approximately 10 million non-redundant genes, which is 2-3 orders of magnitude larger than the human gene complement [8, 22].

As indicated before, the gut microbiota in the healthy adult is dominated by *Firmicutes* and *Bacteroidetes* as well as *Actinobacteria* at the phylum level, with lower proportions of other phyla such as *Proteobacteria* and *Verrucomicrobia* [23, 24]. However, the microbial composition varies along the intestinal tract due to variation in environmental conditions [7]. The small intestine consisting of duodenum, jejunum and ileum is considered a harsh environment for the gut microbiota due to the relatively high oxygen level, presence of bile acids and digestive enzymes, and short transit time. As a result, microbial numbers are only a fraction of the total gut microbiota with 10^3-10^4 per millilitre in duodenum and jejunum, increasing to 10^8 in the ileum [25, 26]. Since it is difficult to access the small intestine for sampling, studies on its residing microbiota are limited. It has been shown that the microbial composition in the small intestine is different from that in the colon, and even different when comparing proximal and distal parts of the small intestine [27, 28]. Microbiota profiling of ileal effluent samples of ileostomists as well as samples taken at proximal sites in the small intestine demonstrated that among others, members of the genera *Streptococcus* and *Veillonella* are predominant [25, 28]. In contrast, samples from the distal small intestine resemble those of the colon, which could be due to colonic reflux [28].

The colon is considered the main fermentation vessel of the human body and is the most densely populated organ with 10^{11} cells per millilitre and approximately 400 millilitres in total. The high density and biomass levels are attributed to the extended transit time and more suitable fermentation conditions in comparison to the small intestine [7, 26]. Most studies in the past decades used faecal samples to characterise the gut microbiota in the colon due to their accessibility. Although a variety of studies have demonstrated that the gut microbiota is individual-specific,

population-level studies observed that the gut microbiota is characterised by certain conserved patterns at a higher organisational level, termed enterotypes [29]. It is evident that such stratification of the microbiota may improve our understanding of gut microbial ecology [30]; however, there is an ongoing debate regarding the identification and stability of the enterotypes. Meta-analyses of microbial composition data indicated that *Faecalibacterium prausnitzii*, *Oscillospira guillermondii* and *Ruminococcus obeum* are the top three taxa shared by adults [31]. Of note is that the faecal microbiota only resembles that of the distal colon, and that differences have been observed between lumen and mucosa-associated microbiota [32, 33]. A study characterising the microbiota associated with human rectal biopsies and mucosal swab samples indicated a higher proportion of *Proteobacteria* and *Actinobacteria* compared to faecal samples. Taxa belonging to these phyla are often described to primarily metabolise peptones and amino acids in the mucus layer, reflecting the selective pressure and adaption to substrates most readily available in this niche [34, 35]. Furthermore, *Akkermansia muciniphila,* an abundant mucin degrader, is thought to act as the gatekeeper of the mucosa maintaining the stability of our gut microbial ecosystem [36-38]. Overall, it is evident that the differences in microbiota composition between subjects and intestinal locations make it challenging to comprehensively study the role of the microbiota and how it is impacted by diet.

Microbial diversity, stability and resilience

The adult gut microbiota remains relatively stable over time, suggesting that it is resilient to environmental perturbations. One hypothesis is that the microbes residing in microhabitats like the colonic crypts, the appendix or the mucus layer serve as reservoirs of microbial diversity and can replenish the gut microbiota after perturbations [39]. Moreover, a deep phylogenetic analysis of gut microbial data in Western adults demonstrated that a limited number of gut microbial taxa show bimodal distributions, being either highly abundant or nearly absent in most individuals. Such bistability may also play a role in maintaining the homeostasis of the gut ecosystem [40].

Despite the resilience of the gut ecosystem, perturbations exceeding its capacity, like the use of antibiotics and extreme dietary changes can disrupt gut microbial composition and function, converting the healthy stable state of gut microbiota into a degraded stable state [41]. Accumulating evidence demonstrated that the human gut microbiota composition differs between healthy subjects and those suffering from disorders like obesity, IBD and CRC [18, 42, 43]. It has been postulated that these disorders may be induced by the consumption of a Western diet via diet-microbiota interactions [7]. However, whether a different microbiota composition is

the cause or the result of disorders and diseases remains unknown in most cases. On the other hand, it was pointed out that compromised individuals have generally a lower gut microbial diversity compared to healthy individuals [7, 41, 44]. Therefore, homeostasis of healthy individuals is hypothesized to be associated with a highly diverse and resilient microbiota [7]. In line with this, obese individuals with a low microbial richness are at increased risk for developing insulin resistance, dyslipidaemia and a more pronounced inflammatory phenotype in comparison with those with higher microbial richness [17, 45].

The transference of microbes from healthy donors to patients (faecal microbiota transplantation, FMT) has been tested as a therapy for a variety of health disorders. The most successful application of FMT has been without doubt in patients suffering from recurrent *Clostridioides difficile* infections [46]. FMT has also been used in individuals suffering from ulcerative colitis (UC) and IBD. Studies have shown that the treatment can relieve symptoms or eradicate disease after one or more transplants [47, 48], indicating that some microbiomes contain health promoting aspects and can pass on their beneficial effects to others. This may be explained by the fact that a more diverse and more resilient microbiota can help compromised individuals to rebuild a stable personalised microbiota restoring intestinal homeostasis. This hypothesis is supported by the recent discovery that donor microbiota richness determines FMT success in IBD [48]. However, not all individuals respond to the FMT. For example studies in UC patients found that FMT induces remission in some individuals, but not all [47, 49]. Moreover, one donor faeces does not suit all individuals. Analysis of the microbiota of responders and non-responders may help elucidating the mechanisms underlying FMT success [50].

Microbial metabolites

Dietary components escaping the digestion and absorption in the small intestine include mostly complex carbohydrates, such as dietary fibre, but also certain proteins and peptides. These are subsequently fermented by the gut microbiota, generating specific metabolites (as shown in Figure 1) including short-chain fatty acids (SCFAs) and gasses such as hydrogen and carbon dioxide [51-53]. Besides diet, the mucin secreted by goblet cells provides a substrate for gut microbes [36, 54].

SCFAs, notably acetate, propionate and butyrate, produced in the intestine can reach as far as the lungs through circulation, and cross the blood-brain barrier. The combined concentration of the three SCFAs acetate, propionate and butyrate is approximately 50-150 mM with a typical ratio of 3:1:1 in the colon [52]. Butyrate is

the main energy source for the epithelial cells and is locally consumed resulting in a lower concentration in the systemic circulation. Propionate that is absorbed from the lumen is transported via the portal vein and subsequently metabolised by the liver. Acetate remains in relatively high concentrations in peripheral blood circulation [52, 55]. Butyrate is formed during fermentation using acetyl-CoA as the starting point via the phosphotransbutyrylase/butyrate kinase or butyryl-CoA:acetate CoA-transferase route, which allows butyrate producers to utilise sugars, lactate and acetate as well as amino acids as substrates [56, 57]. A wide variety of microbes are able to produce butyrate, including e.g. *Faecalibacterium prausnitzii*, *Anaerobutyricum hallii* (recently renamed from *Eubacterium hallii*), *Roseburia intestinalis*, and *Intestinimonas* strain AF211 [57, 58]. Propionate is generated predominantly via the succinate pathway in the colon [52], but the acrylate and propanediol pathways have also been described for gut microbes [57]. Like butyrate, propionate can also be produced by a number of bacterial taxa, which include *Bacteroides* spp. and *Veillonella* spp., among others [52]. As for acetate, it can be produced via acetyl-CoA or via the Wood-Ljungdahl pathway [57], in which acetate is produced via reductive acetogenesis. Acetate can be produced by almost all intestinal microbes. When fermentable carbohydrates are limited, which is typical for Western diets, the gut microbiota switches to utilise less favourable sources such as amino acids. This may result in increased concentrations of branched-chain fatty acids (BCFAs), amines, ammonia, and phenolic compounds, which are considered detrimental [52, 57].

Besides organic acids, fermentation also leads to production of gasses. Hydrogen is one of the main gasses that is produced during microbial fermentation in the gut. The three most common pathways for hydrogen production include the reoxidation of reduced pyridine and flavin nucleotides, the metabolism of formate generated by the cleavage of pyruvate, and the activity of pyruvate:ferredoxin oxidoreductase and hydrogenase [59]. Hydrogen producers include, among others, strains of *Ruminococcus* spp., *Roseburia* spp., *Clostridium* spp., and *Bacteroides* spp. [59]. Hydrogen accumulation by hydrogenogenic microbes increases the partial pressure in the gut, limiting further microbial fermentation thermodynamically. In turn, hydrogen can be removed from the intestine via flatus and can also be transferred into the blood with subsequent excretion via the lungs. Microbial disposal of hydrogen in the gut by hydrogenotrophic microbes has also been described and includes reductive acetogenesis, methanogenesis and sulphate reduction, which are performed by archaea or bacteria that use hydrogen as electron donor for anaerobic respiration [59-61]. Reductive acetogenic bacteria like *Blautia hydrogenotrophica* and *Marvinbryantia formatexigens* can utilise hydrogen and carbon dioxide or formate which results in the generation of acetate [62]. It is estimated that almost one third of acetate in the gut ecosystem is derived from this

activity [63]. The most common methanogenic archaeal species isolated from the human colon is *Methanobrevibacter smithii* that converts hydrogen and carbon dioxide to methane. It is estimated that one third of the Western population carries approximately10^9 CFU/g of methanogens in stool, while individuals who are non-methane producers harbour less than 10^4 CFU/g [64, 65]. Remarkably, in Africans approximately 80% of the population harbours high numbers of intestinal methanogens [66]. Whether this is related to the differences in diets remains speculative. Sulphidogenic bacteria can generate the toxic component hydrogen sulphide from inorganic and organic sulphur containing compounds. Many use sulphate and/or sulphite and hydrogen as the electron acceptor and donor, respectively, although it should be noted that sulphide can also be produced through fermentation of sulphur-containing organic compounds [67]. Hydrogen sulphide was reported to be associated with IBS, IBD and CRC [59]. Sources of intestinal sulphur include host-derived mucin and taurine as well as dietary sulphur-containing components including amino acids.

A vast number of metabolites from dietary sources are produced by the gut microbiota. Some of them are beneficial, but some detrimental [52, 53]. They play a critical role in the connection between the gut microbiota and human health and diseases. Therefore, clarifying how metabolites affect human health and diseases and how diets are associated with metabolite production can be very meaningful but challenging in the future.

The impact of diet on the gut microbiota

Dietary modulation of the gut microbiota

Diet is considered a crucial determinant of gut microbial composition, and a variety of factors may explain why diet acts as the ecological driving force, which include being the energy source of the microbes, affecting the pH of the lumen, regulating gut transit rate and stimulating the secretion of bile acids and other components (Figure 1). First, dietary components are used as energy source, conferring selective growth advantages to specific microbial taxa. Typical Western diets with high fat and protein content are markedly different from non-Western diets, such as traditional African diets which are rich in complex carbohydrates. Hence, the finding that Prevotella is abundantly detected in Africans with non-Western diet, suggests that this taxon plays a significant role in extracting energy from undigested plant-derived carbohydrates [68-70]. Additionally, the conversion of dietary components by the gut microbiota can lead to changes in pH, caused by metabolites such as

SCFAs that can decrease pH in the intestinal lumen. It has been observed that pH values vary from 5.5 in the caecum to 6.5 in the descending colon [55]. An in vitro study using continuous flow fermenters indicated that the Bacteroidetes outcompeted Firmicutes and dominated in the system at pH 6.5, but could not persist at pH 5.5, demonstrating the critical role of pH in shaping gut microbial composition [71]. Another factor that can be a link between diet and microbiota is intestinal transit time (ITT). ITT has an impact on microbial competition since a faster transit favours the survival of fast growing organisms over slow growing ones. A murine model study showed that ITT was accelerated by high dietary fibre intake [72], and the explanation for a faster ITT is that dietary fibre, and especially incompletely fermented fibre, attracts water, increasing digesta mass, which shortens the transit time [73]. Last but not least, secretion of digestive enzymes and other components like bile acids into the intestinal lumen are stimulated by diet intake and is a selective force affecting residing microbes. Bile acids in the colon can act as antibacterial components selecting resistant microorganisms, indirectly making diet a selective force on gut microbial composition and function [74, 75]). Overall, studying the impact of diet on our microbiota can be very complex due to a myriad of actions.
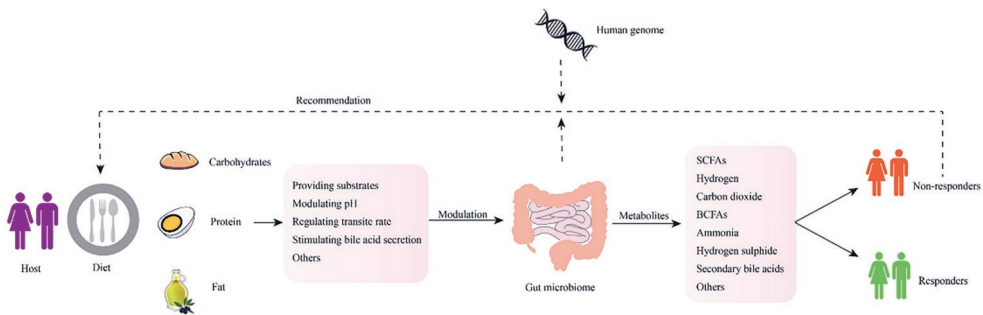
Figure 1: Targets for improving the modulation of gut microbiota as a basis for individualised dietary recommendations. Diet modulates gut microbiota via many factors. Gut microbes convert dietary components into a broad range of small molecule metabolites. However, variable responsiveness to a given diet is seen in different individuals who can be stratified into responders and non-responders. Accordingly, precision diet based on information related to individualised gut microbiome and human genome data is recommended with the ambition to change non-responders into responders.

A range of dietary components have been investigated for their impact on human health through gut microbial functionality (Table 1). The studies can be roughly divided into two classes. The first class examined the effects of long-term dietary patterns on gut microbiota by cross-sectional comparative analysis of individuals

that consistently consumed a specific diet such as Western diet, traditional diet, vegetarian or vegan diets. The second class comprises short-term intervention studies that investigated the impact of diet on gut microbiota by switching from one dietary pattern to another for a certain period of time. These studies allowed to determine whether a specific diet or increased intake of certain macronutrients can lead to variations in gut microbiota composition and/or activity.

Several human short-term intervention studies have indicated that the microbiota can quickly adapt to a change in dietary pattern, which may not only lead to drastic microbiota activity changes, but also to compositional changes [13-15], while the gut microbiota reverted to its original structure when the dietary intervention stopped [14]. Long-term dietary patterns have been linked to distinct gut microbial communities [13]. Major differences in microbiota composition, notably within the *Bacteroidetes* phylum (*Prevotella* in native Africans and *Bacteroides* in African Americans) have been observed when comparing the gut microbiota of Africans consuming a native diet to that of African Americans consuming a Western diet [69]. A recent study showed that, as a consequence of modernisation, a shift occurred from ancient microbial communities with a higher capacity to degrade complex carbohydrates, to microbial taxa that are more suited to metabolise protein and fat [68]. In line with this observation, a mouse model study indicated that long-term adaptation to a Western diet over generations may result in progressive loss of gut microbial species and diversity which cannot be fully recovered by the reintroduction of traditional diets [76]. This is a worrying scenario as Westernisation of our diet, associated with increased risk of gastrointestinal diseases, is increasing world-wide.

Responsiveness to dietary interventions appears to be host dependent, although a number of studies have identified dietary components that may have a universal effect on the gut microbiota. A study with obese men indicated that responsiveness of the gut microbiota to dietary components, and the generated fermentation products, differed drastically between subjects, suggesting that individuals could be stratified into responders and non-responders based on their gut microbial dynamics [77]. Similarly, a dietary intervention study using a 3-day window of consumption of barley kernel-based bread showed that certain individuals gained improvement in glucose metabolism with a corresponding increase of *Prevotella copri* abundance while others did not [78]. Comparative analyses between responders and non-responders are essential for the identification of signature microbes that could predict physiological changes in the host [79]. A recent study validated a comprehensive computational platform, the "community and system-level interactive optimization" (CASINO) toolbox, which was capable of predicting faecal and blood metabolomics data in a dietary interventional study with 45 obese

and overweight individuals, thereby providing a powerful tool to determine diet-induced metabolic changes of the gut microbiome [80]. Similarly, a different study successfully predicted personalised postprandial glycaemic responses to real-life meals by integrating microbial and host datasets using machine-learning algorithms [81]. Predictive studies and algorithms may lead to the design of personalised dietary interventions, which could prospectively convert non-responders into responders using human genome and gut microbiome information (Figure 1) [82].

Gut microbial interactions with dietary macronutrients

The main macronutrients in diet are carbohydrates, fat and protein. As described earlier, after escaping digestion and absorption by the human digestive system, nutrients can be utilized by the gut microbiota, resulting in the generation of a range of metabolites via microbial fermentation and cross-feeding. Composition of the macronutrients thus exerts a considerable influence on gut microbiota composition, activity, and human physiology (Figure 1).

(a) Carbohydrates: Microbiota-accessible carbohydrates are fermented by the resident microbes resulting in the production of SCFAs, notably acetate, propionate and butyrate, and gasses such as hydrogen and carbon dioxide. Manipulating the gut microbial composition and activity by specific dietary carbohydrates, notably non-digestible fibre, is increasingly accepted as a promising approach to benefit human health. A study by Tap and colleagues [83] using 19 healthy volunteers indicated that increased dietary fibre restored richness and stability of the gut microbiota in adults. Moreover, a double-blind, placebo-controlled cross-over study in individuals given a 3-week oral administration of the prebiotic inulin showed that inulin increased the numbers of bifidobacteria and lactobacilli in faecal samples [84]. Not only do the dietary fibres alter gut microbiota composition, but also contribute to human physiology. For example, a 4-week randomized cross-over trial in healthy individuals found that intake of whole grains increased gut microbial diversity, and that this was also associated with the reduction of the postprandial glucose peak and immunological improvements [85]. However, a dietary component-induced change of the microbiota not always results in an altered physiological response. For example, a recent intervention with galacto-oligosaccharides in prediabetic individuals resulted in increased relative abundance of bifidobacteria but did not improve insulin sensitivity or host energy metabolism [86]. It has to be realized that carbohydrate fermentation does not always lead to improvements in the host. For example, FODMAPs, which are usually digested and absorbed in the small intestine, may cause a quick increase of glycaemic concentrations. When FODMAPs reach the colon, they can be rapidly

fermented, leading to a mass production of hydrogen, an associated factor underlying susceptibility in irritable bowel syndrome (IBS) patients [16]. Hence, restriction of FODMAP diets may reduce gastrointestinal symptoms in IBS patients [16, 87].

As indicated above, microbial fermentation of carbohydrates results mainly in the production of SCFAs. However, there is no general consensus on the link between diets and SCFAs since many conflicting results have been published. A positive correlation was observed between diets rich in fibre and high levels of SCFAs [88]. In contrast, another study showed unexpected reductions in faecal SCFA concentrations in vegans compared with omnivores [89], and a trial in which a whole grain-based diet was compared to a red meat-based diet did not show differences in SCFA levels [90]. Reasons for observed variations between studies could be that a minimum intake of dietary fibre is needed to induce measurable alterations in SCFA production, and that SCFA production occurs but is not measurable in faecal samples due to intestinal absorption and subsequent microbial interactions. Other reasons could include the type of carbohydrate used in an intervention, the intestinal location where a certain carbohydrate fermentation takes place as well as the metabolic state of the individual. Overall, these intervention studies confirmed the complex interactions between carbohydrates and gut microbiota, and thus more mechanistic studies are needed to further elucidate how carbohydrates affect gut microbial composition and metabolites, and subsequently human physiology.

(b) Fat: Dietary fat is believed to be degraded and absorbed in the small intestine. Thus, the colonic microbiota is not expected to have significant interactions with this macronutrient directly. Primary bile acids, produced from host cholesterol in the liver and then conjugated with taurine and glycine, are secreted into the small intestine to solubilise lipids, facilitating the digestion and absorption of fat. Secretion of bile acids is stimulated by consumption of high-fat diets, and excessive amounts are metabolised by bile salt hydrolases (BSH) from intestinal microorganisms into secondary bile acids, such as deoxycholic acid and lithocholic acid [91, 92]. The accumulation of secondary bile acids, which are biologically active, may contribute to the development of gallstones, CRC, and other diseases [92-94]. Bile acids can also regulate gut microbial composition due to their antibacterial properties. Research in rats indicated that *Firmicutes* and *Proteobacteria* are generally more resistant to bile salts compared to *Bacteroidetes* [95]. Although these observations have not been consistently reported [96, 97], differences in bacterial resistance to bile acids may in part explain why obese individuals harbour more *Firmicutes* but less *Bacteroidetes* [17].

Many studies have described the effects of high fat diets on host metabolism and the gut microbiota. However, human studies do not show a similar consistency. A 2-week dietary exchange study in which rural Africans were fed a high-fat diet and African Americans a low-fat diet demonstrated that the switch from a high-fat diet to a high-complex carbohydrate diet increased butyrate production and suppressed the synthesis of secondary bile acids [15]. In addition, an intervention study in subjects at increased risk for development of metabolic syndrome indicated that a diet high in mono-unsaturated fat did not affect gut microbial composition, but rather reduced the number of total bacteria, whereas a diet high in saturated fat increased the abundance of *Faecalibacterium prausnitzii* and SCFA concentrations [98]. Therefore, attention should be paid to both the quantity and quality of the dietary fat consumed and how this affects microbiota composition and activity.

(c) Protein: Protein may also escape digestion and absorption in the small intestine, reaching the colon where it is exposed to the colonic microbiota. When carbohydrates become limited substrates for the gut microbiota, fermentation of proteins occurs in the distal colon. Protein fermentation may result in the production of metabolites consisting of sulphur compounds, N-Nitroso compounds, ammonia, heterocyclic amines and organic acids, which are potentially toxic and detrimental to human health [99-101].

Both, short-term and long-term dietary intervention studies in humans indicated that *Bacteroides* is strongly associated with an animal-based diet high in fat and proteins [13, 14]. *In vitro* fermentation studies revealed that the predominant proteolytic bacteria belong to the genera *Bacteroides* and *Propionibacterium* as well as species from to the genera *Streptococcus*, *Clostridium*, *Bacillus* and *Staphylococcus*, as determined by culturing [102]. More recent culture-independent studies using the TIM-2 dynamic colon model indicated that a high-protein diet enriches the genus *Bacteroides* with a concomitant increase in BCFAs, such as *iso*-butyrate and *iso*-valerate [103]. In line with these observations, a 4-week dietary intervention study in obese individuals indicated that a diet high in proteins reduced the abundance of members of the *Roseburia/Eubacterium rectale* group with a significant decrease in beneficial metabolites like butyrate but concomitant increase in the concentration of metabolites such as BCFAs in comparison with the maintenance diet [104]. Due to the production of potentially detrimental protein-derived metabolites, it has been assumed that the intake of high protein is associated with several human diseases including CRC, IBD and cardiovascular diseases [15, 88, 105]. However, we still lack the evidence explaining the relationship between the protein-derived metabolites and human diseases. Therefore, more studies should be carried out to discover the underlying causality.

Explaining the impact of a dietary change mechanistically is challenging. In intervention studies, diets are often calorically matched and as a result, the increase of one component is compensated by the decrease in other. For example, typical Western diets contain lower amounts of fibre and therefore concomitantly the amounts of fat and protein are higher. As a consequence, it remains speculative if the observations in comparative analyses between diets are a direct effect of the carbohydrate, fat or protein content, or a combination of these. Moreover, it could also be that the effect of diet is indirect via bile or other host secretions as a result of the dietary intake. It is evident that there is an urgent need of dedicated studies to unravel the mechanisms underlying the observations in dietary intervention studies.

Table 1: Overview of diet intervention studies in individuals.

Diet	Cohort	Time	Analysis method	Gut microbial response	References
Inulin/Oligofructose (50%/50%, 16 g/d)	30 obese women	3 months	Human Intestinal Tract Chip (HITChip); qPCR	*Bifidobacterium* ↑, *Faecalibacterium prausnitzii* ↑, *Bacteroides intestinalis* ↓, *Bacteroides vulgatus* ↓, *Propionibacterium* ↓	[106]
Inulin (12 g/d)	44 healthy subjects	8 weeks	Sequencing (16S rRNA gene)	*Bilophila* ↓	[107]
Very long-chain inulin (10 g/d)	32 healthy adults	6 weeks	Fluorescent *in situ* hybridisation (FISH)	bifidobacteria ↑, lactobacilli ↑, *Atopobium* ↑, *Bacteroides* ↓, *Prevotella* ↓	[84]
Galacto-oligosaccharides (GOS) supplementation (15 g/d)	44 obese prediabetic individuals	12 weeks	HITChip	*Bifidobacterium* ↑	[86]
Xylooligosaccharides- (XOS-) enriched rice porridge	20 healthy subjects	6 weeks	Medium selection	*Lactobacillus* spp. ↑, *Bifidobacterium* spp. ↑, *Clostridium perfringens* ↓	[108]
Fructooligosaccharides (FOS); Galactooligosaccharides (GOS) (16 g/day)	35 adults	14 days	318 V2 chip	FOS: *Bifidobacterium* ↑, *Phascolarctobacterium* ↓ GOS: *Bifidobacterium* ↑, *Ruminococcus* ↓	[109]
Partially hydrolysed guar gum (PHGG) and fructooligosaccharides (FOS) in biscuits	31 healthy subjects	6 weeks	FISH	bifidobacteria ↑	[110]
Arabinoxylan and resistant starch type 2	19 adults	8 weeks	Sequencing (16S rRNA gene)	Bacterial diversity ↓, *Bifidobacterium* ↑	[111]

2

Diet	Subjects	Duration	Method	Results	Ref
Resistant starch (RS) or non-starch polysaccharides (NSPs) supplementation; weight-loss (WL) diet	14 obese males	3 weeks	HITChip; qPCR	RS diet: *Ruminococcaceae* ↑ NSP diet: *Lachnospiraceae* ↑ WL diet: bifidobacteria ↓	[77]
10 g or 40 g dietary fibre per day	19 healthy adults	6 weeks	Pyrosequencing (16S rRNA gene); qPCR	High dietary fibre: microbiota richness ↑, *Prevotella* ↑, *Coprococcus* ↑	[83]
Barley kernel-based bread (BKB)	39 subjects	3 days	Metagenomics; qPCR	BKB: *Prevotella/Bacteroides* ↑, *Prevotella copri* ↑	[78]
High resistant starch (RS) diet; Weight loss (WL) diet	14 overweight men	10 weeks	Sequencing (16S rRNA gene); qPCR; Denaturing Gradient Gel Electrophoresis (DGGE; 16S rRNA gene)	RS diet: *Ruminococcus bromii* ↑, Uncultured *Oscillibacter* group ↑, *Eubacterium rectale* ↑ WL diets: Uncultured *Oscillibacter* group ↑, *Eubacterium rectale* ↓, *Collinsella aerofaciens* ↓	[112]
Vegetable/fruit juices	20 healthy adults	3 days	Sequencing (16S rRNA gene)	*Cyanobacteria* ↑, *Firmicutes* ↓, *Proteobacteria* ↓, *Bacteroidetes* ↑	[113]
High-vegetable/low-protein diet (HV/LP)	20 healthy adults	12 months	Sequencing (16S rRNA gene)	HV/LP: *Lachnospiraceae* ↑	[114]
Diet rich in whole grain (WG) Diet rich in red meat (RM)	20 healthy adults	10 weeks	DGGE (16S rRNA gene)	WG: *Collinsella aerofaciens* ↑ RM: *Clostridium* spp. ↑	[90]
Whole-grain wheat (WGW); whole-grain rye (WGR); refined wheat (RW)	70 healthy adults	6 weeks	Sequencing (16S rRNA gene)	No effects	[115]

Diet	Subjects	Duration	Method	Results	Ref
Whole-grain foods; fibre-rich rye breads; refined wheat breads	51 Finnish individuals with metabolic syndrome	12 weeks	HITChip; qPCR	Refined wheat breads: *Bacteroides vulgatus* ↓, *B. plebeius* ↓, *Prevotella tannerae* ↓, *Collinsella* ↑, *Clostridium* clusters IV and XI ↑	[116]
Whole grains, traditional Chinese medicinal foods, and prebiotics (WTP diet)	93 obese volunteers	9 weeks	Pyrosequencing (16S rRNA gene)	*Bifidobacteriaceae* ↑, *Enterobacteriaceae* ↓, *Desulfovibrionaceae* ↓,	[117]
Whole-grain barley (WGB); Brown rice (BR); BR+WGB (60 g/day)	28 healthy humans	4 weeks	Pyrosequencing (16S rRNA gene)	All treatments: microbial diversity ↑, *Firmicutes/Bacteroidetes* ratio ↑, *Blautia* spp. ↑; WGB: *Roseburia* spp. ↑, *Bifidobacterium* spp. ↑, *Dialister* spp. ↑	[85]
Whole grain diet (WG); Refined grain diet (RG)	60 Danish adults	8 weeks	Shotgun metagenomics; Sequencing (16S rRNA gene)	No major changes of the gut microbiome WG: *Faecalibacterium prausnitzii* ↑, *Prevotella copri* ↑, and *Bacteroides thetaiotaomicron* ↓	[118]
Whole-grain (WG) wheat; wheat bran	31 individuals	3 weeks	FISH	WG: bifidobacteria ↑, lactobacilli ↑	[119]
WG diet; Refined grain (RG) diet	17 healthy subjects	2 weeks	qPCR	WG: *Clostridium leptum* ↑	[120]
FODMAPs (low 3.5 g/d vs high 23.7 g/d)	27 IBS patients; 6 healthy subjects	21 days	qPCR; DGGE	Low FODMAPs: microbial diversity ↑, total bacterial abundance ↓ High FODMAPs: *Ruminococcus torques* ↑, *Clostridium* cluster XIVa ↑, *Akkermansia muciniphila* ↑	[121]
FODMAPs-restricted diet	41 IBS patients	4 weeks	FISH	bifidobacteria ↓	[87]

Diet	Subjects	Duration	Method	Results	Ref
African style diet (high fibre); Western-style diet (high fat)	20 healthy African Americans; 20 rural Africans	2 weeks	HITChip	Western style diet: *Eubacterium rectale* ↓, *Clostridium symbiosum* et rel ↓, *Oscillospira guillermondii*↓	[15]
High-fat/low-fibre diet; Low-fat/high-fibre diet	98 healthy individuals	10 days	Sequencing (16S rRNA gene)	High-fat: *Bacteroidetes* ↑, *Actinobacteria*↑ Low-fat: *Bacteroidetes* ↓, *Actinobacteria* ↓	[13]
High saturated fat diet (HS); high monounsaturated fat diet (HM)	88 subjects at increased metabolic syndrome risk	28 weeks	FISH	HS: *Faecalibacterium prausnitzii* ↑ HM: total bacteria ↓	[98]
High-fat diet with additional whipping cream	24 healthy men	7 days	Sequencing (16S rRNA gene)	*Bacteroidaceae* ↓, *Betaproteobacteria* ↑	[122]
High-protein/ moderate-carbohydrate diet (HPMC); High-protein / low-carbohydrate diet (HPLC)	17 obese men	8 weeks	FISH	HPMC and HPLC: bacterial numbers ↓ HPLC: *Roseburia/Eubacterium rectale* ↓, *Bacteroides* spp. ↓	[104]
High protein (HP) diet; low protein (LP) diet	20 healthy subjects	4 weeks	DGGE	No effects	[100]
Gluten-free diet (GFD)	10 healthy subjects	1 month	FISH; qPCR	*Clostridium lituseburense* ↓, *Faecalibacterium prausnitzii* ↓, *Bifidobacterium* ↓, *Lactobacillus* ↓, *Bifidobacterium longum* ↓, *Enterobacteriaceae* ↑, *Escherichia coli* ↑	[123]
GFD	21 healthy subjects	4 weeks	Pyrosequencing (16S rRNA gene)	*Veillonellaceae* ↓	[124]

↑: increase of the microbial (relative) abundance; ↓: decrease of the microbial (relative) abundance

Conclusions

Cross-sectional and interventional studies have investigated differences of long-term and short-term dietary changes. Comparative analyses indicated that long-term consumption of traditional versus Western diets are associated with major differences in microbiota composition. On the other hand, short-term dietary interventions, even those that included major dietary changes, had a limited impact on composition but could have significant impact on metabolite production. It is evident that diet is a crucial determinant in the ecology of the gut microbiota, with concomitant effects on human metabolism and physiology. Nevertheless, our knowledge of how diets affect the microbiota in adulthood and what impact this has on human physiology is still in its infancy, mainly because the mechanisms underlying observations involve many factors often dependent on each other. In addition, comparative analyses between studies are hampered by the facts that the setup, the choice of approaches to study the gut microbiota, background of the study participants, duration of the study, and choice of diet components and their amounts differ between studies. Moreover, due to the individuality of microbiota composition, responses towards the same intervention can be drastically different between subjects. This may explain why contrasting observations with similar diets or dietary components have been reported.
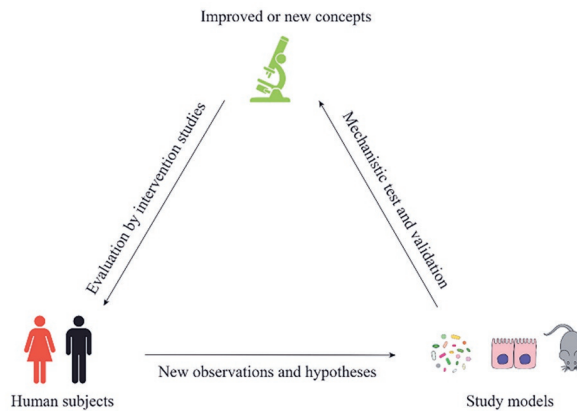
Figure 2. Strategies for investigating the mechanisms of diet-microbiota interactions. Performed prospective studies that observe altered gut microbiota composition and/or activity after the dietary interventions in individuals can be used to generate new observations and hypotheses. Subsequently, relevant experimental models, such as microbial consortia including (defined) synthetic communities, cell lines, intestinal organoids and animal models can be used to test and validate the underlying mechanisms to improve or generate new concepts. These can be further evaluated by using precision dietary interventions.

In order to improve our understanding of how diet impacts gut microbiota and human physiology (Figure 2), prospective studies should be performed to determine whether the gut microbiota is altered after the dietary interventions, which can help to form new hypotheses based on new observations. Then, the generated hypotheses should be tested and validated using microbial consortia including defined (synthetic) communities, cell lines and intestinal organoids, animal models or other effective ways to improve or form new concepts of diet-microbiota interactions [125, 126]. After that, individualised precision interventional studies can be carried out to further evaluate the new concepts. Such microbial research triangle is essential to elucidate whether the gut microbiota is causally linked to host metabolism in humans (Figure 2). Besides this, different approaches with the ambition to predict diet-induced metabolic changes of the human gut microbiome [80] or the personalised postprandial glycaemic response to real-life meals have been reported [81]. These are very promising approaches to move from observations towards subject-specific predictions on the effect of diets on the gut microbiota and human physiology and enable microbiota-focused precision nutrition in the future.

Acknowledgements

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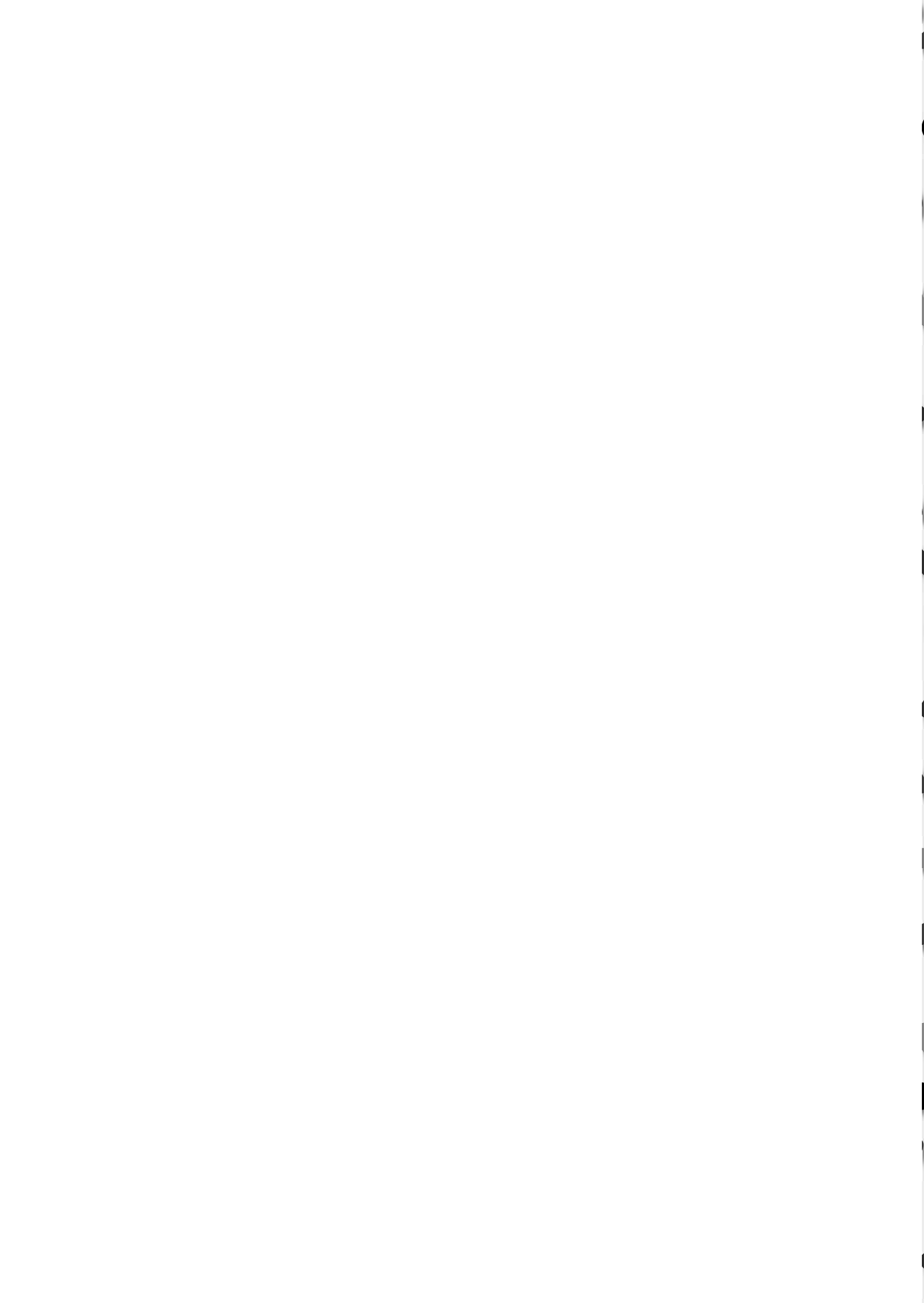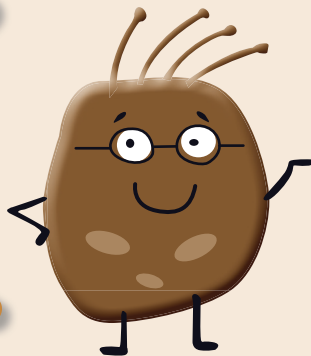
CHAPTER 3

Faecal microbiota signatures are not consistently related to symptom severity in irritable bowel syndrome

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Abstract

Irritable Bowel Syndrome (IBS) is the most prevalent functional bowel disorder, but its pathophysiology is still unknown. Although a microbial signature associated with IBS severity has been suggested, its association with IBS severity still remains largely unknown. This study aimed to assess longitudinal dynamics of faecal microbiota and short chain fatty acids (SCFAs) in different IBS severity groups, and study the association with stool pattern, diet, depression, anxiety and quality of life (QoL). A longitudinal study was performed, including n=91 IBS patients and n=28 matched controls. All participants collected faecal samples for microbiota composition and SCFA analysis and completed validated questionnaires regarding IBS severity, stool pattern, depression, anxiety and IBS-QoL at two timepoints T1 and T2 with four weeks in-between. Diet was assessed at T1. Over time, 36% of IBS patients changed in severity group, and 53% changed in predominant stool pattern. The largest proportion of microbiota variation was explained by the individual (R^2 = 70.07%). Microbiota alpha diversity and composition, and SCFAs did not differ between IBS severity groups, nor between IBS and controls. Relative abundances of *Bifidobacterium, Terrisporobacter* and *Turicibacter* consistently differed between IBS and controls, but not between IBS severity groups. Large dynamics over time were observed in the association of microbiota composition with questionnaire data where IBS symptom severity was associated at T1 but not at T2. In conclusion, faecal microbiota and SCFA signatures were not consistently associated with IBS severity over time, indicating the importance of repeated sampling in IBS research.

Keywords: Irritable bowel syndrome; faecal microbiota composition; short chain fatty acids; severity

Introduction

Irritable bowel syndrome (IBS) is the most commonly diagnosed functional gastrointestinal disorder, with a global prevalence around 11% [1]. IBS reduces quality of life (QoL) and increases health care costs [2]. Several factors have been associated with IBS, such as alterations in the gut-brain axis [3], visceral hypersensitivity [4], increased intestinal permeability [5] and altered faecal microbiota composition [6, 7]. Different studies have been performed to identify microbial signatures in IBS patients, but a general consensus in IBS-related profiles is lacking [8]. This inconsistency could be due to the large individual variation in microbiota composition and cohort-specific characteristics [9], as well as cross-sectional study designs. Moreover, there is large variation in symptom severity and stool pattern within and between individuals with IBS [5], and instability in faecal microbiota over time [10, 11]. Furthermore, studies often do not include other covariates like diet and psychological state, which can be different in IBS and are associated with faecal microbiota [8, 12, 13].

Recently, Tap et al (2017) were the first to explore IBS symptom severity related to the microbiota, and they cross-sectionally identified a microbial signature of 90 operational taxonomic units (OTUs) associated with IBS severity [14], which provides a new research direction to investigate microbial signatures with taking IBS severity into consideration. Although a recent study by Mars and colleagues longitudinally identified species-level taxa associated to severity of symptoms in diarrhoea-predominant IBS [15], the consistency of microbial signatures associated with IBS symptom severity over time still remains unknown. Moreover, alterations in faecal short-chain fatty acids (SCFAs), including acetate, propionate and butyrate, have also been observed between IBS and controls [16]. These alterations were associated with bloating, abdominal pain and QoL in IBS [17]. However, the association of SCFAs with IBS severity and its consistency over time are unknown.

Therefore, we investigated the dynamics of faecal microbiota composition and SCFA levels in different IBS severity groups compared to controls, and the association with stool pattern, diet, depression, anxiety and QoL over a period of four weeks. These dynamics and associations were also investigated between IBS and controls. We hypothesised that over time, IBS severity would demonstrate a regression to the mean, while faecal microbiota signatures associated with severity would remain stable.

Methods

This was an observational longitudinal study with two timepoints (T1 and T2) with four weeks in-between, and included IBS patients and controls who were matched for age, gender and Body Mass Index (BMI) at T1 (no significance at group level). All participants signed an informed consent. The study was approved by the Medical Ethics Committee of Wageningen and was registered at Clinicaltrials.gov (NCT03720314, https://clinicaltrials.gov/ct2/show/NCT03720314).

Study participants

Participants were recruited using the Wageningen University subject database, and recruitment calls on websites and social media. Participants were aged 18-65 years, lived near Wageningen and had a BMI between 18.5-30.0 kg/m^2. IBS patients had to fulfil the Rome IV criteria or had to be diagnosed with IBS by a physician. Exclusion criteria were presence of any other gastrointestinal or systemic diseases, antibiotics use < 3 months before study start, pregnancy or breastfeeding. We aimed to include 100 IBS patients and 30 matched controls at T1, to be able to detect a difference of 3.6 ± 4.9% in similarity of microbiota over time [10]. After T1, the 30 IBS patients with the least symptoms and 30 with the most severe symptoms were selected for T2, to assess the regression to the mean hypothesis and faecal microbiota dynamics. Controls completed both timepoints.

Faecal microbiota profiling

Participants collected a faecal sample at both timepoints. After collection, the faecal material was immediately stored in the participants' home freezer. Faecal samples were transported on dry ice by research staff to the laboratory on average within 1.1 ± 1.2 days, where it was stored immediately at -80 ºC until further analysis.

Faecal microbiota composition was determined by sequencing the V4 region of the 16S ribosomal RNA (rRNA) gene (Illumina Hiseq2500, 150bp paired end). As previously described, 0.25 g faeces (wet weight) was used for DNA isolation with the Repeated Bead Beating method [18]. Subsequently, DNA was purified using the Maxwell® 16 Total RNA system (Promega, Madison, WI, USA) with the 16 Tissue LEV Total RNA purification Kit Cartridge (XAS1220). Amplification was performed in duplicate with uniquely barcoded primers [19] 515F (5'-GTGYCAGCMGCCGCGGTAA-3') [20] and 806R (5'-GGACTACNVGGGTWTCTAAT-3') [21]. Reaction conditions and library preparation were performed as described previously [19]. Afterwards, the libraries

were purified with the CleanPCR kit (CleanNA, Waddinxveen, the Netherlands), and sent to Eurofins Genomics Germany GmbH (Konstanz, Germany) for sequencing. NG-Tax 2.0 was used to process the raw sequencing data for Amplicon Sequencing Variant (ASV) picking with default settings and for taxonomic assignments using the SILVA database (version 128) [22, 23]. Sequence data was submitted to the European Nucleotide Archive with accession number PRJEB44533.

SCFA profiling

SCFAs were measured as described previously with minor modifications [24]. In total, 0.4 g of faeces (wet weight) were mixed thoroughly with 1.6 mL ultrapure water to extract SCFAs (acetate, propionate, and butyrate). Subsequently, the mixture was centrifuged (21,130 × g, 10 mins) to get the supernatant. Afterwards, 0.4 mL supernatant was added to 0.6 mL 10 mM DMSO as the internal standard in 0.1 N H_2SO_4 solution, and analysed by High-Performance Liquid Chromatography (HPLC, LC-2030C, Shimazu, Kyoto, Japan) with a Shodex SH1821 column (Showa Denko K.K., Tokyo, Japan).

Questionnaires

Both IBS patients and controls completed all questionnaires at T1 and T2 for comparison. IBS severity was assessed using the validated IBS Symptom Severity Score (IBS-SSS), which was used to classify severity (no symptoms ≤ 75; mild = 76-175; moderate = 176-300, and severe > 300 IBS) [25]. Both the continuous severity score and severity grouping were used in the analysis. QoL was assessed with the 34-item IBS-QoL, which gave a score for total IBS-QoL, and subscales dysphoria, interference with activity, body image, health worry, food avoidance, social reaction, sexual life and relationship [26]. Participants completed the Hospital Anxiety and Depression Score (HADS) [27]. A score ≥ 8 indicated substantial depressive or anxious symptoms [28]. Furthermore, the predominant stool pattern of the previous week was assessed by letting participants rank their stools of the week before sampling from most to least frequent, using the seven types of the validated Bristol Stool chart [29]. Participants also indicated the Bristol stool scale of the faecal sample. Habitual dietary intake of the previous month was assessed at T1 using a semi-quantitative 83-item Food Frequency Questionnaire [30, 31]. Dietary intake was calculated using the Dutch Food Composition table [32]. Furthermore, IBS patients were asked if they were currently following the Fermentable Oligo-, Di-, Monosaccharides, And Polyols (FODMAP) diet. Participants were instructed to keep their diet similar during the study period.

Statistical analyses

Microbiota data were analysed in R version 4.0.0 [33] and questionnaire data in SPSS version 25 (Armonk, NY, USA: IBM Corp.). Continuous data are presented as mean ± standard deviation, or median and interquartile range when skewed. Categorical data are presented as counts and percentages. Differences in the questionnaire data between IBS and controls were tested with an independent sample T-test or Mann-Whitney U test when not normally distributed. Differences in questionnaire data between IBS subgroups and controls were tested with a one-way Analysis of Variance (ANOVA), or a Wilcoxon test when not normally distributed, with Bonferroni corrected post-hoc testing. An unpaired Wilcoxon test was used to test differences in SCFAs between IBS subgroups and controls, or between IBS and controls. Differences for categorical data were assessed using chi-square tests. Associations of acetate, propionate and butyrate (dependent variable) with questionnaire data (independent variables) were determined using linear mixed models.

Alpha diversity (within sample diversity) and beta diversity (between sample diversity) were calculated at ASV level using Phyloseq [34]. Alpha diversity metrics ASV richness and Shannon diversity were calculated. To visualize beta diversity, Principle Coordinate analysis (PCoA) based on unweighted (considering presence/absence of ASVs) and weighted (considering ASVs and their relative abundance) Unifrac [35] distances was performed. An unpaired Wilcoxon test was used to compare genus-level taxa between IBS and controls, or between IBS subgroups and controls at both timepoints. The p-values for multiple pairwise tests were corrected for multiple testing using Benjamini-Hochberg false-discovery rate (FDR). The Vegan package [36] was used to assess the association of microbiota composition with questionnaire and dietary variables, using Permutational Multivariate Analysis of Variance (PERMANOVA).

A (corrected) p-value ≤ 0.05 was considered statistically significant, and 0.05 ≤ (corrected) p-value < 0.1 was considered a trend.

Results

A total of n=91 IBS and n=30 controls participated, with n=55 IBS and n=28 controls included for longitudinal analyses (Figure 1). Baseline characteristics differed between IBS and controls for IBS-SSS (p < 0.001), IBS-QoL (p < 0.001), anxiety (p = 0.001) and depression (p = 0.004, Table 1). When data were stratified

for IBS severity and compared with controls, IBS-QoL, anxiety and depression remained different (p-values < 0.05). Distribution of the predominant stool patterns was not different between IBS severity groups. There was no significant difference in dietary intake of energy, fat, carbohydrates, polysaccharides, dietary fibre, alcohol or water between IBS and controls (Supplementary Table 1). However, IBS patients had a lower intake of protein, maltose and lactose. Moreover, 22% of IBS patients followed a FODMAP diet for whom lactose intake was significantly lower than those not following a FODMAP diet (p = 0.007).
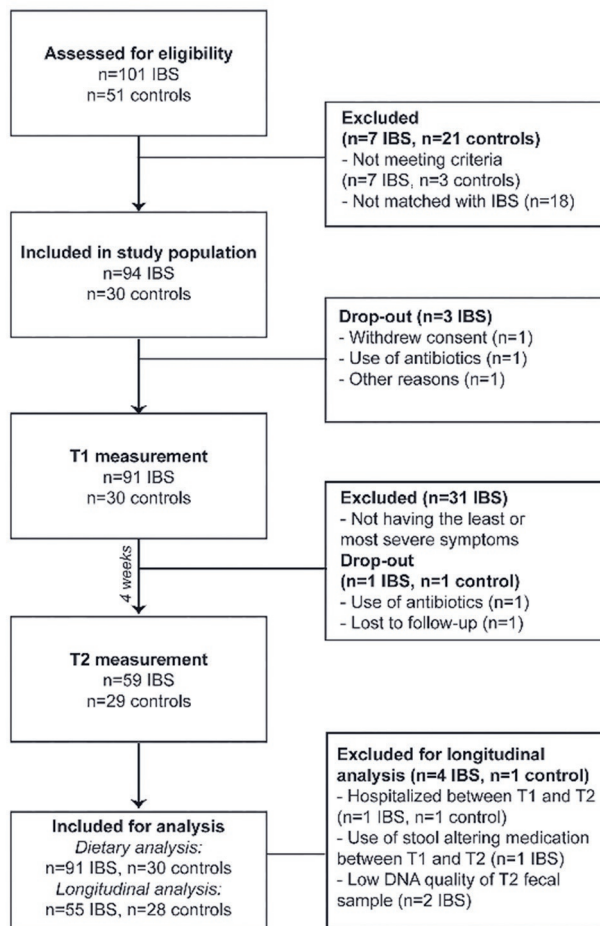

Figure 1: Flowchart of participant recruitment

Table 1 Baseline characteristics of the study population

	IBS (n=91)	Control (n=30)	p values
Age, years	41.7 ± 14.4	39.4 ± 16.9	0.500
Gender n (%) male	19 (21)	4 (13)	0.361
BMI, kg/m^2	22.9 ± 2.9	23.2 ± 3.1	0.566
IBS-SSS	150 (110 – 230)	55 (27 – 90)	**0.000**
Severity groups n (%)			**0.000**
No symptoms (≤75)	7 (8)	19 (63)	
Mild (76-175)	48 (53)	11 (37)	
Moderate (176-300)	24 (26)	0 (0)	
Severe (>300)	12 (13)	0 (0)	
Predominant stool pattern n (%)			0.183
Constipation	26 (29)	14 (47)	
Diarrhoea	32 (35)	6 (20)	
Mixed	19 (21)	4 (13)	
Unspecified	14 (15)	6 (20)	
IBS-QoL	72.2 ± 16.8	98.6 ± 3.6	**0.000**
Anxiety			
Score	6.0 (3.0 – 10.0)	4.0 (2.8 – 6.0)	**0.001**
Substantial anxious symptoms n (%)[†]	35 (38)	3 (10)	**0.004**
Depression			
Score	2.0 (1.0 – 5.0)	(0.0 – 2.2)	**0.004**
Substantial depressive symptoms n (%)[†]	9 (10)	1 (3)	0.258

Data presents mean ± standard deviation or median (interquartile range) when skewed.
Abbreviations: BMI, Body Mass Index; IBS-SSS, IBS Symptom Severity Score; IBS-QoL, IBS Quality of Life.
[†]Based on the Hospital Anxiety and Depression Score (HADS) cut-off > 8

Instability of IBS symptom severity over time

Instability of the IBS severity score was observed over time (Figure 2A). The severity score decreased by ≥ 50 in 14 IBS patients and increased by ≥ 50 in 9 IBS patients (Figure 2B). Furthermore, over time, 20 (36%) of the IBS patients changed in severity groups (Supplementary Figure 1A). For other IBS symptoms, a large variation over time was also observed: 29 (53%) of the IBS patients changed in their predominant stool patterns (Supplementary Figure 1B). Total IBS-QoL (Δ = 2.4, p = 0.028), and sub-scores dysphoria (Δ = 3.1, p = 0.032), body image (Δ = 3.9, p = 0.004) and impact on relationship (Δ = 4.1, p = 0.010) increased, while other IBS-QoL sub-scores, anxiety and depression remained stable over time.
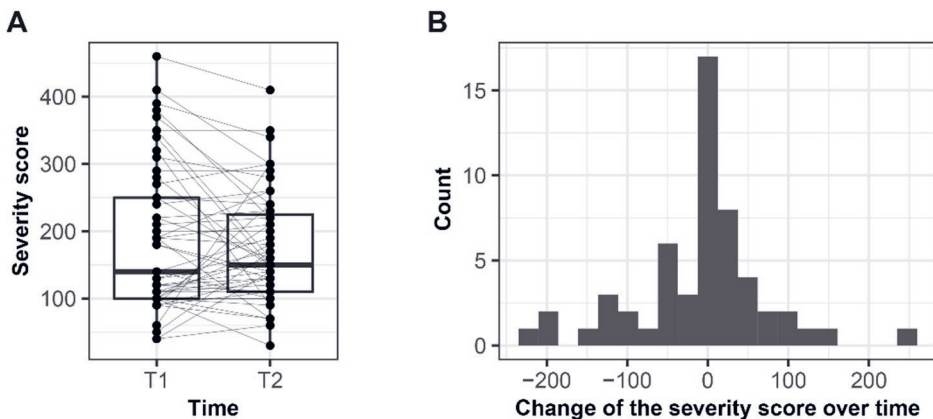

Figure 2: Instability of severity score over time. Dynamics of IBS symptom severity score over time *(A)*, and values were presented as interquartile with a boxplot. Samples taken at different timepoints are connected by solid lines per subject. Distribution of change of the severity score over time with a histogram *(B)*.

No difference in microbiota alpha and beta diversity between IBS severity groups and controls

ASV richness and Shannon diversity were not different between IBS and controls, and between IBS with different predominant stool patterns and controls nor between IBS severity groups and controls (Figure 3A and 3B, Supplementary Figure 2) at both timepoints. This indicates the number and distribution of microbial ASVs were similar between these groups. PERMANOVA based on unweighted

and weighted Unifrac distance (Figure 3F) revealed no significant difference between IBS severity groups and controls nor between IBS with different predominant stool patterns and controls. Between IBS and controls, the unweighted Unifrac based observation was similar, while based on weighted Unifrac, a trend at T1 (p = 0.073) and T2 (p = 0.064) was observed (Figure 3C). This indicates that the relative abundance of microbial taxa played a role in the differences of microbiota composition between IBS and controls, rather than the presence or absence of the microbial taxa. Longitudinally, the change in faecal microbiota composition in IBS and controls was not different (Figure 3D, p = 0.27), indicating that the temporal stability of the microbiota of IBS patients was similar to that of controls. Moreover, microbiota variation over time was not associated with a change in severity score (Figure 3E).
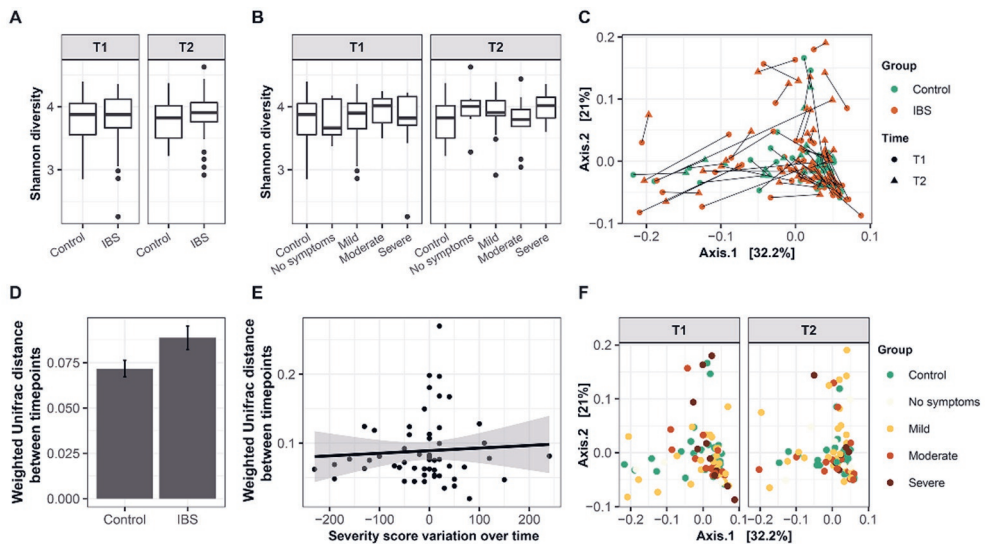

Figure 3: Analysis of faecal microbiota alpha and beta diversity for IBS patients and controls and severity groups over time. Shannon diversity displayed as interquartile with boxplot, stratified for IBS and controls (*A*), symptom severity groups and controls (*B*). PCoA of microbiota composition based on weighted Unifrac distances, stratified for IBS and controls. Samples taken at different timepoints are connected by solid lines per subject (*C*). Comparison of microbiota composition stability based on weighted Unifrac distances over time between IBS patients and controls. Values are presented as mean±standard error (*D*). Linear model indicated no association between the changed severity score and weighted Unifrac distance of microbiota composition over time (*E*). PCoA of microbiota composition based on weighted Unifrac distances, stratified for symptom severity groups and controls (*F*).

Differences in genus level taxa between IBS and controls were not associated with IBS symptom severity

Relative abundances were different for ten genus level taxa at T1 and eight at T2 between IBS and controls (Figure 4A, Supplementary Table 2). However, of those only *Bifidobacterium*, *Terrisporobacter* and *Turicibacter* were consistently different over time. The relative abundance of *Bifidobacterium* was lower in IBS compared to controls (p_{T1} = 0.0003; p_{T2} = 0.0003). In contrast, the relative abundances of *Terrisporobacter* (p_{T1} = 0.010; p_{T2} = 0.004) and *Turicibacter* (p_{T1} = 0.042; p_{T2} = 0.013) were consistently higher in IBS patients over time. However, these taxa were not associated with IBS severity (Figure 4B, Supplementary Table 3). The relative abundances of five genus level taxa at T1 and seven at T2 were significantly different within the severity groups or between severity groups and controls. Remarkably, one uncultured taxon within the *Tenericutes* phylum (p = 0.021) and *Prevotellaceae*_UCG-001 (p = 0.021) were significantly higher in severe IBS patients at T2 compared to controls. However, none of these taxa were consistently different between IBS severity groups. In addition, the change of the genus-level taxa over time was not correlated with the change of the severity score over time (Supplementary Table 4). Next to IBS severity, we also assessed associations between faecal microbiota composition and predominant stool patterns in IBS over time. Some genus-level taxa were associated with predominant stool patterns, such as *Alistipes* with constipation. However, in line with the IBS severity observations, these associations were only observed at a single timepoint, and not consistent over time (Supplementary Table 5).
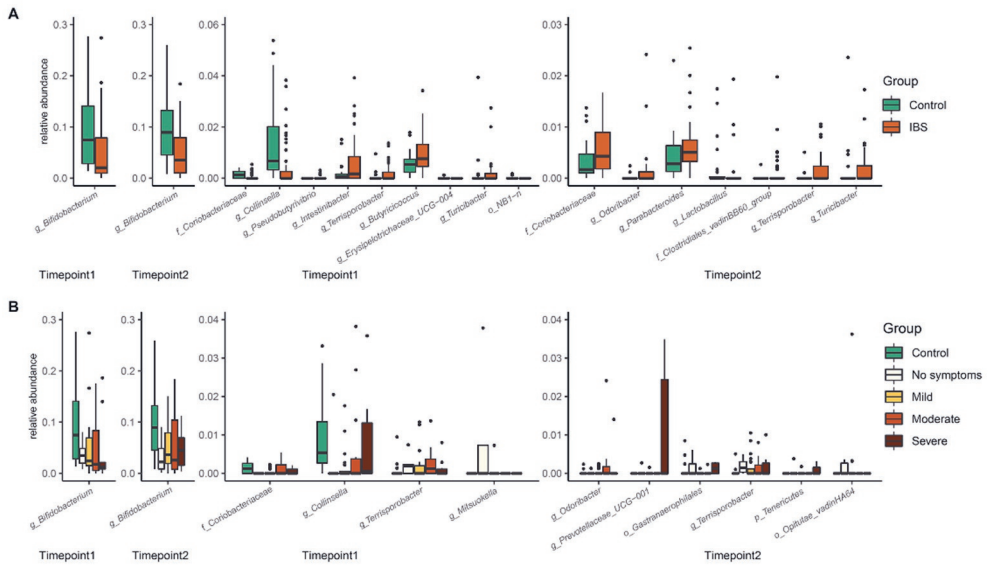
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Figure 4: Genus level taxa that significantly differed in relative abundance between IBS patients and controls (*A*), or between severity groups and controls (*B*). Data are presented as interquartile with boxplot.

Dynamics of the association between questionnaire data and microbiota composition over time

The largest proportion of microbiota variation was explained by the individual ($R^2 =$ 70.07%), when data of both timepoints was included. Age was significantly and consistently associated with microbiota composition at T1 ($R^2 = 2.75\%$) and T2 ($R^2 = 3.63\%$, Figure 5). IBS explained a stable proportion of microbiota variation over time ($R^2_{T1} = 2.41\%$, $R^2_{T2} = 2.30\%$). At T1, Bristol stool scale and symptom severity score were significantly associated to microbiota composition, and explained, respectively, the first and second largest proportion of microbiota variation out of all participant characteristics ($R^2_{Bristol\ stool} = 3.69\%$, $R^2_{Severity} = 3.17\%$). However, at T2 the proportion of variation explained by Bristol stool scale and symptom severity score decreased, and was not significant anymore. This indicates large dynamics of explained microbiota variation by participant characteristics over time. We did not observe an association between dietary intake and microbiota variation. Moreover, no correlations were found between macronutrient, lactose or maltose intake and relative abundance of *Bifidobacterium, Terrisporobacter* and *Turicibacter*.
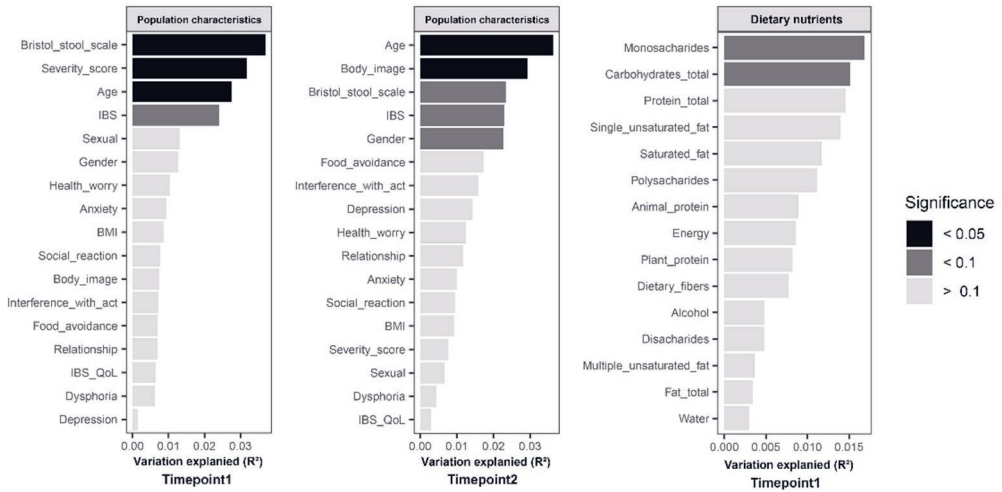
Figure 5: Large dynamics in explained variation of microbiota composition based on weighted Unifrac distances by population characteristics and dietary nutrients.

No difference in SCFAs between IBS, controls and severity groups

Large within and between person variation in SCFA levels was observed at both timepoints (Figure 6), but no differences were found between IBS severity groups or IBS and controls. When subjects were stratified based on the predominant stool pattern, acetate was consistently higher in diarrhoea-predominant IBS compared to patients with constipation ($p_{T1} = 0.000$; $p_{T2} = 0.008$). Linear mixed model analysis revealed that acetate, propionate and butyrate were not associated with psychological factors over time (Supplementary Table 6). No correlations were observed between diet and SCFAs, except for lactose intake and butyrate (r = -0.182, p = 0.046, Supplementary Table 7).

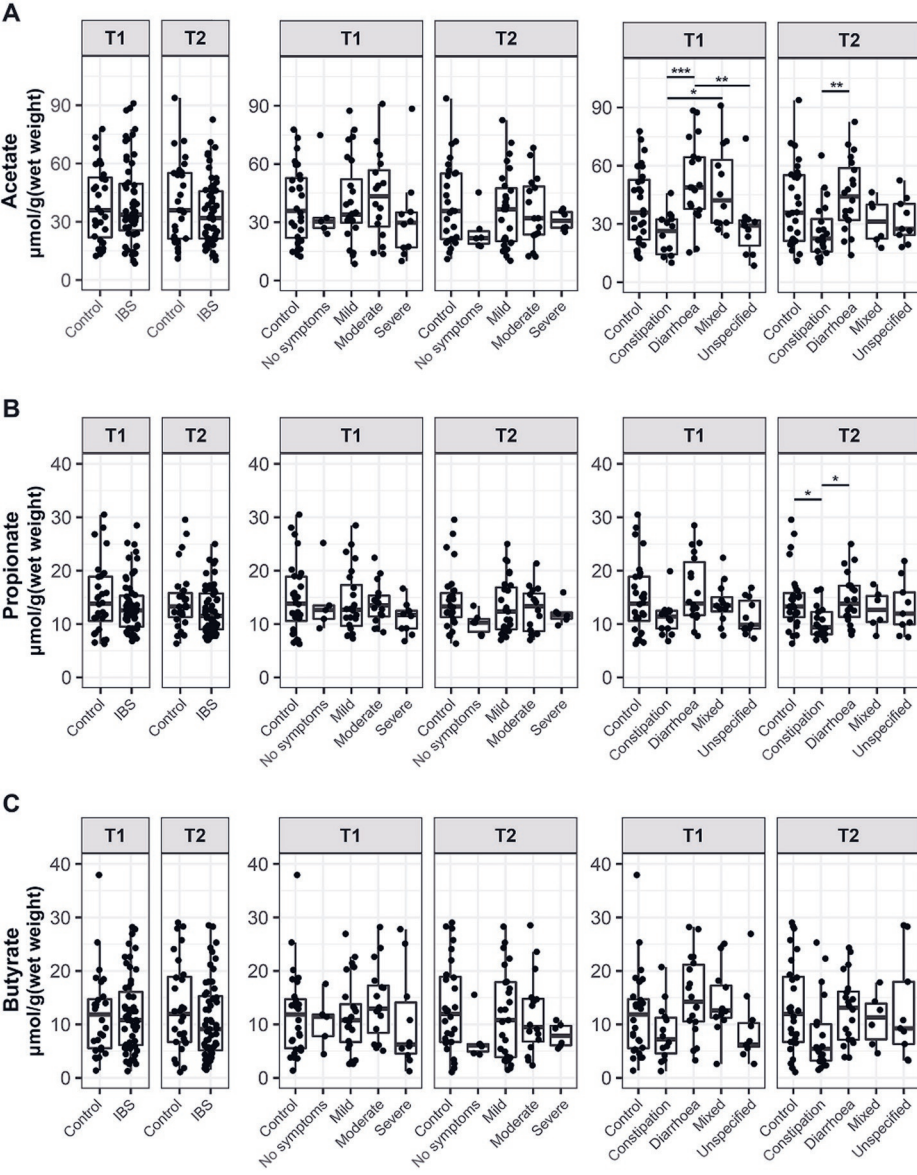**Figure 6:** No differences in faecal acetate (*A*), propionate (*B*), and butyrate (*C*) between controls and IBS, and severity groups over time, but lower acetate levels in constipation-predominant IBS patients. Values were presented as interquartile with boxplot. Significance between groups was tested by Wilcoxon. * $p < 0.05$; ** $p < 0.01$.

Discussion

We performed a longitudinal study to examine the dynamics of faecal microbiota and SCFA levels between IBS severity and controls over time. Moreover, we assessed the association between faecal microbiota and SCFAs with stool pattern, IBS-QoL, anxiety, depression and diet. We did not find any difference in microbial alpha diversity, composition or SCFAs between the control and IBS severity groups. Multiple taxa were significantly different in relative abundance between IBS and controls at different timepoints, but consistent differences were only observed for *Bifidobacterium, Terrisporobacter* and *Turicibacter*. Moreover, consistently lower acetate levels were found in only constipation-predominant IBS patients. Longitudinal analysis showed a large within and between subject variation in IBS severity, predominant stool pattern, and their association with microbiota composition.

Our results showed no significant difference of faecal microbial alpha diversity and composition in IBS, indicating that the number, distribution and phylogenetic identity of microbial ASVs were similar between IBS and controls. This is in line with some studies [14, 37], but not all [38-41]. These conflicting results could be due to different methods used for the analysis, variations in discriminative power of different 16S rRNA gene regions, variations in inclusion and exclusion criteria, as well as heterogeneity of IBS cohorts [8]. Although the individuality explained the largest proportion of microbiota variation when looking at genus level taxa, we observed several consistent microbial signatures associated with IBS over time. Lower relative abundance of *Bifidobacterium* in IBS patients has been reported in previous studies with a cross-sectional study design [8], which we found to be consistent over time. Remarkably, we found both *Bifidobacterium* and lactose consumption significantly lower in IBS patients compared to controls. Lactose has been suggested as one of the carbon sources of *Bifidobacterium* [42, 43]. However, the relative abundance of *Bifidobacterium* and lactose were not correlated in our study. Interestingly, *Bifidobacterium* supplements have been reported to improve IBS symptoms [44, 45], however, relief of symptoms was not always associated with an increase in relative abundance of *Bifidobacterium* [46]. This supports our observation that *Bifidobacterium* was not associated with symptom severity, but with IBS itself. Remarkably, we observed consistently higher relative abundance of *Terrisporobacter* and *Turicibacter* in IBS. Both taxa are thought to regulate the biosynthesis and release of serotonin and may play a role in IBS pathophysiology [47-50]. As these taxa have not been associated with IBS before, this finding may provide potential targets for future research.

Due to the accessibility without invasive procedures, faecal samples were the most commonly used to explore the role of gut microbiota in IBS [8]. It is evident that faecal samples only represent the end of the colon, and previous studies have shown that comparing samples from small intestine and colon provides relevant insights into the microbiota at other locations in the intestine of IBS patients [37, 51]. Hence, our study cannot exclude that potential key microbes at other locations in relation to IBS are overlooked. Nevertheless, longitudinal studies require repeated sampling without disturbing the intestine, and taking samples from other locations is nearly impossible without invasive procedures.

In our study, we found some taxa to be associated with IBS severity at one of the two timepoints. However, differences were not consistent over time. IBS symptom severity itself changed drastically within four weeks, and the explained variance of microbiota composition by severity also indicated large dynamics over time. Moreover, we did not find any correlation between the change of the severity score over time with the change of genus-level taxa in relative abundance over time, which indicates that IBS symptom severity seems not the reason causing changes in the relative abundance of faecal microbial taxa. A cross-sectional study by Tap et al (2017) [14] and a longitudinal study by Mars et al (2020) [15] have suggested that microbial signatures are associated with IBS symptom severity. However, the dynamics of the microbial signatures over time has not been determined in these two studies. Our study shows that the microbial signature associated with symptom severity is not stable longitudinally. Therefore, caution is needed in identifying signatures based on cross-sectional comparisons, which may change over time.

Increasing evidence indicates that IBS symptoms and microbiota composition are associated with carbohydrate intake [52, 53], and especially the FODMAPs [54, 55]. In our study, we did not find an association between microbiota composition and carbohydrate intake. Furthermore, after comparing the microbiota composition between IBS patients following the FODMAP diet and those who did not, no difference was found. This might be due to the large dietary differences within the FODMAP diet and between studies. Further studies assessing the effects of carbohydrates on IBS symptoms and microbiota are needed.

The main microbial metabolites, SCFAs, have been suggested as a biomarker of IBS [16, 56]. However, approximately 80% of SCFAs produced in the gut are absorbed, and therefore not found in faecal samples [57], which may limit the effectiveness of faecal SCFAs as a biomarker. In our study, we did not find differences in SCFA levels between IBS and controls, while we confirmed that acetate was consistently lower in constipation-predominant IBS compared to diarrhoea-predominant IBS [16]. This might be explained by the shorter transit time

in diarrhoea, leaving less time for absorption of SCFA in the gut, as shown in people with slow colonic transit [58].

Our study is strengthened by the longitudinal design, which enabled us to assess dynamics of faecal microbiota and SCFAs associated with IBS severity. Moreover, we assessed diet and psychological factors, which are altered in IBS, thus giving a more complete overview of the IBS patient. However, due to our observational study design we cannot determine causality. In addition, given the large variability over a short period of time in symptom severity scores, maintaining consistently equal sized groups of severity was challenging.

In conclusion, consistent faecal microbiota and SCFA signatures associated with IBS severity were not found. Interestingly, the relative abundances of the genera *Bifidobacterium*, *Terrisporobacter*, and *Turicibacter* were consistently different between IBS and controls over time, giving directions for future explorations. The importance of inclusion of multiple timepoints was demonstrated by the large within and between person variation of observed IBS severity, stool pattern and their association with faecal microbiota composition over time. Hence, conclusions of single-timepoint studies in the past should be reconsidered, and future studies are highly recommended to take time-dynamics into account.

Potential competing interest: the authors report no conflict of interest.

Ethics: The study was approved by the medical ethics committee of Wageningen (18/06) and conducted according to the declaration of Helsinki. All participants signed a written informed consent. The study was registered at Clinicaltrials.gov under number NCT03720314 on October 25th 2018.
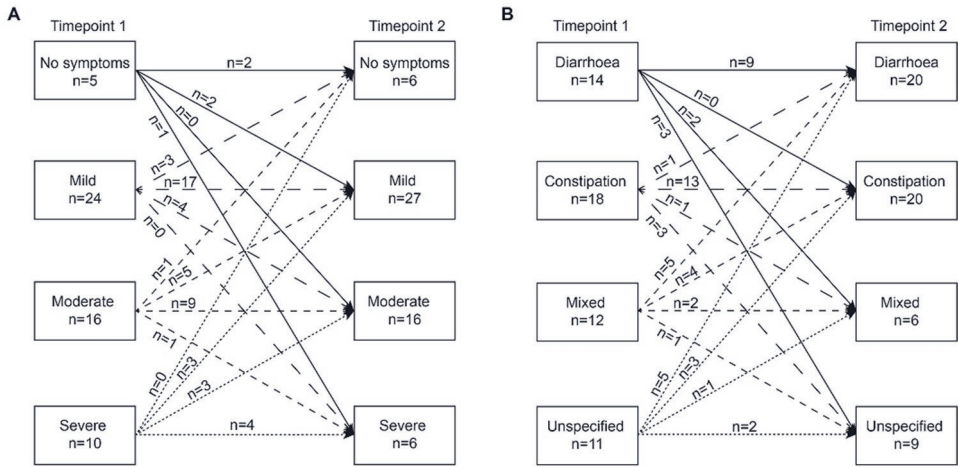
Supplementary Figure 1: Dynamics of IBS symptom severity groups *(A)* and predominant stool patterns *(B)* over time.
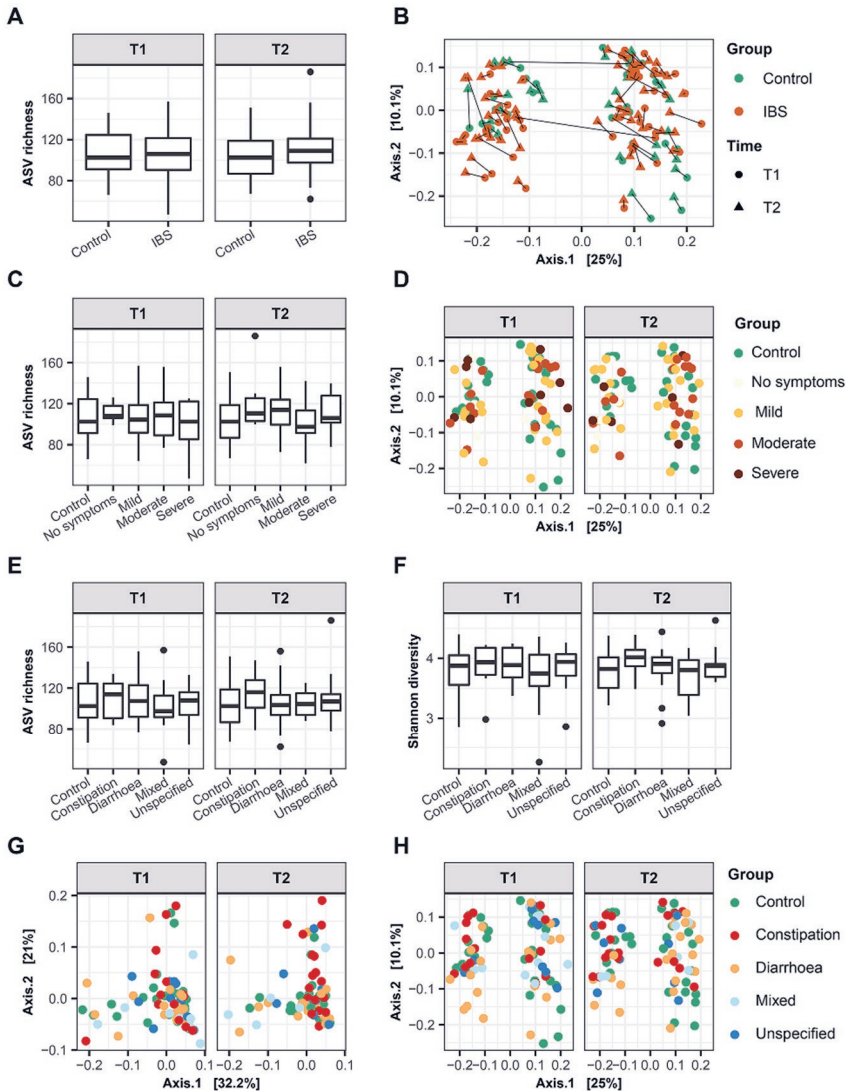
Supplementary Figure 2: Comparison in faecal microbiota alpha and beta diversity between IBS patients and controls, severity groups, subgroups based on predominant stool pattern. Significance between groups was tested by Wilcoxon for alpha diversity, and PEMMANOVA for beta diversity. Comparison of ASV richness between IBS and control *(A)*. PCoA of microbiota composition based on unweighted Unifrac distances, stratified for IBS and controls *(B)*. Comparison of ASV richness between severity groups and control *(C)*. PCoA of microbiota composition based on unweighted Unifrac distances, stratified for symptom severity groups and controls *(D)*. Comparison of ASV richness between subgroups based on predominant stool pattern *(E)*. Comparison of Shannon diversity between subgroups based on predominant stool pattern *(F)*. PCoA of microbiota composition based on weighted Unifrac distance, stratified for subgroups based on predominant stool pattern *(G)*. PCoA of microbiota composition based on unweighted Unifrac distances, stratified for subgroups based on predominant stool pattern *(H)*.

Supplementary Table 1 Dietary intake of the study population at the first timepoint

	IBS (n=91)	Control (n=30)	p value
Energy (kcal)	1942 ± 587	2165 ± 673	0.083
Protein (g)	70.1 ± 21.4	80.2 ± 25.8	**0.036**
Total fat (g)	82.2 ± 29.2	90.6 ± 34.8	0.197
Saturated fat (g)	27.2 ± 11.8	31.2 ± 13.0	0.118
Total Carbohydrates (g)	205 ± 65	226 ± 64	0.137
Glucose (g)	9.8 ± 4.3	8.8 ± 4.2	0.295
Fructose (g)	13.6 ± 5.9	12.8 ± 5.4	0.565
Lactose (g)	4.6 (2.0 – 10.1)	11.1 (7.0 – 19.0)	**0.000**
Maltose (g)	2.0 (1.2 – 2.8)	2.6 (2.0 – 3.6)	**0.042**
Saccharides (g)	25.2 ± 9.2	27.4 ± 12.0	0.314
Polysaccharides (g)	120 ± 49	135 ± 46	0.154
Dietary fibre (g)	24.8 ± 8.3	26.6 ± 7.5	0.309
Alcohol (g)	3.2 (0.5 – 9.8)	2.8 (0.0 – 11.8)	0.123
Water (g)	2603 ± 696	2517 ± 651	0.550
Following the FODMAP diet n (%)	20 (22)	Not applicable	

Data are presented as mean ± standard deviation or median (interquartile range) when skewed. Differences between groups are tested with an independent sample t-test or Mann-Whitney U test when skewed. The definition of each nutrient is according to the Dutch Food Composition table[55]. In line with the Dutch Food Composition table, dietary fibre is not included in the calculation of total carbohydrates but treated as a separate category. Abbreviation: IBS; Irritable Bowel Syndrome

Supplementary Table 2: Comparison of genus level taxa in relative abundance between IBS patients and controls over time

	Taxa at genus level	Control (mean ± SD)	IBS (mean ± SD)	p values
T1	g__Bifidobacterium	0.0955 ± 0.0747	0.0452 ± 0.0562	**0.000**
	f__Coriobacteriaceae;g__	0.0014 ± 0.0014	0.0005 ± 0.0011	**0.001**
	g__Collinsella	0.0147 ± 0.0161	0.0041 ± 0.0089	**0.000**
	g__Pseudobutyrivibrio	0.0000 ± 0.0000	0.0003 ± 0.0007	**0.036**
	g__Intestinibacter	0.0019 ± 0.0037	0.0060 ± 0.0086	**0.043**
	g__Terrisporobacter	0.0005 ± 0.0019	0.0020 ± 0.0034	**0.010**
	g__Butyricicoccus	0.0057 ± 0.0045	0.0091 ± 0.0066	**0.018**
	g__Erysipelotrichaceae_UCG-004	0.0001 ± 0.0003	0.0000 ± 0.0000	**0.048**
	g__Turicibacter	0.0018 ± 0.0075	0.0018 ± 0.0046	**0.042**
	c__Mollicutes;o__NB1-n;f__;g__	0.0001 ± 0.0004	0.0000 ± 0.0000	**0.048**
T2	g__Bifidobacterium	0.1021 ± 0.0702	0.0498 ± 0.0451	**0.000**
	f__Coriobacteriaceae;g__uncultured	0.0034 ± 0.0037	0.0066 ± 0.0106	**0.041**
	g__Odoribacter	0.0001 ± 0.0005	0.0012 ± 0.0038	**0.028**
	g__Parabacteroides	0.0041 ± 0.0047	0.0072 ± 0.0072	**0.010**
	g__Lactobacillus	0.0018 ± 0.0044	0.0007 ± 0.0031	**0.031**
	f__Clostridiales_vadinBB60_group;g__	0.0001 ± 0.0005	0.0011 ± 0.0031	**0.046**
	g__Terrisporobacter	0.0002 ± 0.0010	0.0015 ± 0.0027	**0.004**
	g__Turicibacter	0.0013 ± 0.0046	0.0024 ± 0.0055	**0.013**

Data are presented as mean ± SD. The population was used for analysis including 55 IBS patients and 28 controls at both timepoints. Abbreviations: IBS, Irritable Bowel Syndrome; SD, standard deviation. For visibility, here only genera with significance between IBS patients and controls were shown.

Supplementary Table 3: Comparison of genus level taxa in relative abundance between severity groups and controls over time

Taxa at genus level	Con (mean ± SD)	Nosym (mean ± SD)	Mild (mean ± SD)	Mod (mean ± SD)	Sev (mean ± SD)	p values (Con vs NS)	p values (Con vs Mild)	p values (Con vs Mod)	p values (Con vs Sev)	p values (NS vs Mild)	p values (NS vs Mod)	p values (NS vs Sev)	p values (Mild vs Mod)	p values (Mild vs Sev)	p values (Mod vs Sev)
T1															
g__Bifidobacterium	0.0955 ± 0.0747	0.0386 ± 0.0282	0.0482 ± 0.0618	0.0449 ± 0.0520	0.0418 ± 0.0653	0.260	**0.030**	**0.034**	**0.030**	0.854	0.854	0.733	0.854	0.546	0.854
f__Coriobacteriaceae; g__	0.0014 ± 0.0014	0.0000 ± 0.0000	0.0003 ± 0.0007	0.0010 ± 0.0017	0.0006 ± 0.0009	0.164	**0.016**	0.392	0.331	0.419	0.367	0.367	0.367	0.419	0.606
g__Collinsella	0.0147 ± 0.0161	0.0041 ± 0.0092	0.0017 ± 0.0042	0.0054 ± 0.0114	0.0076 ± 0.0119	0.083	**0.000**	**0.005**	0.151	1.000	0.797	0.617	0.617	0.233	0.617
g__Terrisporobacter	0.0005 ± 0.0019	0.0023 ± 0.0030	0.0018 ± 0.0037	0.0027 ± 0.0037	0.0012 ± 0.0026	0.112	0.311	**0.032**	0.450	0.461	0.966	0.461	0.407	0.931	0.450
g__Mitsuokella	0.0000 ± 0.0000	0.0090 ± 0.0164	0.0003 ± 0.0015	0.0000 ± 0.0000	0.0000 ± 0.0000	**0.006**	0.417	NA	NA	**0.038**	**0.038**	0.086	0.518	0.561	NA
T2															
g__Bifidobacterium	0.1021 ± 0.0702	0.0332 ± 0.0344	0.0512 ± 0.0408	0.0540 ± 0.0578	0.0483 ± 0.0415	0.055	**0.028**	0.066	0.119	0.696	0.918	0.808	0.918	0.971	0.971
g__Odoribacter	0.0001 ± 0.0005	0.0002 ± 0.0005	0.0004 ± 0.0008	0.0023 ± 0.0059	0.0023 ± 0.0057	0.702	0.383	**0.031**	0.702	0.869	0.578	1.000	0.383	1.000	0.702
g__Prevotellaceae_U CG-001	0.0000 ± 0.0000	0.0005 ± 0.0011	0.0001 ± 0.0003	0.0000 ± 0.0000	0.0112 ± 0.0174	0.084	0.419	NA	**0.021**	0.354	0.226	0.470	0.470	0.066	0.066
o__Gastranaerophilal es;f__uncultured_bact erium;g__	0.0006 ± 0.0019	0.0016 ± 0.0026	0.0000 ± 0.0002	0.0001 ± 0.0006	0.0014 ± 0.0015	0.292	0.434	0.738	0.088	0.073	0.191	1.000	0.783	**0.015**	0.073
g__Terrisporobacter	0.0002 ± 0.0010	0.0019 ± 0.0020	0.0013 ± 0.0027	0.0015 ± 0.0024	0.0023 ± 0.0041	**0.008**	0.095	0.065	0.173	0.334	0.663	0.836	0.836	0.836	0.966
p__Tenericutes;c__;o __;f__;g__	0.0000 ± 0.0000	0.0006 ± 0.0016	0.0001 ± 0.0003	0.0000 ± 0.0000	0.0009 ± 0.0014	0.084	0.419	NA	**0.021**	0.354	0.226	0.753	0.529	0.066	0.066
o__Opitutae_vadinHA 64;f__uncultured_bact erium;g__	0.0000 ± 0.0000	0.0012 ± 0.0019	0.0016 ± 0.0070	0.0000 ± 0.0000	0.0000 ± 0.0000	**0.016**	0.177	NA	NA	0.212	0.077	0.212	0.212	0.426	NA

Data are presented as mean ± SD. The population was used for analysis including 55 IBS patients and 28 controls at both timepoints. Abbreviations: IBS, Irritable Bowel Syndrome; Con, Control; Mod, Moderate; NS, No symptoms; Sev: Severe; SD, standard deviation; NA: not available. For visibility, here only genera with significance between severity groups and controls were shown.

3

Supplementary Table 4: Correlation of the change of genus level taxa in relative abundance with the change of IBS severity score over time

Taxa at genus level	Spearman rank	p values
g__Methanobrevibacter	-0.062	0.967
g__Methanosphaera	-0.037	0.976
f__Thermoplasmatales_Incertae_Sedis;g__uncultured	-0.172	0.943
g__Actinomyces	0.091	0.967
g__Arcanobacterium	0.172	0.943
g__Varibaculum	-0.129	0.967
g__Bifidobacterium	0.071	0.967
g__Rothia	-0.129	0.967
f__Coriobacteriaceae;g__	-0.096	0.967
g__Collinsella	0.091	0.967
g__Coriobacteriaceae_UCG-002	-0.154	0.943
g__Coriobacteriaceae_UCG-003	-0.127	0.967
g__Eggerthella	-0.292	0.929
g__Enterorhabdus	0.196	0.943
g__Gordonibacter	-0.296	0.929
g__Olsenella	-0.143	0.967
g__Senegalimassilia	-0.036	0.977
g__Slackia	0.290	0.929
f__Coriobacteriaceae;g__uncultured	0.160	0.943
o__Bacteroidales;f__;g__	0.103	0.967
f__Bacteroidaceae;g__	-0.172	0.943
g__Bacteroides	-0.025	1.000
f__Bacteroidales_S24-7_group;g__	0.072	0.967
f__Bacteroidales_S24-7_group;g__uncultured_bacterium	0.006	1.000
g__Barnesiella	0.058	0.967
g__Butyricimonas	-0.015	1.000
g__Coprobacter	-0.012	1.000
g__Odoribacter	-0.050	0.967
g__Parabacteroides	-0.065	0.967
g__Porphyromonas	-0.108	0.967
f__Porphyromonadaceae;g__uncultured	-0.161	0.943
f__Prevotellaceae;g__	-0.005	1.000
g__Alloprevotella	-0.021	1.000
g__Paraprevotella	-0.034	0.977
g__Prevotella	-0.141	0.967
g__Prevotella_2	-0.103	0.967
g__Prevotella_7	0.075	0.967
g__Prevotella_9	-0.060	0.967
g__Prevotellaceae_NK3B31_group	-0.048	0.967
g__Prevotellaceae_UCG-001	0.157	0.943
f__Prevotellaceae;g__uncultured	-0.055	0.967
g__Alistipes	0.060	0.967
g__Rikenellaceae_RC9_gut_group	-0.194	0.943
o__Bacteroidales;f__uncultured;g__	0.223	0.943
o__Bacteroidales;f__uncultured;g__uncultured_bacterium	-0.207	0.943
f__Flavobacteriaceae;g__uncultured	-0.219	0.943
c__Chloroplast;o__;f__;g__	-0.169	0.943
c__Chloroplast;o__uncultured_eukaryote;f__;g__	-0.060	0.967
o__Gastranaerophilales;f__;g__	-0.110	0.967
o__Gastranaerophilales;f__gut_metagenome;g__	-0.172	0.943
o__Gastranaerophilales;f__uncultured_bacterium;g__	-0.188	0.943
o__Gastranaerophilales;f__uncultured_organism;g__	-0.103	0.967
g__Elusimicrobium	-0.151	0.943
g__Staphylococcus	0.232	0.943

g__Enterococcus	0.154	0.943
g__Lactobacillus	-0.015	1.000
g__Pediococcus	0.000	1.000
g__Leuconostoc	0.056	0.967
g__Lactococcus	-0.076	0.967
g__Streptococcus	-0.307	0.929
o__Clostridiales;f__;g__	-0.120	0.967
g__Christensenellaceae_R-7_group	-0.041	0.967
f__Christensenellaceae;g__uncultured	0.052	0.967
g__Clostridium_sensu_stricto_1	0.062	0.967
f__Clostridiales_vadinBB60_group;g__	0.082	0.967
f__Clostridiales_vadinBB60_group;g__gut_metagenome	0.207	0.943
f__Clostridiales_vadinBB60_group;g__uncultured_bacterium	0.107	0.967
f__Clostridiales_vadinBB60_group;g__uncultured_organism	0.072	0.967
g__Defluviitaleaceae_UCG-011	0.174	0.943
g__Eubacterium	-0.077	0.967
g__Parvimonas	-0.108	0.967
o__Clostridiales;f__Family_XIII;g__Family_XIII_AD3011_group	0.252	0.943
o__Clostridiales;f__Family_XIII;g__Family_XIII_UCG-001	0.263	0.943
o__Clostridiales;f__Family_XIII;g__Mogibacterium	0.159	0.943
f__Lachnospiraceae;g__	0.048	0.967
g__Acetitomaculum	0.247	0.943
g__Anaerosporobacter	-0.056	0.967
g__Anaerostipes	-0.047	0.967
g__Blautia	0.052	0.967
g__Butyrivibrio	0.034	0.977
g__Coprococcus_1	-0.045	0.967
g__Coprococcus_2	0.063	0.967
g__Coprococcus_3	0.113	0.967
g__Dorea	-0.068	0.967
g__Eisenbergiella	0.039	0.969
g__Fusicatenibacter	-0.045	0.967
g__Howardella	0.154	0.943
g__Hungatella	-0.134	0.967
g__Lachnoclostridium	-0.096	0.967
g__Lachnospira	-0.075	0.967
g__Lachnospiraceae_FCS020_group	-0.166	0.943
g__Lachnospiraceae_ND3007_group	0.023	1.000
g__Lachnospiraceae_NK4A136_group	-0.048	0.967
g__Lachnospiraceae_NK4B4_group	-0.127	0.967
g__Lachnospiraceae_UCG-001	-0.164	0.943
g__Lachnospiraceae_UCG-003	-0.108	0.967
g__Lachnospiraceae_UCG-004	0.101	0.967
g__Lachnospiraceae_UCG-008	0.149	0.943
g__Marvinbryantia	0.175	0.943
g__Pseudobutyrivibrio	0.057	0.967
g__Roseburia	0.018	1.000
g__Sellimonas	0.000	1.000
g__Shuttleworthia	0.056	0.967
g__Tyzzerella	-0.194	0.943
g__Tyzzerella_3	-0.083	0.967
g__Tyzzerella_4	0.085	0.967
g__[Bacteroides]_pectinophilus_group	0.014	1.000
g__[Eubacterium]_eligens_group	0.187	0.943
g__[Eubacterium]_hallii_group	0.074	0.967
g__[Eubacterium]_ruminantium_group	-0.053	0.967
g__[Eubacterium]_ventriosum_group	-0.100	0.967
g__[Eubacterium]_xylanophilum_group	-0.036	0.977

3

g__[Ruminococcus]_gauvreauii_group	-0.060	0.967
g__[Ruminococcus]_gnavus_group	-0.194	0.943
g__[Ruminococcus]_torques_group	-0.118	0.967
f__Lachnospiraceae;g__uncultured	0.145	0.967
g__Peptococcus	-0.045	0.967
f__Peptococcaceae;g__uncultured	0.043	0.967
f__Peptostreptococcaceae;g__	0.188	0.943
g__Intestinibacter	0.202	0.943
g__Terrisporobacter	0.245	0.943
f__Ruminococcaceae;g__	0.007	1.000
g__Anaerotruncus	-0.067	0.967
g__Butyricicoccus	0.088	0.967
g__Faecalibacterium	-0.121	0.967
g__Flavonifractor	-0.188	0.943
g__Oscillibacter	0.027	1.000
g__Oscillospira	-0.090	0.967
g__Ruminiclostridium_1	-0.121	0.967
g__Ruminiclostridium_5	-0.067	0.967
g__Ruminiclostridium_6	-0.053	0.967
g__Ruminiclostridium_9	-0.306	0.929
g__Ruminococcaceae_NK4A214_group	0.071	0.967
g__Ruminococcaceae_UCG-001	0.129	0.967
g__Ruminococcaceae_UCG-002	0.077	0.967
g__Ruminococcaceae_UCG-003	-0.101	0.967
g__Ruminococcaceae_UCG-004	0.079	0.967
g__Ruminococcaceae_UCG-005	0.208	0.943
g__Ruminococcaceae_UCG-008	0.112	0.967
g__Ruminococcaceae_UCG-009	-0.017	1.000
g__Ruminococcaceae_UCG-010	-0.162	0.943
g__Ruminococcaceae_UCG-013	-0.100	0.967
g__Ruminococcaceae_UCG-014	-0.096	0.967
g__Ruminococcus_1	0.205	0.943
g__Ruminococcus_2	-0.017	1.000
g__Subdoligranulum	-0.301	0.929
g__[Eubacterium]_coprostanoligenes_group	0.095	0.967
f__Ruminococcaceae;g__uncultured	0.095	0.967
g__Catenibacterium	0.045	0.967
g__Catenisphaera	0.089	0.967
g__Erysipelatoclostridium	-0.306	0.929
g__Erysipelotrichaceae_UCG-003	0.053	0.967
g__Erysipelotrichaceae_UCG-004	0.103	0.967
g__Faecalicoccus	0.000	1.000
g__Faecalitalea	0.219	0.943
g__Holdemanella	-0.149	0.943
g__Solobacterium	-0.001	1.000
g__Turicibacter	0.027	1.000
g__[Clostridium]_innocuum_group	-0.238	0.943
f__Erysipelotrichaceae;g__uncultured	-0.173	0.943
g__Acidaminococcus	0.006	1.000
g__Phascolarctobacterium	0.047	0.967
g__Succiniclasticum	-0.018	1.000
g__Allisonella	0.052	0.967
g__Dialister	0.048	0.967
g__Megamonas	0.061	0.967
g__Megasphaera	0.007	1.000
g__Mitsuokella	-0.223	0.943
g__Veillonella	-0.018	1.000
g__Victivallis	-0.089	0.967

o__Victivallales;f__vadinBE97;g__uncultured_bacterium	-0.181	0.943
o__Victivallales;f__vadinBE97;g__uncultured_rumen_bacterium	0.079	0.967
f__Rhodospirillaceae;g__uncultured	-0.077	0.967
g__Parasutterella	-0.074	0.967
g__Sutterella	0.174	0.943
f__Comamonadaceae;g__	-0.219	0.943
g__Oxalobacter	-0.115	0.967
g__Bilophila	0.114	0.967
g__Desulfovibrio	-0.096	0.967
g__Succinivibrio	-0.026	1.000
f__Enterobacteriaceae;g__	0.107	0.967
g__Haemophilus	-0.029	1.000
g__Cloacibacillus	-0.219	0.943
g__Synergistes	-0.172	0.943
p__Tenericutes;c__;o__;f__;g__	-0.040	0.969
g__Anaeroplasma	-0.160	0.943
o__Mollicutes_RF9;f__;g__	0.183	0.943
o__Mollicutes_RF9;f__gut_metagenome;g__	-0.042	0.967
o__Mollicutes_RF9;f__uncultured_bacterium;g__	0.093	0.967
k__Bacteria;p__Tenericutes;c__Mollicutes;o__NB1-n;f__;g__	-0.219	0.943
c__Mollicutes;o__NB1-n;f__gut_metagenome;g__	0.232	0.943
c__Mollicutes;o__NB1-n;f__uncultured_bacterium;g__	0.000	1.000
c__Mollicutes;o__NB1-n;f__uncultured_bacterium;g__	0.151	0.943
o__Opitutae_vadinHA64;f__uncultured_bacterium;g__	0.052	0.967
g__Akkermansia	0.044	0.967

The population was used for analysis including 55 IBS patients. Abbreviations: IBS, Irritable Bowel Syndrome.

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Supplementary Table 5: Comparison of genus level taxa in relative abundance between IBS patients with different predominant stool patterns and controls over time

	Taxa at genus level	CT (mean ± SD)	CS (mean ± SD)	DH (mean ± SD)	MX (mean ± SD)	US (mean ± SD)	p values (CT vs CS)	p values (CT vs DH)	p values CT vs MX	p values (CT vs US)	p values (CS vs DH)	p values (CS vs MX)	p values CS vs US	p values (DH vs MX)	p values (DH vs US)	p values (MX vs US)
T1	g__Bifidobacterium	0.0955 ± 0.0747	0.0337 ± 0.0376	0.0483 ± 0.0588	0.0299 ± 0.0315	0.0716 ± 0.0836	**0.015**	**0.029**	**0.015**	0.360	1.000	0.881	0.416	0.943	0.360	0.360
	g__Collinsella	0.0147 ± 0.0161	0.0047 ± 0.0101	0.0039 ± 0.0070	0.0017 ± 0.0041	0.0062 ± 0.0133	**0.006**	**0.003**	**0.002**	**0.008**	0.946	0.857	0.946	0.857	0.946	0.946
	f__Ruminococcaceae;g__uncultured	0.0041 ± 0.0095	0.0123 ± 0.0177	0.0053 ± 0.0109	0.0019 ± 0.0022	0.0089 ± 0.0146	**0.040**	0.936	0.844	0.844	0.088	**0.040**	0.563	0.844	0.844	0.844
	g__Terrisporobacter	0.0005 ± 0.0019	0.0019 ± 0.0030	0.0038 ± 0.0048	0.0005 ± 0.0013	0.0008 ± 0.0015	0.119	**0.002**	0.794	0.454	0.329	0.329	0.454	0.070	0.119	0.710
	g__Turicibacter	0.0018 ± 0.0075	0.0021 ± 0.0053	0.0029 ± 0.0063	0.0010 ± 0.0015	0.0003 ± 0.0008	0.321	**0.025**	0.268	0.594	0.300	0.814	0.268	0.330	0.064	0.268
	f__Coriobacteriaceae;g__uncultured	0.0038 ± 0.0031	0.0092 ± 0.0139	0.0051 ± 0.0032	0.0017 ± 0.0018	0.0062 ± 0.0038	0.545	0.236	0.084	0.129	1.000	0.084	0.779	0.026	0.492	**0.026**
T2	g__Bifidobacterium	0.1021 ± 0.0702	0.0479 ± 0.0416	0.0511 ± 0.0489	0.0267 ± 0.0242	0.0665 ± 0.0534	**0.017**	**0.017**	**0.017**	0.412	0.892	0.599	0.599	0.599	0.635	0.599
	g__Barnesiella	0.0048 ± 0.0058	0.0119 ± 0.0119	0.0039 ± 0.0056	0.0066 ± 0.0065	0.0034 ± 0.0023	**0.026**	0.587	0.587	0.957	**0.026**	0.525	0.094	0.525	0.605	0.525
	g__Odoribacter	0.0001 ± 0.0005	0.0025 ± 0.0060	0.0001 ± 0.0004	0.0007 ± 0.0012	0.0007 ± 0.0011	**0.049**	0.826	0.173	0.153	0.109	0.826	0.826	0.267	0.247	0.944
	g__Alistipes	0.0079 ± 0.0069	0.0182 ± 0.0142	0.0078 ± 0.0072	0.0099 ± 0.0116	0.0089 ± 0.0071	**0.037**	0.976	0.982	0.976	**0.037**	0.207	0.159	0.976	0.976	0.976
	f__Clostridiales_vadinBB60_group;g__	0.0001 ± 0.0005	0.0023 ± 0.0048	0.0002 ± 0.0009	0.0000 ± 0.0000	0.0008 ± 0.0018	**0.018**	0.809	0.777	0.214	0.056	0.214	0.556	0.777	0.362	0.455
	g__Anaeroplasma	0.0000 ± 0.0000	0.0025 ± 0.0076	0.0000 ± 0.0000	0.0002 ± 0.0005	0.0000 ± 0.0000	**0.043**	NA	0.087	NA	0.069	0.621	0.161	0.145	NA	0.322
	g__Allisonella	0.0001 ± 0.0004	0.0000 ± 0.0000	0.0001 ± 0.0003	0.0007 ± 0.0008	0.0003 ± 0.0007	0.469	0.856	**0.014**	0.200	0.469	**0.012**	0.091	**0.030**	0.256	0.469
	f__Peptostreptococcaceae;g__	0.0066 ± 0.0075	0.0137 ± 0.0124	0.0044 ± 0.0035	0.0113 ± 0.0133	0.0042 ± 0.0049	0.065	0.660	**0.446**	0.611	**0.016**	0.493	0.065	0.180	0.617	0.223

Data are presented as mean ± SD. The population was used for analysis including 55 IBS patients and 28 controls at both timepoints. Abbreviations: IBS, Irritable Bowel Syndrome; CT, Control; CS, Constipation; DH, Diarrhoea; Mixed; MX; US, Unspecified; SD, standard deviation; NA: not available. For visibility, here only genera with significance between IBS patients with different predominant stool patterns and controls were shown.

Supplementary Table 6 Mixed model analysis between faecal SCFAs and psychological and IBS characteristics

	Acetate		Propionate		Butyrate	
	Estimate (95% CI)	*p-value*	*Estimate (95% CI)*	*p-value*	*Estimate (95% CI)*	*p-value*
Psychological characteristics						
Depression	-0.28 (-1.32; 0.76)	0.589	-0.01 (-0.31; 0.28)	0.920	-0.02 (-0.41; 0.37)	0.919
Anxiety	-0.24 (-0.98; 0.49)	0.524	-0.07 (-0.27; 0.14)	0.538	-0.00 (-0.28; 0.28)	0.995
Total IBS-QoL	0.03 (-0.15; 0.20)	0.766	0.03 (-0.02; 0.09)	0.177	-0.00 (-0.07; 0.07)	0.981
IBS characteristics						
IBS-SSS	-0.00 (-0.03; 0.03)	0.820	-0.01 (-0.01; 0.00)	0.183	0.00 (-0.01; 0.01)	0.742
Predominant stool pattern		**0.000**		**0.000**		**0.024**
Constipation	-3.8 (-12.7; 5.2)	0.406	-0.9 (-2.9; 1.0)	0.359	-2.5 (-5.7; 0.6)	0.117
Diarrhea	14.5 (5.5; 23.5)	**0.002**	3.4 (1.4; 5.4)	**0.001**	0.18 (-1.4; 5.1)	0.267
Mixed	9.9 (-0.4; 20.3)	0.059	1.6 (-0.6; 3.8)	0.162	1.8 (-1.9; 5.4)	0.337
Unspecified	Ref		Ref		Ref	

Mixed model analysis was done using scaled identify scale and identifying time as repeated factor and subject as random factor. SCFAs are dependent variables, psychological factors and IBS characteristics were put in the model as the independent variables as a fixed main effect. The longitudinal population was used for analysis, including 55 IBS patients and 28 controls. Abbreviations: IBS, Irritable Bowel Syndrome; IBS-QoL; IBS quality of life; IBS-SSS, IBS Symptom Severity Score; IBS-C, IBS constipation predominant; IBS-D, IBS diarrhea predominant; IBS-M, IBS alternating between constipation and diarrhea; IBS-U, IBS unspecified type.

Supplementary Table 7 Spearman correlations between SCFAs and dietary intake

|	Acetate		Propionate		Butyrate	
	Spearman rank	*p-value*	*Spearman rank*	*p-value*	*Spearman rank*	*p-value*
Energy (kcal)	0.038	0.678	0.026	0.777	-0.015	0.872
Total protein (g)	0.019	0.835	0.027	0.768	-0.050	0.587
Plant protein (g)	0.035	0.707	-0.002	0.981	0.009	0.923
Animal protein (g)	0.007	0.937	-0.026	0.781	-0.063	0.495
Total fat (g)	0.036	0.693	0.016	0.858	-0.020	0.829
Saturated fat (g)	0.042	0.645	0.038	0.682	-0.019	0.835
Single unsaturated fat (g)	0.046	0.617	0.017	0.856	0.001	0.990
Multiple unsaturated fat (g)	0.054	0.558	0.021	0.818	0.002	0.978
Total carbohydrates (g)	0.031	0.736	0.037	0.691	0.003	0.974
Monosaccharides (g)	-0.031	0.738	-0.066	0.473	-0.072	0.436
Lactose (g)	-0.135	0.140	-0.094	0.306	-0.182	**0.046**
Polysaccharides (g)	0.089	0.329	0.118	0.196	0.079	0.389
Dietary fibre (g)	0.003	0.977	-0.094	0.306	-0.027	0.773
Water (g)	-0.009	0.922	-0.137	0.134	-0.077	0.401
Alcohol (g)	0.116	0.205	0.077	0.399	0.058	0.524

Data includes 91 IBS patients and 30 controls. SCFA levels at the first timepoint are compared to dietary intake.

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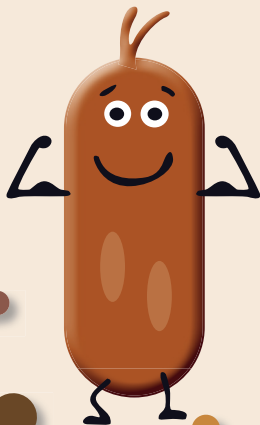
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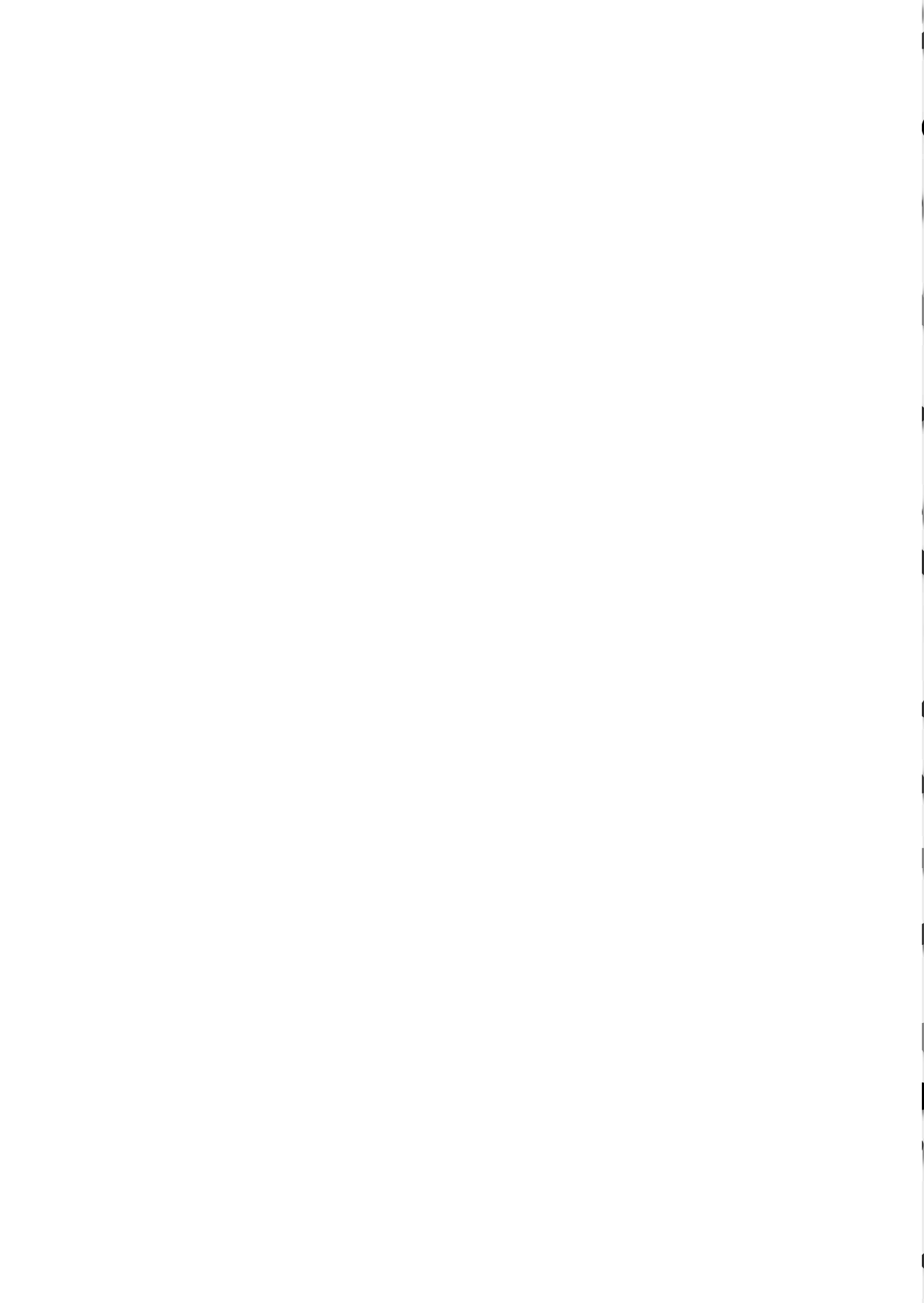
CHAPTER 4

Methanogen levels significantly associate with faecal microbiota composition and alpha diversity in healthy adults and irritable bowel syndrome patients

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Abstract

Hydrogenotrophic microbes, primarily including the three functional groups methanogens, sulphate reducing bacteria and reductive acetogens, use hydrogen as an energy source and play an important role in maintaining the hydrogen balance in gut ecosystems. A distorted hydrogen balance has been associated with irritable bowel syndrome (IBS). However, the role of hydrogenotrophic functional groups in maintaining this balance in healthy adults (HA) and IBS patients remains largely unknown. This study aims to assess the distribution and stability of hydrogenotrophic functional groups in HA and IBS patients, and their association with total microbiota composition and IBS symptoms, such as severity score, depression, anxiety and quality of life. A two-timepoint study with four weeks in between was performed with n=55 IBS patients and n=27 HA, who completed the whole study. Analysis of microbiota composition revealed that methanogens showed a bimodal distribution across samples. A high-level methanogen microbiota was consistently associated with higher alpha diversity, and its composition was significantly different from that of individuals with low-level methanogens. In general, associations of methanogen levels with microbial community characteristics were stronger in IBS patients than in HA. The differences in copy numbers of genes indicative of total bacteria (16S rRNA gene) and acetogens (acsB) between HA and IBS patients, and their correlations with IBS symptom severity, anxiety, depression and QoL were sampling time-dependent. Hydrogenotrophic functional groups coexisted in the human gut and did not show negative abundance correlations with each other in HA and IBS patients. These findings suggest that methanogens in the gut have a strong association with microbiota alpha diversity and composition, and that identifying a robust microbial signature of IBS and its symptoms related to hydrogenotrophic microbes requires a longitudinal study design.

Keywords: Hydrogenotrophic functional groups; qPCR; faecal microbiota composition; sulphate-reducing bacteria; acetogens

Introduction

The human gut microbiota, comprising hundreds to thousands of microbial species, and it ferments dietary fibres that escape digestion and absorption, which results in the production of metabolites, such as short–chain fatty acids, carbon dioxide and hydrogen [1-3]. Hydrogen is one of the main gases produced by hydrogen-producing microbes (hydrogenogens) with an estimated daily production of more than 13 litres in people consuming a typical Western diet [4]. Hydrogen accumulation leads to a high partial pressure that would inhibit the regeneration of the coenzyme NAD^+ from NADH, and thermodynamically restrict further microbial fermentation and growth [5, 6]. The critical disposal of hydrogen during bacterial fermentation is done via hydrogen-consuming microbes (hydrogenotrophs) [4], which decrease the partial pressure of hydrogen and maintain the hydrogen balance in gut ecosystems [7]. The collection of hydrogenotrophic microbes in the human gut, collectively called hydrogenotrophic microbiota, consists of three major functional groups, namely methanogens that use hydrogen and carbon dioxide producing methane, reductive acetogens that use hydrogen and carbon dioxide producing acetate, and sulphate-reducing bacteria (SRB) that use hydrogen and sulphate producing hydrogen sulphide [4, 6].

It has been reported that hydrogenotrophic microbes are associated with Irritable bowel syndrome (IBS) [5, 6]. IBS is the most commonly diagnosed functional gastrointestinal disorder with an estimated 11% of people being affected globally [8]. IBS is characterized by symptoms of abdominal pain typically accompanied by bloating and alterations in stool pattern [9], which reduces the Quality of Life (QoL), work productivity, and increases health costs [8]. Although the aetiology of IBS is still unclear, a distorted hydrogen metabolism is suggested to play a role in IBS [5, 6]. Accumulating hydrogen is considered to be associated with IBS symptoms abdominal pain and bloating, and a higher hydrogen concentration in exhaled breath has been observed in IBS patients compared to healthy adults (HA) [6, 10, 11]. A decreased colonic transit motility is considered to be associated with a higher methane concentration and methanogen count in both healthy people and IBS patients [12, 13], and the severity of constipation in IBS has been reported to be correlated with a higher exhaled breath methane concentration [14]. Higher SRB numbers have been found in constipated-IBS patients compared to HA [16], and hydrogen sulphide produced by SRB has been linked to the modulation of pain-related signals, implicating a potential role of hydrogen sulphide in the primary IBS symptom of abdominal pain [15]. Although these studies mainly compared the hydrogenotrophic functional groups between HA and IBS patients, the association of hydrogenotrophic functional groups with microbiota composition, IBS symptoms, such as severity, depression, anxiety and QoL, and their relationships with each

other remain largely unknown. Moreover, although gut microbiota composition and IBS symptoms such as severity scores have shown fluctuations over time [17-19], the stability of hydrogenotrophic functional groups and their roles in HA and IBS patients have not been investigated.

Therefore, we performed a two-timepoint study over a period of four weeks, aiming to assess the distribution and stability of hydrogenotrophic functional groups in HA and IBS patients, and their association with IBS symptoms, such as symptom severity, depression, anxiety and QoL over time.

Materials and Methods

The details of the study design, subject recruitment, questionnaire data collection and faecal microbiota composition profiling are described in **Chapter 3**. Briefly, this was an observational study including two timepoints (T1 and T2) with four weeks in between. Ninety-one IBS patients and 30 healthy adults who were matched for age, gender and Body Mass Index (BMI) were recruited. All participants signed an informed consent. This study was approved by the medical ethics committee of Wageningen and registered at Clinicaltrials.gov (NCT03720314).

Study design

A subset of subjects who completed both timepoints (n=27 HA and n=55 IBS patients) were included in this study (Figure 1). Recruited subjects were aged 18-65 years, and had a BMI between 18.5-30.0 kg/m^2. IBS patients fulfilled the Rome IV criteria or were diagnosed with IBS by a physician. IBS patients and HA that had any other gastrointestinal or systemic diseases, antibiotics use less than three months before the study started, pregnancy or breastfeeding were excluded. Questionnaires were completed by HA and IBS patients at both timepoints for comparison. The validated IBS Symptom Severity Score (SSS) was used to assess IBS severity [20]. The 34-item IBS-QoL was used to assess QoL, which gave a score for total IBS-QoL, and subscales dysphoria, interference with activity, body image, health worry, food avoidance, social reaction, sexual life and relationship [21]. The Hospital Anxiety and Depression Score [22] was completed by subjects with a score ≥8 indicating substantial depressive or anxious symptoms [23]. The seven types of the validated Bristol Stool chart were used to assess the Bristol stool scale of the faecal sample [24]. A semi-quantitative 83-item Food Frequency Questionnaire was used to assess the habitual dietary intake of the month preceding T1 [25, 26]. The Dutch Food Composition table was used to calculate

the dietary intake [27]. Subjects were instructed to keep their diet similar during the study period. Faecal samples were collected at both timepoints. Faecal microbiota composition was determined based on the V4 region sequence of the bacterial and archaeal 16S ribosomal RNA (rRNA) gene (Illumina Hiseq2500, 150bp paired end). Total bacteria, methanogens, acetogens and SRB were quantified by quantitative Polymerase Chain Reaction (qPCR).

Total bacteria and hydrogenotrophic functional group profiling

Total bacteria was quantified by determining the 16S rRNA gene copy number, while methanogens, SRB, and acetogens were quantified by determining the copy number of the functional genes *mcrA*, *dsrA* and *acsB*, respectively (Table 1), which are representative for their respective metabolic pathways [4, 28]. Standards for quantification included the amplified 16S rRNA gene of *Escherichia coli* JM109 for total bacteria and genomic DNA isolated from *Desulfovibrio piger* DSM 749 , *Blautia hydrogenotrophica* DSM 10507 and *Methanobrevibacter smithii* DSM 861 for *dsrA*, *acsB* and *mcrA*, respectively. Standards were serially ten-fold diluted with nuclease-free water. Sample DNA concentration was adjusted to 1 ng/µL with nuclease-free water for qPCR.

All qPCRs were carried out in triplicate with an iCycler iQ real-time detection system (Bio-Rad Laboratories BV). Each reaction mixture with a total volume of 10 µL contained 5 µL 2× iQ SYBR green (Bio-Rad Laboratories B.V.), 2 ng DNA template, forward and reverse primers and nuclease-free water. For each group, the primers selection and annealing temperature were chosen as listed in Table 1.

Table 1: List of the qPCR reactions in this study

Group	Gene	Primers	Annealing temp (°C)	Standard	References
Total bacteria	16S rRNA	Uni331 F Uni797 R	60°C	*E. coli* JM109	[29]
Methanogens	*mcrA*	qmcrA-F qmcrA-R-d	60°C	*M. smithii* DSM 861	[30]
SRB	*dsrA*	DSR1-F DSR-R	60°C	*D. piger* DSM 749	[31]
Acetogens	*acsB*	ACS_f ACS_r	52°C	*B. hydrogenotrophica* DSM 10507	[32]

SRB: Sulphate-reducing bacteria; *mcrA*: subunit of Methyl Coenzyme M Reductase genes; *dsrA*: subunit of dissimilatory (bi)sulphite reductase genes; *acsB*: subunit of acetyl-CoA synthase genes.

Faecal microbiota composition analysis

Faecal microbiota composition was determined by sequencing the V4 region of the 16S rRNA gene, and NG-Tax 2.0 was used to process the raw sequence data for Amplicon Sequencing Variant (ASV) picking with default settings and for taxonomic assignments as described in **Chapter 3**. Alpha diversity (within-sample diversity) and beta diversity (between-sample diversity) were calculated at the ASV level using Phyloseq [33]. For Alpha diversity metrics, ASV richness, Shannon diversity and phylogenetic diversity were calculated. To visualize beta diversity, Principle Coordinate Analysis (PCoA) based on unweighted (considering presence/absence of ASVs) and weighted (considering ASVs and their relative abundance) Unifrac [34] distances, and Bray-Curtis dissimilarity was performed. Subjects were stratified into high-level methanogens (HM) or low-level methanogens (LM) based on if methanogens (genus *Methanobrevibacter* and *Methanosphaera*) were detected in the 16S rRNA gene amplicon data with a threshold of 0.1% (HM, methanogen relative abundance ≥ 0.1%; LM, methanogen relative abundance < 0.1%). Linear Discriminant Analysis (LDA) Effect Size (LEfSe) was used to determine the differences in microbial gene-level taxa between LM and HM subjects [35].

Statistical analysis

Continuous data were presented as mean ± standard deviation, or median and interquartile range when skewed. Categorical data were presented as counts and percentages. Differences between HA and IBS patients or between HM and LM were tested with one-way Analysis of Variance (ANOVA), or Mann-Whitney U test when not normally distributed. Differences for categorical data were assessed using a Pearson's Chi-square test. The significance of the correlation between total bacteria and hydrogenotrophic functional groups, the correlation between hydrogenotrophic functional groups and questionnaire data, and the correlation between the functional genes and alpha diversity metrics were determined using Spearman's rank correlation coefficient. All of the data was analysed in R version 4.0.0 [36].

All of the p-values for the multiple pairwise tests were corrected using Benjamini-Hochberg false-discovery rate (FDR). A p-value (or corrected p-value) < 0.05 was considered to indicate statistical significance, and a trend was considered when 0.05 ≤ p-value (or corrected p-value) < 0.1.
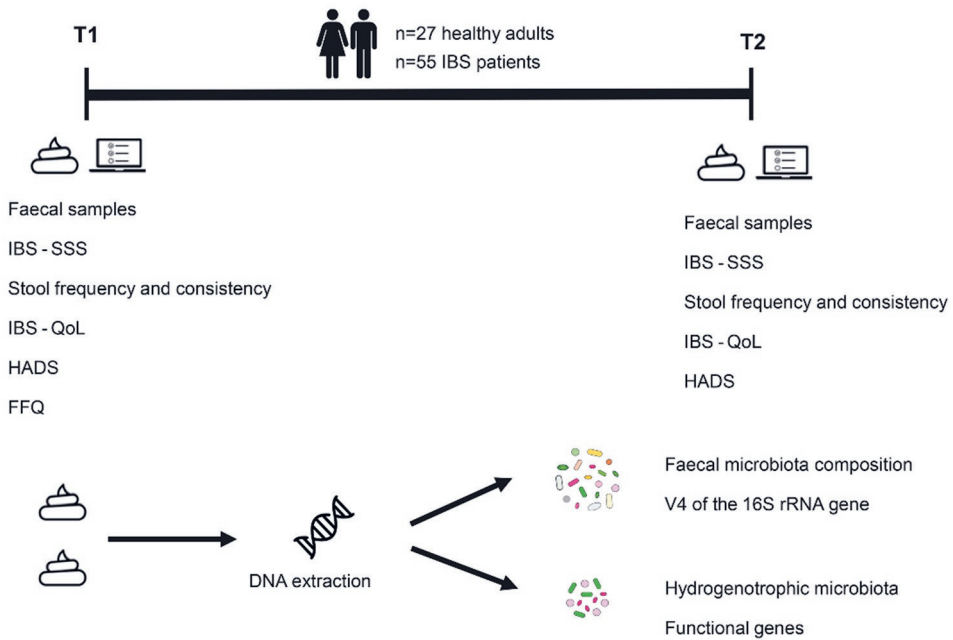
Figure 1: Schematic overview of the study design. 55 IBS patients and 27 healthy adults were included in this study. Faecal samples and questionnaire data were collected. Faecal microbiota composition was analysed by sequencing the V4 region of the 16S rRNA gene, and three different groups of hydrogenotrophic microbiota (methanogens, sulphate-reducing bacteria, acetogens) were quantified by quantitative PCR targeting representative functional genes. IBS: Irritable Bowel Syndrome; SSS: Symptom Severity Score; QoL: Quality of Life Questionnaire; HADS: Hospital Anxiety and Depression score; FFQ: Food Frequency Questionnaire.

Results

For this study a total of 27 HA and 55 IBS patients were compared at two timepoints with four weeks in between. The age, gender and BMI of HA and IBS patients were matched and thus were not significantly different (Table 2). Baseline characteristics differed between HA and IBS patients for IBS-SSS ($p < 0.001$), IBS-QoL ($p < 0.001$), anxiety ($p < 0.05$) and depression ($p < 0.05$), while no difference in the Bristol stool scale was observed between HA and IBS patients. IBS patients had a lower intake of lactose ($p < 0.05$) and maltose ($p < 0.05$), but a higher trend for glucose intake ($p = 0.059$) (Supplementary Table 1)**.** A lower trend of protein intake ($p = 0.073$) in IBS patients was observed compared to HA. No significant difference was observed in dietary intake of energy, fat, carbohydrates, polysaccharides, dietary fibre, alcohol or water between HA and IBS patients.

Table 2: Baseline characteristics of the study population with 55 IBS patients and 27 healthy adults (HA)

	IBS (n=55)	HA (n=27)	p values
Age (years)	42.0 (26.0 – 52.5)	35.0 (22.5 – 38.4)	0.590
Gender n (%) male	9 (16.4)	3 (11.1)	0.764
BMI (kg/m^2)	22.8 ± 2.8	23.3 ± 3.0	0.508
IBS-SSS	140 (100 – 20)	60 (30 – 90)	**0.000**
Bristol stool scale	3 (3 – 4)	4 (3 – 6)	0.270
IBS-QoL	75.7 (57.4 – 85.3)	99.3 (98.9 – 100.0)	**0.000**
Anxiety score	7.0 (4.0 – 11.5)	4.0 (3.0 – 6.0)	**0.003**
Depression score	2.0 (1.0 – 5.5)	(0.5 – 2.5)	**0.025**

Data are presented as mean ± standard deviation or median (interquartile range) when skewed. Age, IBS-SSS, Bristol stool scale, IBS-QoL, anxiety score and depression score were tested with the Mann-Whitney U test. BMI was tested with an independent sample t-test. Gender was tested with a Pearson's Chi-square test. Abbreviations: BMI, Body Mass Index; IBS-SSS, IBS Symptom Severity Score; IBS-QoL, IBS Quality of Life.

Differences in total bacteria and acetogens between healthy adults and IBS patients are sampling time dependent

Differences in copy numbers of genes representative of total bacteria and hydrogenotrophic functional groups within individuals were observed over time (Figure 2A). Total bacterial 16S rRNA gene copies significantly decreased over time in both HA (p < 0.001) and IBS patients (p < 0.001). A much lower *acsB* copy number (p < 0.001) at T2 was observed compared to that at T1 in HA. In contrast, *acsB* copy numbers remained stable over time in IBS. Neither for *dsrA* nor for *mcrA*, any significant differences were observed between timepoints in either HA or IBS patients. When comparing gene copy numbers between HA and IBS patients at each timepoint, we observed a higher copy number of total bacterial 16S rRNA genes and *acsB* in HA (p < 0.001) at T1, while these differences were not observed at T2 (Figure 2B). For copy numbers of *mcrA* and *dsrA*, no differences were found between HA and IBS at both timepoints. Stratification of IBS patients into subgroups based on the predominant stool patterns demonstrated that only total bacteria (p < 0.001) and *acsB* (p < 0.01) were significantly lower in constipation-predominant IBS compared to HA at T1 (Supplementary Table 2, Supplementary Figure 1).

Correlations between hydrogenotrophic functional groups and IBS symptoms are sampling time-dependent

The demographic data and symptoms measured in HA and IBS patients did not change during the study, except for IBS-QoL sub-indices Dysphoria, Body image, and Relations in IBS patients (Supplementary Table 3). Spearman's correlations of the IBS symptoms with the copy number of total bacterial 16S rRNA genes, and genes indicative of the different hydrogenotrophic functional groups indicated that total bacterial 16S rRNA gene and *acsB* were negatively correlated with IBS symptom severity, anxiety, and depression, but positively correlated with IBS-QoL and its sub-indexes at T1 (Figure 3). However, we did not observe any of these correlations at T2. For the demographic data, only age was positively correlated with *mcrA* at T1, and the Bristol stool scale was positively correlated with counts of total bacterial 16S rRNA genes and *acsB* at T2. However, when only IBS patients were included in the correlation analyses, *mcrA* and *acsB* were positively correlated with age and Bristol stool scale, respectively, at both timepoints, while other correlations were only significant at one of the two timepoints (Supplementary Figure 2). Overall, these observations indicate that the correlations between hydrogenotrophic functional groups and study population characteristics varied over time and were depended on the selection of the population as well.
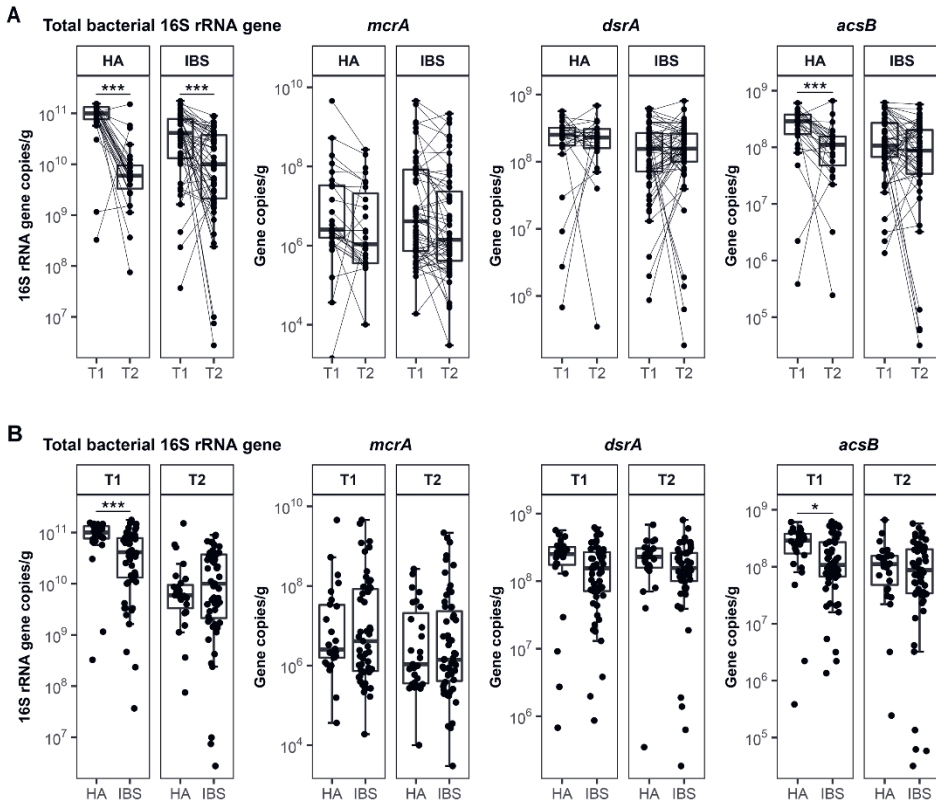
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Figure 2: Box plots showing the stability of total bacterial 16S rRNA gene counts and hydrogenotrophic functional groups over time (A), and timepoint specific comparisons between healthy adults (HA) and IBS patients (B). Samples taken at different timepoints are connected by solid lines per subject. Differences between groups were tested with the Mann-Whitney U test. * $p < 0.05$; ** $p < 0.01$. *** $p < 0.001$.
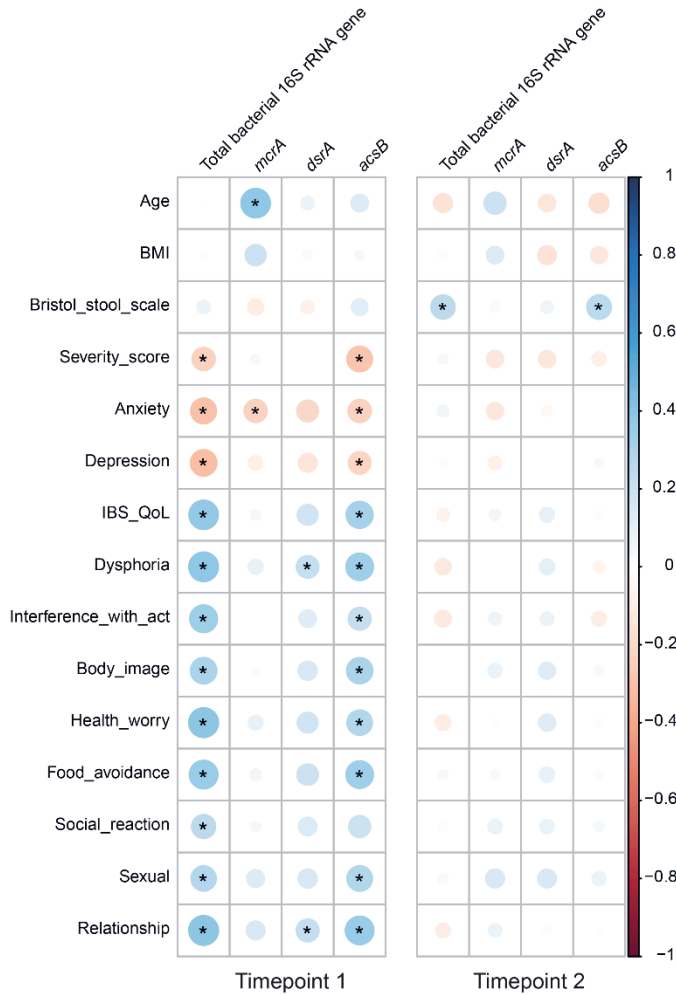
Figure 3: Spearman's correlation analyses of copy numbers of total bacterial 16S rRNA genes and functional genes representative of the different hydrogenotrophic functional groups with population characteristics over time. This analysis includes 55 IBS patients and 27 healthy adults. Significant correlations (p < 0.05) are indicated with an asterisk. Abbreviations: BMI, Body Mass Index; IBS-QoL, IBS Quality of Life.

Methanogen levels have a strong association with faecal microbiota alpha diversity and composition

Spearman correlation was performed between alpha diversity metrics (phylogenetic diversity, ASV richness and Shannon diversity), and total bacteria and hydrogenotrophic functional groups in both HA and IBS patients at both timepoints (Figure 4). Remarkably, *mcrA* was positively correlated with phylogenetic diversity, ASV richness and Shannon diversity in both HA and IBS patients at both timepoints. In contrast, no significant correlation was observed between faecal microbiota alpha diversity indices and *dsrA*. As for *acsB*, a negative correlation with phylogenetic diversity and ASV richness was observed at T2 in IBS, however, this was not observed at T1.
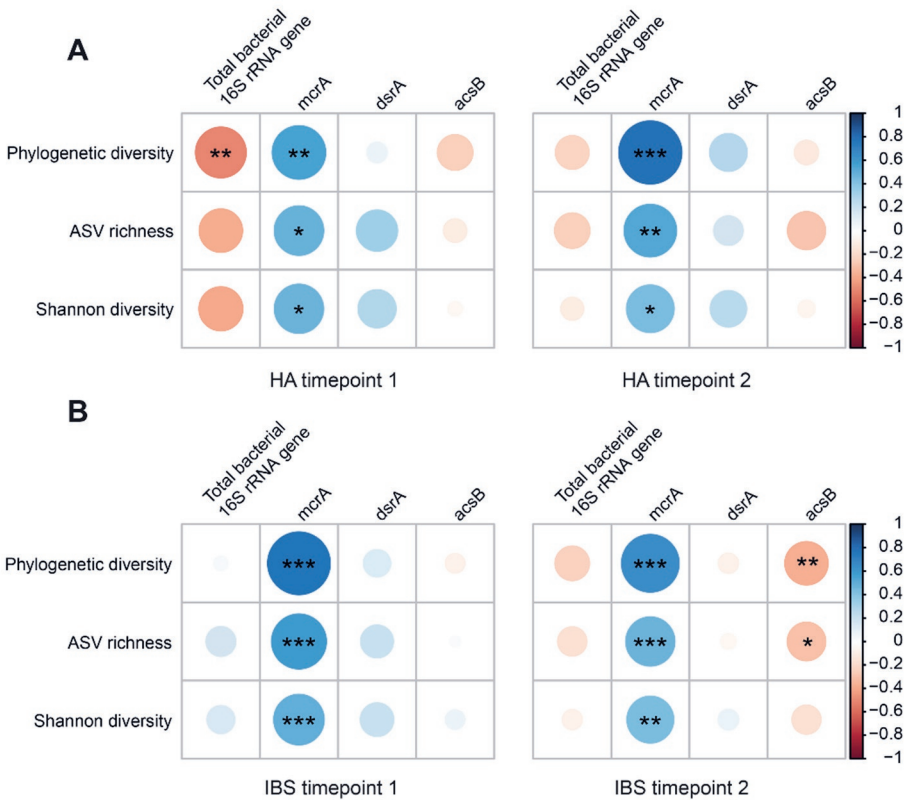

Figure 4: Spearman's correlation between hydrogenotrophic functional groups and the microbial alpha diversity metrics phylogenetic diversity, ASV richness and Shannon diversity in HA (A) and IBS patients (B) over time. P-values were highlighted with different asterisks: * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$. HA: healthy adults.

The abundance of the genus *Methanobrevibacter* in the human gut shows a bimodal distribution, which means that this genus is either very abundant or nearly absent [37]. Despite the limited number of measurements in this study, the abundance distribution of *mcrA* showed two peaks in both HA and IBS patients, which confirmed that methanogens had a bimodal distribution in our study populations (Supplementary Figure 3). In contrast, this bimodality was not observed with the functional genes indicative for SRB or acetogens. To investigate how methanogens were associated with total microbiota composition, subjects were stratified into high-level methanogens (HM) or low-level methanogens (LM) based on if methanogens (genus *Methanobrevibacter* and *Methanosphaera*) were detected in the 16S rRNA gene amplicon data with a threshold of 0.1% (HM, methanogen relative abundance ≥ 0.1%; LM, methanogen relative abundance < 0.1%). The ratio of subjects with HM or LM did not differ over time. In addition, no difference in HM-LM ratio was found between HA and IBS patients at both timepoints (Supplementary Table 4). As expected, this stratification resulted in a significant difference in *mcrA* between HM and LM (Supplementary Figure 4). In contrast, no significant abundance differences were observed in total bacterial 16S rRNA genes, nor the genes indicative of SRB and acetogens.

Remarkably, the stratification of subjects based on methanogen level indicated a strong association with microbiota alpha diversity indices and microbiota composition (Figure 5). Both HA and IBS patients with HM had a higher phylogenetic diversity (Figure 5A, $p < 0.001$) compared to those with LM at both timepoints, which is expected because methanogens as an independent domain (archaea) play an important role in determining the phylogenetic distance within the algorithm. Nevertheless, even without considering the phylogenetic distance, ASV richness (Figure 5B, $p_{T1} < 0.001$, $p_{T2} < 0.001$) and Shannon diversity (Figure 5C, $p <.001$, $p < 0.001$) were significantly higher in the IBS patients with HM than in those with LM at both timepoints as well. In contrast, for ASV richness and Shannon diversity, we did not observe a significant difference between HM and LM in HA. In addition, PERMANOVA revealed significant differences between the faecal microbiota of subjects with HM and those with LM based on unweighted (Figure 5D, $p < 0.001$, $R^2 = 23.27\%$) and weighted Unifrac (Figure 5E, $p < 0.001$, $R^2 = 7.83\%$) distance, as well as Bray-Curtis dissimilarity (Figure 5F, $p < 0.001$, $R^2 = 3.73\%$) with samples from both HA and IBS patients included. As could be expected, a larger effect based on UniFrac distances that take phylogeny into account was observed for differences between HM and LM. Similar observations were found when HA and IBS patients were separately analysed, however, significant differences between HM and LM based on Bray-Curtis dissimilarity were only found in IBS at both timepoints (Supplementary Figure 5, $p_{T1} < 0.001$, $R^2_{T1} = 5.00\%$, $p_{T2} < 0.001$, $R^2_{T2} = 5.98\%$), whereas this was only observed at T1 in HA

($p_{T1} < 0.05$, $R^2_{T1} = 5.21\%$, $p_{T2} = 0.079$, $R^2_{T2} = 5.02\%$), indicating that methanogen levels have a stronger association with the microbiota composition in IBS patients than in HA. To further determine in more detail which taxa were associated with HM and LM, a LEfSe analysis was performed (Figure 5G, 5H, Supplementary Figure 6). This indicated that besides methanogens, the family *Christensenellaceae* was consistently associated with HM over time in both HA and IBS patients. In HA the family-level taxon *Ruminococcaceae* and genus *Phascolarctobacterium* showed a higher relative abundance in HM, whereas the relative abundances of the family *Veillonellaceae* and genus *Dialister* were higher in LM. However, this was not observed in IBS patients.
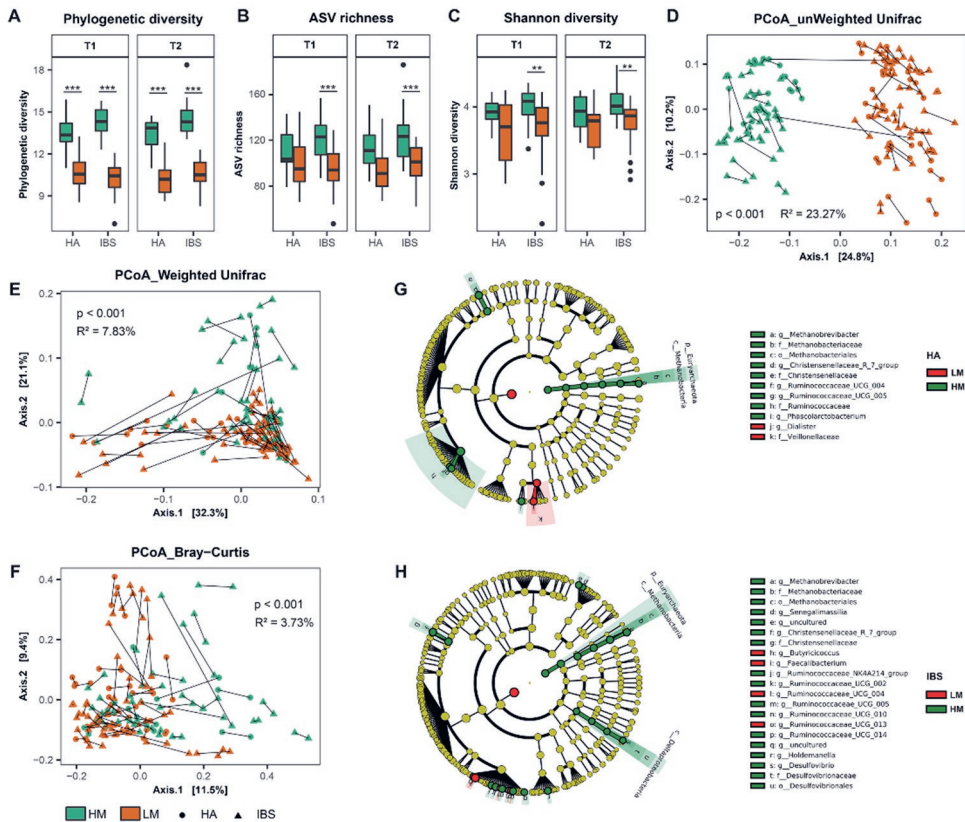
Figure 5: Stratification of subjects (27 healthy adults and 55 IBS patients) based on methanogen-level shows a distinct microbiota composition associated with HM. Significance was observed in phylogenetic diversity (A), ASV richness (B), and Shannon diversity (C) between HM and LM in IBS patients at both timepoints. Mann-Whitney U test was used to test for significance. PCoA of microbiota composition was carried out based on unweighted (D) and weighted (E) Unifrac distances, and Bray-Curtis dissimilarity (F). PERMANOVA showed that the microbiota composition of HM is different from that of LM in IBS at both timepoints. Samples taken at different timepoints are connected by solid lines per subject. Cladogram representing the genera that were significantly different between HM and LM in healthy adults (G) and IBS patients (H) is presented using the linear discriminant analysis effect size (LEfSe) method. HM and LM were assigned as class. Timepoint 1 and timepoint 2 were assigned as subclass. Taxa or nodes highlighted in red and green were significantly more abundant in LM or HM, respectively. Nomenclature of microbial genus-level taxa is based on the highest achievable taxonomic resolution at phylum, class, order, family or genus level. * p < 0.05; ** p < 0.01, *** p < 0.001. HM: High-level methanogens; LM: Low-level methanogens.

Hydrogenotrophic functional groups did not show negative abundance correlations with each other

Remarkably, no negative correlations were found between the different hydrogenotrophic functional groups in either HA or IBS patients over time (Figure 6). Total bacterial 16S rRNA gene copy numbers were positively correlated with *acsB* at both timepoints in HA and IBS patients. Total bacterial 16S rRNA gene and *dsrA* were positively correlated at both timepoints in IBS patients, while in HA, the positive correlation was only observed at T2. No correlation was observed between the bacterial 16S rRNA gene and *mcrA*. As for the copy number of *acsB*, it was positively correlated with *dsrA* in both HA and IBS patients at both timepoints. In contrast, *acsB* was not correlated with *mcrA*, and *mcrA* was not correlated with *dsrA* in HA, while they were positively correlated in IBS patients at T1, but not at T2. Collectively, these findings indicate that the hydrogenotrophic functional groups may co-exist and the fact that no negative correlations were observed may suggest that these hydrogenotrophic functional groups did not compete with each other in the gut ecosystems of HA and IBS patients.
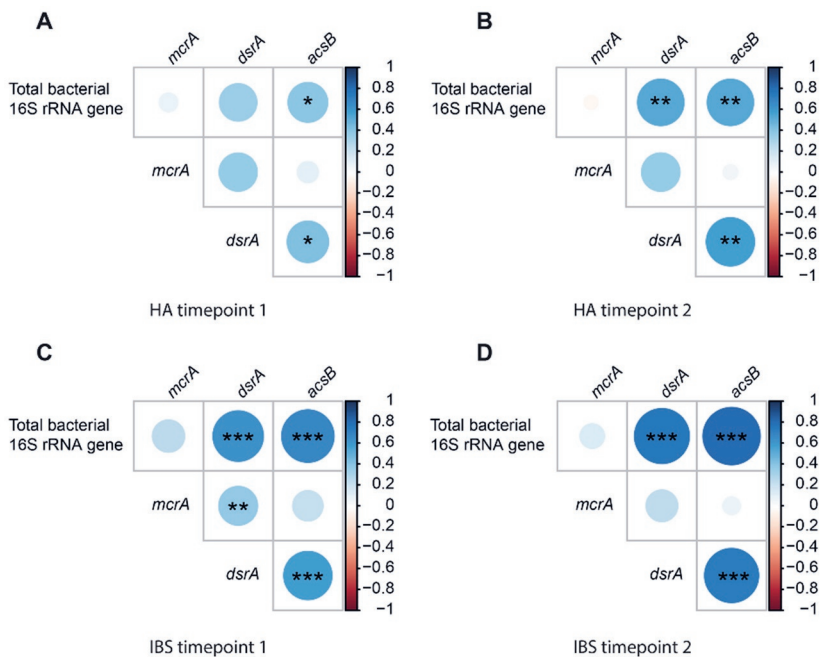

Figure 6: Correlation between total bacteria and hydrogenotrophic functional groups. Subjects are stratified into HA (A, at T1; B, at T2) and IBS (C, at T1; D, at T2) with two timepoints. Statistical significance was determined using the Spearman method.   * p < 0.05; ** p < 0.01, *** p < 0.001.

Discussion

In this study, we compared the abundance of total bacteria and three different hydrogenotrophic functional groups, namely methanogens, SRB and acetogens, between HA and IBS patients at two timepoints spanning a period of four weeks, and showed an association of methanogen levels with microbiota alpha diversity and composition. Moreover, we investigated the correlation of hydrogenotrophic functional groups with each other and IBS symptoms, such as severity, depression, anxiety and QoL. We mainly found that total bacteria and acetogens showed differences between HA and IBS patients and correlations with IBS symptoms, such as severity score, anxiety, depression and QoL, albeit depending on the time of sampling. The different hydrogenotrophic functional groups coexisted and were not found to negatively correlate, suggesting that they may not compete in the human gut. Higher faecal methanogen levels were significantly associated with higher microbiota alpha diversity and showed compositional differences compared to low methanogen levels and more so in IBS patients than in HA.

Cross-sectional studies have been commonly used to identify microbial signatures associated with IBS [38]. Previously, no difference in total bacterial abundance was observed between IBS patients and healthy controls [39]. However, using a two-timepoint comparison in our study, the difference in total bacterial abundance between HA and IBS patients varied over the short four-week period included in this study. Besides total bacterial abundance, we also observed differences between timepoints in the abundance of hydrogenotrophic functional groups within individuals. In addition, we observed the abundances of total bacteria and reductive acetogens were correlated with IBS symptom severity and other IBS symptoms, such as anxiety, depression and QoL at T1, but this was not the case at T2, indicating that correlations at a single timepoint can be coincidental as has been shown previously [40] and in **Chapter 3**. This highlights that snapshot cross-sectional comparisons or correlations may not reliably identify microbial signatures in chronic diseases without a longitudinal sampling process.

It has been considered that different hydrogenotrophic functional groups may compete because all of them use hydrogen as an energy source [4, 5]. To support this, a mutually exclusive relationship between methanogens and SRB has been suggested, where SRB are rarely detected in the gut microbiota of methane excretors that have a significantly higher number of methanogens than non-methane excretors, while the gut microbiota of non-methane excretors harbours higher numbers of SRB [41]. However, other studies did not observe such mutual exclusivity, and no significant relationship was found between methanogens and SRB [42, 43]. To this end, our study did not show negative abundance correlations

4

between any of the three hydrogenotrophic functional groups over time, neither in HA nor in IBS patients. An explanation why they coexist could be that hydrogen supply is not a limiting factor or that hydrogen is considered essential to methanogens, whereas SRB and reductive acetogens are more metabolically flexible. SRB can also use other compounds, such as lactate in the absence of hydrogen, and thereby become a hydrogen-producer [44, 45]. Reductive acetogens are not restricted to hydrogen as an energy source either and are instead also able to ferment carbohydrates [46, 47]. Considering the complexity of gut ecosystems, including nutrient supply, variable environmental conditions throughout the gut as well as the metabolic flexibility of some of the hydrogenotrophic microbes, the interactions between hydrogenotrophic microbes in the gut are difficult to decipher [48]. Therefore, insights into the interactions between the three hydrogenotrophic functional groups are needed, and can be obtained for example by co-culturing them *in vitro* or co-colonisation *in vivo*.

Hydrogen metabolism differs between IBS patients and HA, and it has been reported that hydrogen production is higher in IBS patients than healthy controls, suggesting that bacterial fermentation may be an important factor in IBS pathogenesis [49]. Interestingly, in this study, compared to HA, methanogens were found to have a stronger association with faecal microbiota alpha diversity and composition in IBS patients. Hence it could be that higher hydrogen production increases the association of microbiota composition with methanogens in IBS. However, due to the lack of hydrogen and methane data in this study, this remains speculative and requires further investigation.

Methanobrevibacter is the dominant methanogenic genus in the human gut ecosystem, using hydrogen and carbon dioxide for the production of methane. A bimodal distribution of *Methanobrevibacter* was reported [37], which is in line with our finding that methanogens quantified via *mcrA* showed a bimodal distribution as well. In addition, based on the detectable breath methane, people can be stratified into methane excretors with a significantly higher number of methanogens ($\sim 10^9$ CFU/g) and non-methane excretors with significantly lower methanogen counts ($\sim 10^4$ CFU/g) in stool [4, 50, 51]. Remarkably, in this study stratifying subjects into HM and LM showed that subjects with HM harboured a faecal microbiota with a higher alpha diversity and a distinct microbiota composition compared to subjects with LM. Since this was also observed with algorithms that do not include phylogenetic distance, these observations cannot be explained solely by the fact that phylogenetically distinct methanogenic archaea differed in prevalence and abundance, but is also related to differences with respect to other, bacterial, taxa. Hydrogen accumulation leads to a higher hydrogen partial pressure that would inhibit the regeneration of the coenzyme NAD^+ from NADH, and thermodynamically

restrict further microbial fermentation and growth [5, 6]. Hydrogenotrophic microbes play an essential role in reducing the hydrogen partial pressure and facilitating the fermentation process. We speculate that methanogens contribute strongly to the hydrogen partial pressure and thereby facilitate fermentation capacity for a diverse microbiota. This is supported by the findings that cellulose-degrading bacterial communities in the human gut differ between individuals according to the presence or absence of methanogens [52]. When looking at taxa associated with HM or LM, we observed that the family *Christensenellaceae* was consistently associated with HM in both HA and IBS patients. Co-culturing the species from the *Christensenellaceae* and *M. smithii* indicated a syntrophic relationship between *Christensenella* and *Methanobrevibacter* via interspecies hydrogen transfer [53]. Hence, the consistent cooccurrence of both taxa observed in this study likely reflects their syntrophic interaction in the intestine with HM, which may affect total microbiota composition.

In conclusion, an HM microbiota was consistently associated with higher alpha diversity, and its composition was significantly different from that of LM. Since we observed a consistent co-occurrence between *M. smithii* and *Christensenellaceae*, we speculate on syntrophic interactions between these taxa to play an important role in the HM ecosystem. Fluctuations in time of the abundance of hydrogenotrophic functional groups, IBS symptoms as well as their correlations suggest a longitudinal sampling process is necessary to identify a reliable microbial signature. The coexistence and non-negative correlations between the abundances of different hydrogenotrophic functional groups, as well as the differed associations with microbiota diversity and composition in HA and IBS patients, implicate that the interactions between hydrogenotrophic functional groups are complex and need further elucidation in gut ecosystems.

Acknowledgements
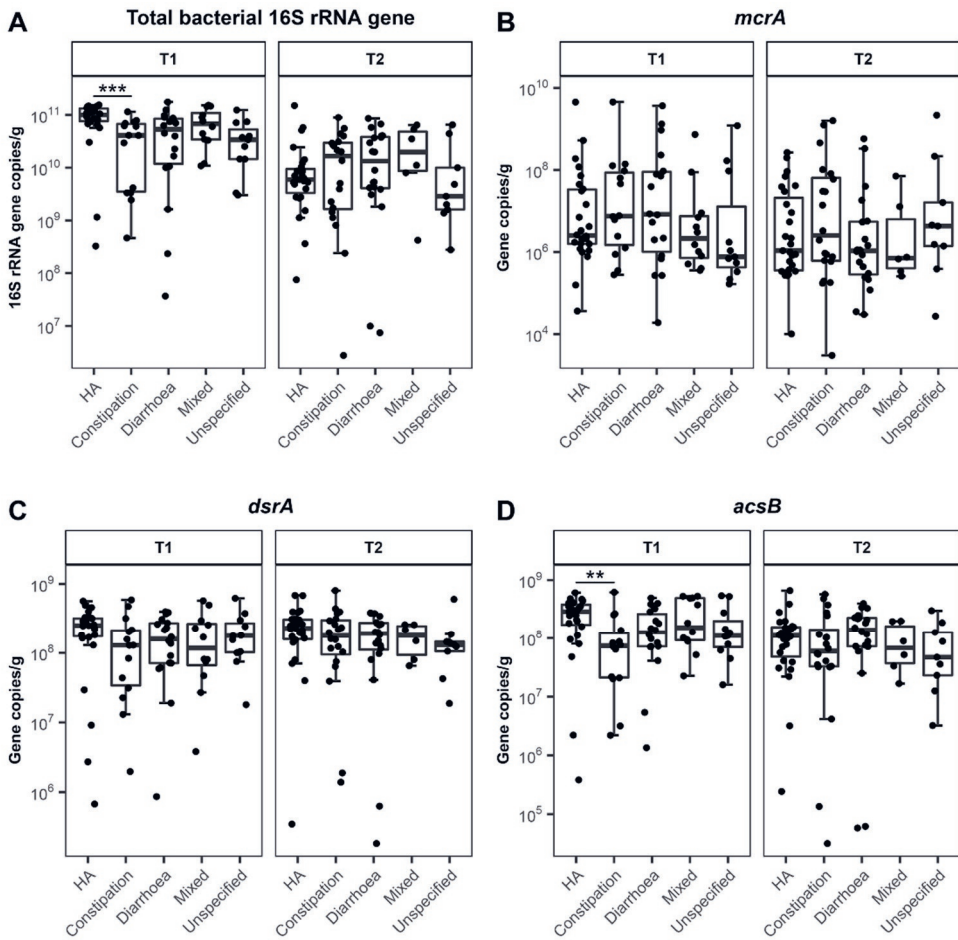
Supplementary Figure 1: Comparison of total bacterial 16S rRNA gene (A) and genes indicative of hydrogenotrophic functional groups (B, *mcrA*, methanogens; C, *dsrA*, sulphate-reducing bacteria; D, *acsB*, acetogens) between IBS subgroups based on predominant stool patterns and healthy adults over time. Values were presented as interquartile with boxplot. Significance between groups was tested with the Mann-Whitney U test. * $p < 0.05$; ** $p < 0.01$.
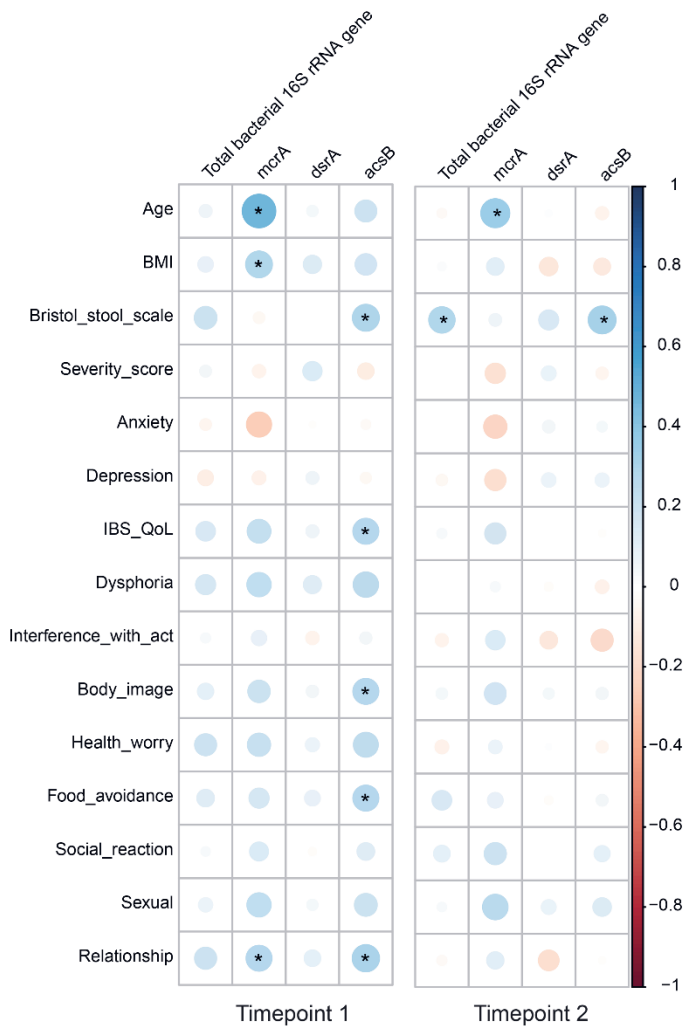
Supplementary Figure 2: Spearman's correlation analyses of total bacterial 16S rRNA gene and hydrogenotrophic functional groups with population characteristics over time. This analysis only includes 55 IBS patients. Significant correlations ($p < 0.05$) are indicated with an asterisk. Abbreviations: BMI, Body Mass Index; IBS-QoL, IBS Quality of Life.
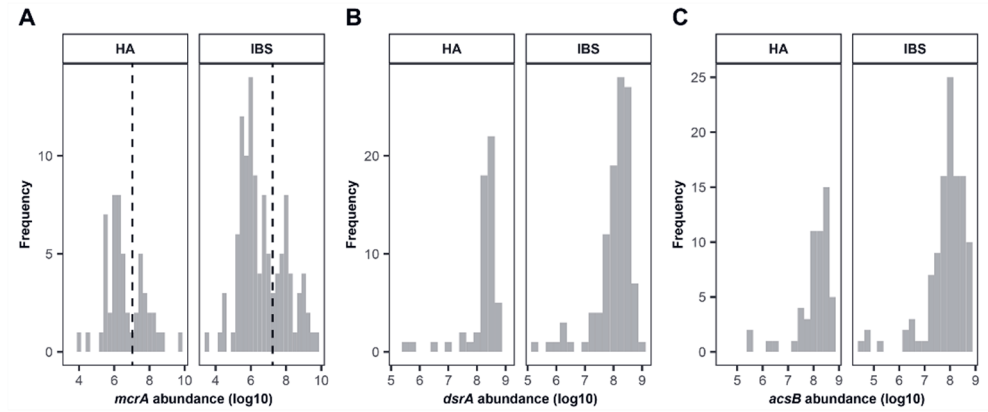
Supplementary Figure 3: Logarithmic abundance distribution of the hydrogenotrophic functional groups in HA and IBS patients. (A) *mcrA* showed two distinct peaks, indicating a bimodality of methanogens, which is not found in the logarithmic abundance distributions of (B) *dsrA* and (C) *acsB*.
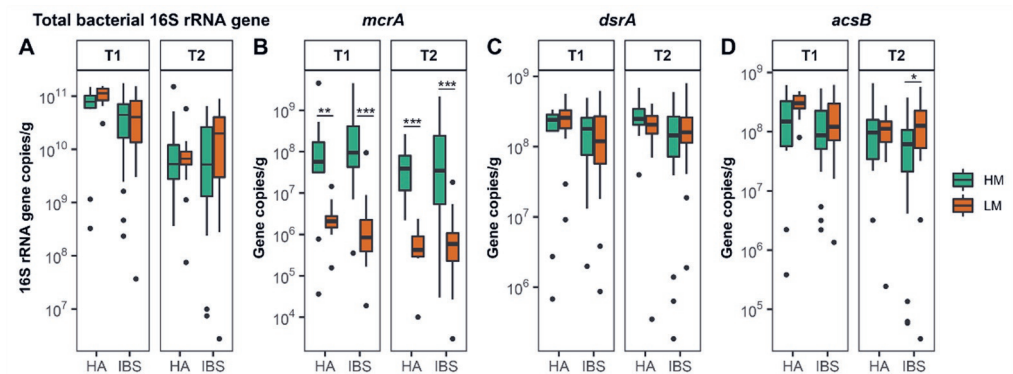
Supplementary Figure 4: Comparison of total bacteria (A) and hydrogenotrophic functional groups (B, *mcrA*; C, *dsrA*; D, *acsB*) between HM and LM in HA and IBS patients over time. Data were displayed as interquartile with boxplot. Mann-Whitney U test was used to test the significance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. HM, High-level methanogens; LM, Low-level methanogens. HA, healthy adults.
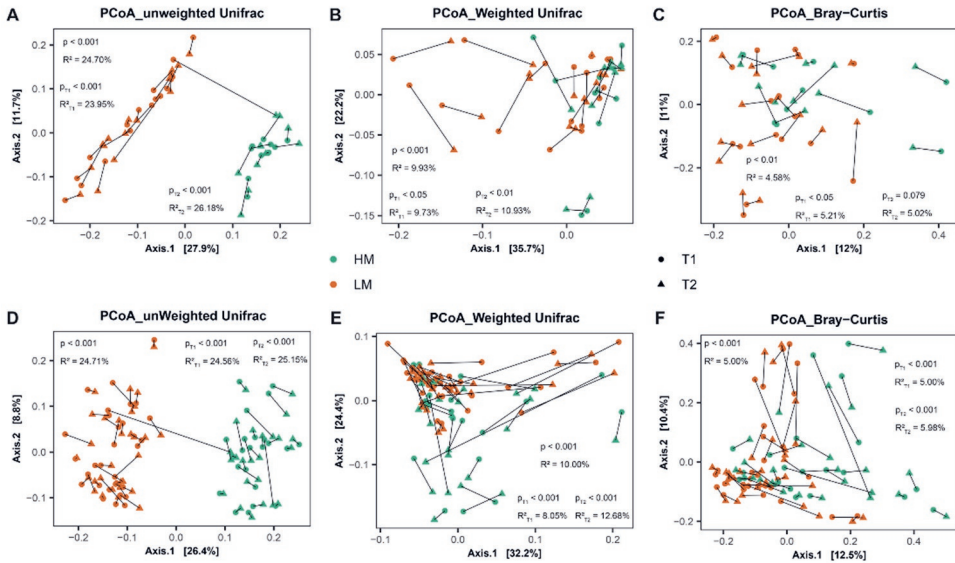
Supplementary Figure 5: PCoA of microbiota composition in HA (A, B, C) and IBS (D, E, F) stratified into HM and LM based on unweighted and weighted Unifrac distances, and Bray-Curtis dissimilarity, respectively. Significant differences between HM and LM in HA and IBS were determined using PERMANOVA with two-timepoint samples included or at each timepoint, respectively. Samples taken at different timepoints are connected by solid lines per subject. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$. HM, High-level methanogens; LM, Low-level methanogens; HA, healthy adults.
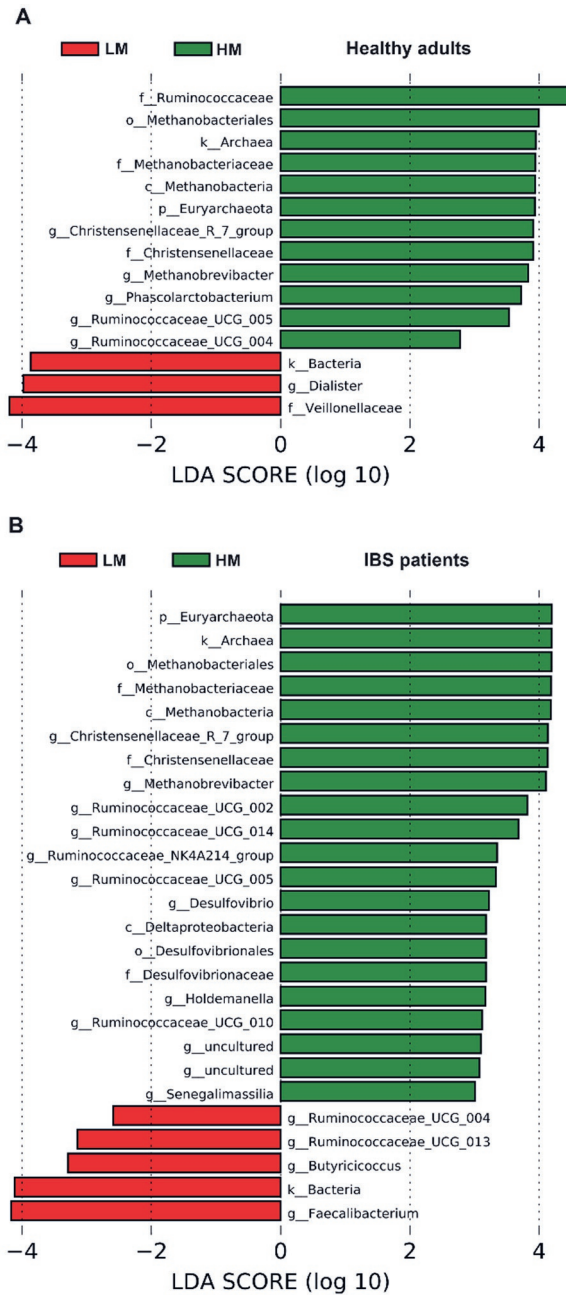
Supplementary Figure 6: Histogram of the linear discriminant analysis (LDA) scores for differentially abundant microbial clades between HM and LM in HA (A) and IBS patients (B). Negative (red bars) LDA scores represent microbial groups over-abundant in LM, while positive (green bars) represent bacterial groups over-abundant in HM. HM, High-level methanogens; LM, Low-level methanogens. HA, healthy adults.

Supplementary Table 1: Dietary intake of the study population

	IBS (n=55)	HA (n=27)	p values
Energy (kcal)	1895.0 (1626.9 - 2157.2)	2105.2 (1699.3 - 2449.7)	0.180
Protein (g)	67.8 (59.2 – 77.4)	77.4 (66.2 – 92.4)	**0.073**
Total fat (g)	76.6 (65.1 – 101.8)	82.7 (67.3 – 100.0)	0.590
Saturated fat (g)	26.8 (18.1-32.0)	31.2 (23.1-35.4)	0.200
Total Carbohydrates (g)	207.7 (181.3 – 244.0)	228.3 (177.8 – 269.8)	0.260
Glucose (g)	9.7 (7.1 – 12.9)	8.6 (6.0-10.8)	**0.059**
Fructose (g)	14.6 ± 6.0	12.8 ± 5.5	0.164
Lactose (g)	6.4 (2.2 – 12.3)	11.3 (7.7 – 19.2)	**0.011**
Maltose (g)	1.8 (1.2 – 2.6)	2.6 (2.1 – 3.6)	**0.004**
Saccharose (g)	26.2 (19.0 – 33.9)	24.0 (18.5 – 38.5)	0.690
Polysaccharides (g)	120.7 (96.1 – 135.8)	130.1 (106.9 – 152.0)	0.190
Dietary fiber (g)	24.0(20.34 – 31.27)	24.2 (21.6 – 30.4)	0.800
Alcohol (g)	2.3 (0.0 – 5.7)	3.3 (0.1 – 10.7)	0.270
Water (g)	2704.0 ± 705.9	2472.0 ± 668.1	0.154

Twenty-seven healthy adults and fifty-five IBS patients were included for the comparative analysis. Data are presented as mean ± standard deviation or median (interquartile range) when skewed. Differences between groups were determined with an independent sample t-test or Mann-Whitney U test when skewed. The definition of each nutrient is according to the Dutch Food Composition table [27]. In line with the Dutch Food Composition table, dietary fibre is not included in the calculation of total carbohydrates but treated as a separate category. Abbreviation: IBS; Irritable Bowel Syndrome

Supplementary Table 2: Stratification of IBS patients based on predominant stool patterns

Timepoint 1				Timepoint 2			
Constipation	Diarrhoea	Mixed	Unspecified	Constipation	Diarrhoea	Mixed	Unspecified
18 (32.7%)	14 (25.5%)	12 (21.8%)	11 (20.0%)	20 (36.4%)	20 (36.4%)	6 (10.9%)	9 (16.4%)

Fifty-five IBS patients were included in the analysis. Data were presented with numbers and ratios in the bracket.

Supplementary Table 3: Dynamics of IBS symptoms over time

	HA			IBS patients		
	Timepoint 1	**Timepoint 2**	**p values**	**Timepoint 1**	**Timepoint 2**	**p values**
Age (years)	35.0 (22.5 – 38.4)	35.0 (22.5 – 38.4)	NA	42.0 (26.0 – 52.5)	42.0 (26.0 – 52.5)	NA
BMI (kg/m²)	23.3 ± 3.0	23.3 ± 3.0	1.00	22.8 ± 2.8	22.8 ± 2.8	1.00
IBS-SSS	60 (30 – 90)	90 (50 - 90)	0.20	140.0 (100.0 – 250.0)	150.0 (110.0 – 225.0)	0.47
Bristol stool scale	3 (3 – 4)	3 (3 – 4)	0.61	4 (3 – 6)	4 (3 – 6)	0.63
Anxiety score	4.0 (3.0 – 6.0)	3.0 (2.0 – 6.0)	0.71	7.0 (4.0 – 11.5)	7.0 (3.5 – 11.0)	0.60
Depression score	2.0 (0.5 – 2.5)	1.0 (0.0 – 2.0)	0.13	2.0 (1.0 – 5.5)	3.0 (1.0 – 6.0)	0.66
IBS-QoL	99.3 (98.9 – 100.0)	100 (98.5 – 100.0)	0.60	75.7 (57.4 – 85.3)	77.2 (65.8 – 86.0)	0.10
Dysphoria	100.0 (100.0 – 100.0)	100.0 (100.0 – 100.0)	0.59	78.1 (56.3 – 90.6)	81.3 (65.6 – 92.2)	**0.03**
Interference_with_act	100.0 (100.0 – 100.0)	100.0 (100.0 – 100.0)	1.00	75.0 (60.7 – 85.7)	78.6 (67.9 – 87.5)	0.11
Body_image	100.0 (96.9 – 100.0)	100.0 (100.0 – 100.0)	0.37	75.0 (56.3 – 87.5)	81.3 (62.5 – 90.7)	**0.00**
Health_worry	100.0 (100.0 – 100.0)	100.0 (100.0 – 100.0)	0.82	75.0 (58.3 – 91.7)	75.0 (58.3 – 87.5)	0.59
Food_avoidance	100.0 (100.0 – 100.0)	100.0 (100.0 – 100.0)	0.34	50.0 (33.3 – 75.0)	50.0 (33.3 – 75.0)	0.88
Social_reaction	100.0 (96.9 – 100.0)	100.0 (100.0 – 100.0)	0.28	81.3 (62.5 – 87.5)	81.3 (62.5 – 93.8)	0.86
Sexual	100.0 (100.0 – 100.0)	100.0 (100.0 – 100.0)	1.00	75.0 (50.0 – 100.0)	75.0 (50.0 – 100.0)	0.36
Relationship	100.0 (100.0 – 100.0)	100.0 (100.0 – 100.0)	1.00	83.0 (66.7 – 91.7)	83.0 (75.0 – 91.7)	**0.04**

Data presents mean ± standard deviation or median (interquartile range) when skewed. BMI was tested with an independent sample t-test. Age, IBS-SSS, Bristol stool scale, anxiety score, depression score, IBS-QoL and its subscales (dysphoria, interference with act, body image, health worry, food avoidance, social reaction, sexual and relationship) were tested with Mann-Whitney U test. Abbreviations: BMI, Body Mass Index; IBS-SSS, IBS Symptom Severity Score; IBS-QoL, IBS Quality of Life. NA: not available

Supplementary Table 4: Subjects with HM or LM in HA and IBS patients.

	Timepoint 1				Timepoint 2			
	Total	IBS patients	Healthy adults	p values	Total	IBS patients	Healthy adults	p values
HM n (%)	33 (40.24%)	23 (41.82%)	10 (37.04%)	0.861	35 (42.68%)	24 (43.64%)	11 (40.74%)	0.991

Twenty-seven healthy adults and fifty-five IBS patients are included in the analysis. Subjects were stratified into low-level methanogens (LM) and high-level methanogens (HM) based on the relative abundance of methanogens with a threshold of 0.1%. Data were presented with a number and the ratio in the bracket. The ratio difference between HA and IBS patients was tested with a Pearson's Chi-square test. Abbreviations: HA, healthy adults; IBS, irritable bowel syndrome; HM, High-level Methanogens.

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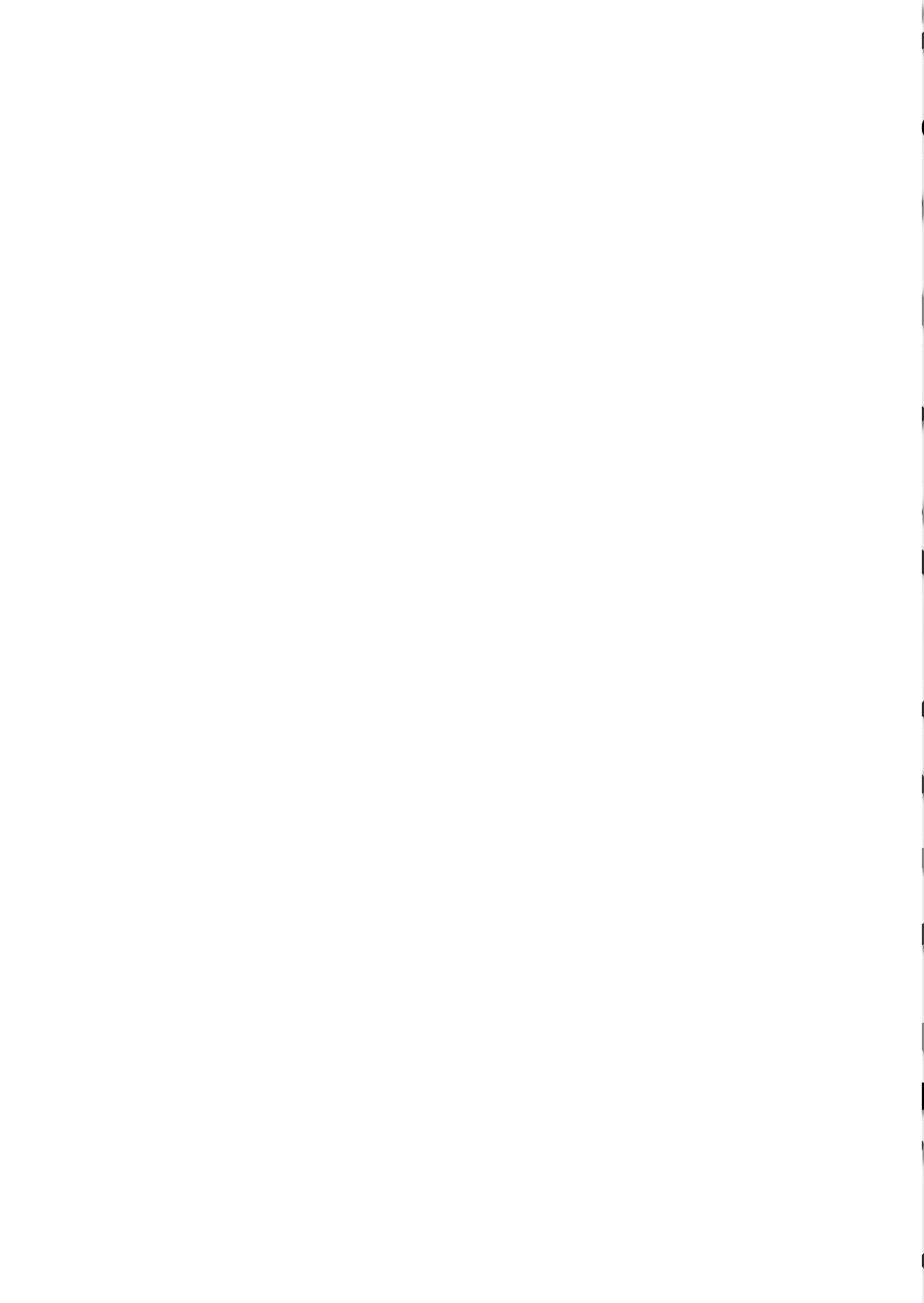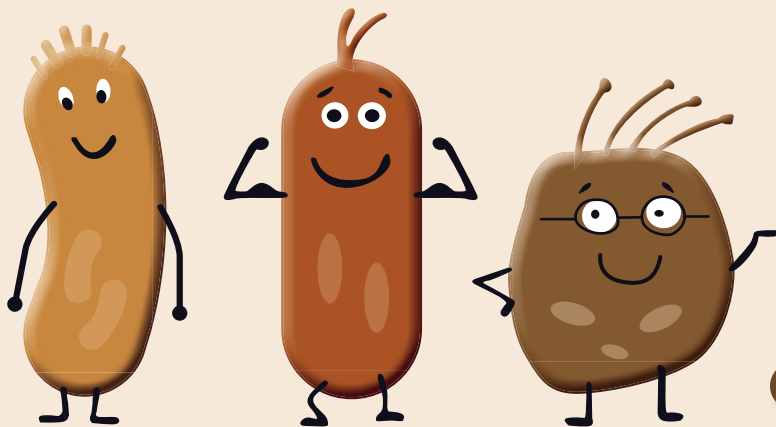
CHAPTER 5

Coculturing with *Blautia hydrogenotrophica* or *Desulfovibro piger* shortens the lag phase of *Methanobrevibacter smithii* under hydrogenotrophic conditions

Taojun Wang, Nils Leibrock , Caroline M. Plugge, Hauke Smidt, Erwin G. Zoetendal

Abstract

In the human gut three functional groups of hydrogenotrophic microbes have been described, namely methanogens, reductive acetogens and sulphate-reducing bacteria. These play an important role in disposing of hydrogen and indirectly contributing to microbial fermentation. Competition has been suggested between these three functional groups because all of them can use hydrogen as an electron donor and energy source. However, information on how these three hydrogenotrophic functional groups interact with each other when cultivated together is still lacking. Therefore, we performed this *in vitro* study to culture the reductive acetogen *Blautia hydrogenotrophica*, the sulphate reducer *Desulfovibro piger* and the methanogen *Methanobrevibacter smithii* singly, in binary cocultures or all together in a triculture, aiming to provide insights into their interactions and growth performance. We found that these three species coexisted and did not compete for hydrogen. The presence of *B. hydrogenotrophica* and *M. smithii* did not affect the growth and sulphate reduction of *D. piger*, whereas the presence of *D. piger* inhibited the growth of *B. hydrogenotrophica* and *M. smithii* and their metabolite production, which might be associated with high concentrations of hydrogen sulphide produced during sulphate reduction. Remarkably, the growth lag phase of *M. smithii* was shortened by coculturing with *B. hydrogenotrophica* and *D. piger*, concomitantly with rapid methane production. However, cocultures of *B. hydrogenotrophica* and *M. smithii* produced less acetate than the monoculture of *B. hydrogenotrophica*. The direct effect of different formate and acetate concentrations on *M. smithii* was also investigated. Although higher formate and acetate concentrations did not stimulate the growth of *M. smithii*, formate was rapidly used by *M. smithii* when acetate concentrations were higher. Overall, these findings suggest that these three hydrogenotrophic microbes coexist and do not compete for hydrogen under hydrogenotrophic conditions and that their interactions vary depending on the metabolites in gut ecosystems.

Keywords: coculture; hydrogenotrophic functional groups; hydrogen; formate; acetate; hydrogen sulphide

Introduction

The gut microbiota comprises a wide variety of microbial species with the ability to ferment dietary fibres and other complex substrates that escape digestion and absorption, resulting in the production of metabolites, such as short chain fatty acids, carbon dioxide and hydrogen [1-3]. The accumulation of hydrogen thermodynamically restricts further microbial fermentation and growth [4-6]. Hydrogenotrophic microbiota comprises microbes that use hydrogen as the electron donor for their anaerobic respiration and play an important role in maintaining the hydrogen balance in gut ecosystems [4, 7]. Moreover, hydrogenotrophic microbiota has been suggested to play an important role in human health, such as constipation [8], irritable bowel syndrome [9, 10], inflammatory bowel disease [11], colorectal cancer [3] and obesity [6, 12].

Hydrogenotrophic microbiota in humans consists of three major functional groups, namely methanogens that use hydrogen and carbon dioxide producing methane, reductive acetogens that use hydrogen and carbon dioxide producing acetate, and sulphate-reducing bacteria (SRB) that use hydrogen and sulphate producing hydrogen sulphide [6]. *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* are the two most common methanogenic species isolated from the gut, and *M. smithii* is the dominant archaeal species in human gut ecosystems with a prevalence up to 95.7% [13]. SRB that colonise the guts of ~50% of humans show greater taxonomic diversity than methanogens with *Desulfovibro piger* described as the most common species [14]. Reductive acetogens are phylogenetically diverse and utilise the Wood-Ljungdahl metabolic pathway to produce acetate. It has been estimated that 33% of acetate is produced this way in the gut [15]. *Blautia hydrogenotrophica* is the most well-known and studied reductive acetogenic species [5].

Competition between the three hydrogenotrophic functional groups is considered because all of them use hydrogen as an energy source [4, 5]. Thermodynamically, sulphate reduction is more favourable with a Gibbs free energy change of -152.2 kJ mol^{-1}, compared to methanogenesis and acetogenesis with Gibbs free energy changes of -131 kJ mol^{-1} and -95 kJ mol^{-1}, respectively [16]. It was reported that SRB and methanogens are mutually exclusive, and SRB are rarely detected in the gut microbiota of so-called methane excretors, which are subjects that have a significantly higher number of methanogens in the gut than non-methane excretors. In contrast, the gut microbiota of non-methane excretors harbours a higher concentration of SRB [17]. However, mutual exclusivity is not always found, and several studies have reported that no significant relationship was observed between methanogens and SRB [18, 19]. It has been reported that lower

acetogenesis was found in the presence of methanogens, and that inhibition of methanogens concomitantly led to higher acetate production in faecal cultures [20]. Although these previous studies indicate that the three hydrogenotrophic functional groups impact each other in the gut microbiota, detailed insight into the specific interactions between the three hydrogenotrophic functional groups remains largely lacking.

Therefore, we performed this study to coculture the hydrogenotrophic microbial species *M. smithii, D. piger*, and *B. hydrogenotrophica* with each other *in vitro*, aiming to understand how these three species affect each other's growth and metabolic activity. We also investigated the direct effect of formate and acetate concentrations on the growth and metabolites of *M. smithii* in this study, due to the fact that formate was detected when *M. smithii* was cocultured with *B. hydrogenotrophica* or *D. piger* and acetate was consistently produced by *B. hydrogenotrophica*, and both metabolites could serve as substrates for *M. smithii* [21].

Materials and Methods

Study set up

To study the interactions between the three hydrogenotrophic species, *Blautia hydrogenotrophica* DSM-10507 (B), *Desulfovibro piger* DSM-749 (D) and *Methanobrevibacter smithii* DSM-11795 (M) were cultured in duplicate in monoculture, in binary cocultures: *B. hydrogenotrophica* and *D. piger* (BD), *B. hydrogenotrophica* and *M. smithii* (BM), *D. piger* and *M. smithii* (DM), and all of them together (BDM) (Figure 1, Experiment one) using a basal medium as previously described [22] with some modifications that included the addition of 2 mM sodium acetate, 20 mM sodium sulphate, 1 g/L yeast extract (OXOID) and 1 g/L tryptone (OXOID). Cultivation was performed in 30 mL serum bottles containing 10 mL medium. The bottle headspace consisted of a mixture of H_2 and CO_2 (80:20, v/v; 1.7 atm). Individual precultures of the three species were prepared prior to the inoculations. Growth was followed using optical density (OD) measurement at 600 nm with a spectrophotometer (OD600 DiluPhotometer, Implen GmbH, Munich, Germany). Precultures were used to inoculate monocultures, binary cocultures, and tricultures at an $OD_{600} = 0.01$ for each species in the medium. Cultures were incubated at 37°C with 150 rpm.
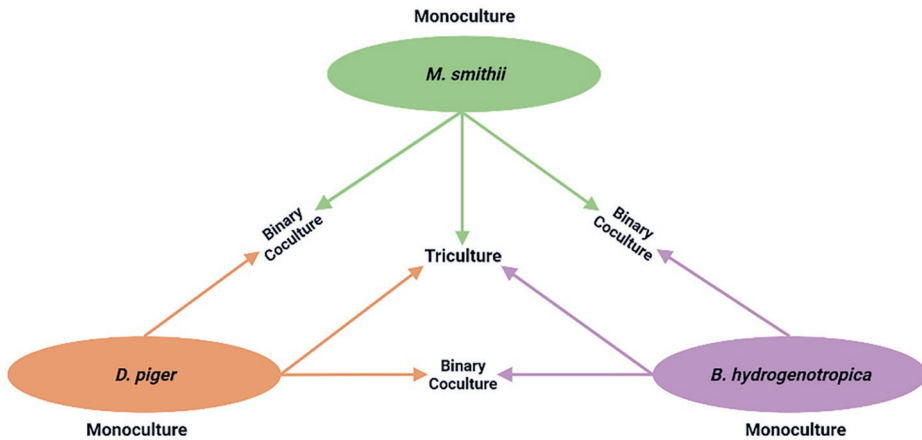
Figure 1: Schematic overview of the experiments to study the interactions between the three hydrogenotrophic species *B. hydrogenotrophica*, *D. piger* and *M. smithii* (Experiment one).

To study the effect of formate and acetate concentrations on the growth of *M. smithii*, it was cultivated in the same medium and under the same conditions with the following modifications (Figure 2, Experiment two): no modification (2 mM acetate, 0 mM formate, 1.7 atm H_2-CO_2 in the headspace; LANF-H_2); addition of 20 mM acetate without modification of the headspace (HANF-H_2); addition of 15 mM formate with the headspace flushed with a mixture of N_2-CO_2 (80:20, v/v; 1.7 atm; LAF-N_2,); addition of 20 mM acetate and 15 mM formate with the headspace flushed with a mixture of N_2-CO_2 (80:20, v/v; 1.7 atm; HAF-N_2); addition of 20 mM acetate and 15 mM formate without modification of the headspace (HAF-H_2).
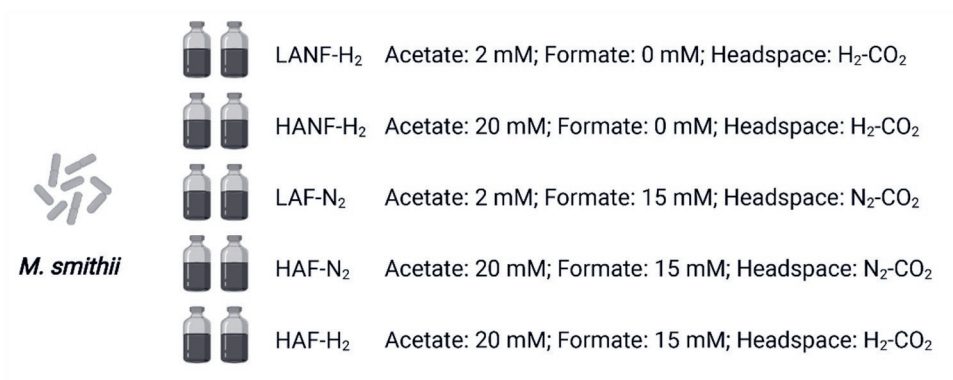
Figure 2: Schematic overview of the experiments to determine the impact of different acetate and formate concentrations on the growth of *M. smithii* (Experiment two).

Sampling and analytical methods

For both Experiment one and two, 0.2 mL gas samples were taken using a 1 mL syringe from the headspace of the serum bottle and were analysed immediately by gas chromatography (GC). 1 mL culture medium was taken at each sampling timepoint and subsequently centrifuged at 4 °C at maximum speed (21130 × g) for 10 min to separate the microbial biomass and supernatant. Afterwards, 200 µL supernatant was mixed with 50 µL of $ZnCl_2$ solution ($ZnCl_2$: 50 g/L; 0.2 mL/L acetic acid), and the remaining supernatant and pellet were stored at -20 °C for further analysis.

For Experiment one, all of the samples were taken at 0 h, 10 h, 24 h, 34 h, 48 h and 72 h except for the monoculture of *M. smithii* for which samples were taken at 0 h, 24 h, 48 h, 58 h, 72 h and 96 h due to its slower growth compared to other cultures. For Experiment two, samples were taken at 0 h, 24 h, 36 h, 48 h, 69 h and 92 h for all incubations.

Hydrogen and methane concentrations were determined using a Compact GC 4.0 (Global Analyser Solutions, Breda, the Netherlands) equipped with a molsieve 5A column, operated at 100 °C coupled to a Carboxen 1010 pre-column. Detection was done via a Thermal Conductivity Detector. Argon was used as carrier gas with a flow rate of 5 mL/min and pressure of 325 kPa. The software Chromeleon (Version 7.2, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used for data processing.

Formate and acetate in the supernatant were measured via High-Pressure Liquid Chromatography (HPLC) equipped with a Shodex SH1821 column (Showa Denko K.K., Tokyo, Japan) operated at 45 °C. 0.4 mL supernatant was added to 0.6 mL 10 mM DMSO as the internal standard in 0.1 N H_2SO_4 solution. 0.01 N H_2SO_4 was used as eluent with a flow rate of 1 mL/min. Detection was done via a refractive index detector. The software Chromeleon (Version 7.2, Thermo Fisher Scientific) was used for the data processing.

Sulphate was measured via Ion Chromatography (IC, ICS-2100, Thermo Fisher Scientific) using a Dionex IonPac AS16 column, operated at 30 °C. 30 µL supernatant was added to 970 µL ultra-pure water with 0.5 mM sodium iodide as the internal standard. Ultra-pure water was used as the eluent with a flow rate of 0.1 mL/min. Detection was done via an electrochemical IC detector. The software Chromeleon (Version 7.2, Thermo Fisher Scientific) was used for data processing.

Hydrogen sulphide was quantified via the methylene-blue method as described previously [23].

Quantitative Polymerase Chain Reaction (qPCR) analysis

The microbial count for each species was determined using qPCR for Experiment one. DNA extraction was performed via the repeated beat-beating method as described previously [24] with a small modification. Briefly, 300 µL of Stool Transport and Recovery (STAR) buffer (Roche Diagnostics, United States) was mixed with each pellet for the first beat-beating, and 200 µL of the STAR buffer was added for the second bead-beating. DNA concentration was adjusted to 1 ng/µL with nuclease-free water for qPCR.

The three microbial species were quantified by qPCR of their 16S rRNA genes. A standard template for each hydrogenotrophic species was generated using purified PCR products. The 16S rRNA gene PCR products for *B. hydrogenotrophica* and *D. piger* were obtained using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') with the DNA extracted from *B. hydrogenotrophica* DSM-10507 and *D. piger* DSM-749 as template, respectively [25]. The 16S rRNA gene PCR product for *M. smithii* was obtained using the primers A109F (5'- ACKGCTCAGTAACACGT -3') and Arch1492R (5'- GGCTACCTTGTTACGACTT -3') with the DNA extracted from *Methanobrevibacter smithii* DSM-11795 as template [26, 27]. All PCR products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific). DNA for all standards was serially ten-fold diluted with nuclease-free water.

All qPCRs were carried out in triplicate with an iCycler iQ real-time detection system (Bio-Rad Laboratories BV). Each reaction mixture with a total volume of 10 µL contained 5 µL 2× iQ SYBR green (Bio-Rad Laboratories B.V.), 2 ng DNA template, forward and reverse primers, and nuclease-free water. Primers and qPCR conditions for each species are listed in Table 1.

Table 1: List of the qPCR assays to quantify the hydrogenotrophic species

Microbial species	Primers	Primer concentration	Annealing temp (°C)	References
Blautia hydrogenotrophica	g-Blau-F	300 nM	55 °C	[28]
DSM-10507	g-Blau-R	300 nM		
Desulfovibro piger	DSV691-F	300 nM	62 °C	[29]
DSM-749	DSV826-R	300 nM		
Methanobrevibacter smithii	Smit.16S-740F	300 nM	60 °C	[13]
DSM-11795	Smit.16S-862R	300 nM		

Statistical analysis

All data were analysed in R version 4.0.0 [30] and presented as mean ± standard deviation. Differences between the groups including monocultures, binary cocultures and tricultures or between time points were tested with a one-way Analysis of Variance (ANOVA). All p-values for pairwise tests were corrected using Benjamini-Hochberg false-discovery rate (FDR). A p-value < 0.05 was considered statistically significant.

Results

Methane production is stimulated by *B. hydrogenotrophica* in the early phase of incubation, but inhibited by *D. piger* in the late phase

Hydrogen was consumed by all of the three hydrogenotrophic species over time (Figure 3A). For monocultures, *D. piger* and *B. hydrogenotrophica* consumed hydrogen much faster than *M. smithii* in the early phase of incubation, and the amount of hydrogen in the bottle remained stable after 24 h. In contrast, after an initial lag phase with little hydrogen consumption during the first 24 h, *M. smithii* consumed hydrogen at a steady rate throughout the 96 h incubation. The hydrogen in the binary BD and DM cocultures showed a similar trend as the monoculture of *D. piger* over time. In contrast, the BM coculture continued to consume hydrogen also after 24 h with near depletion of hydrogen (0.03 mmol left) at 72 h. As for the BDM triculture, hydrogen consumption was fast in the first 24 h, however, after 24 h, the residual amount of hydrogen remained stable, which indicates that the presence of *D. piger* limits the hydrogen consumption in the late phase of incubation.

As expected methane was only detected in the cultures containing *M. smithii* (Figure 3B). The monoculture of *M. smithii* had a very small amount of methane produced (0.006 mmol) in the first 24 h. Afterwards, the methane rapidly increased at a constant rate throughout the remainder of the 96 h incubation. For the DM coculture, only a very small amount of methane (0.003 mmol at 24 h) was observed during the whole incubation. In contrast, the BM coculture, as well as the BDM triculture, showed faster methane production in the first 10 h of incubation compared to the monoculture of *M. smithii*. After 10 h, methane continued to be rapidly produced in the BM coculture at a similar rate as the monoculture of *M. smithii*. However, in the BDM culture, methane production stopped after 34 h, indicating that the presence of *D. piger* inhibited the methane production in the late phase of incubation.

Sulphate reduction by *D. piger* is not affected by *B. hydrogenotrophica* and *M. smithii*

D. piger converted sulphate into sulphide (Figure 4). As expected, sulphate was not used in the monocultures of *B. hydrogenotrophica* and *M. smithii*, nor in their binary coculture (BM) (Figure 4A), and sulphide was not detected (Figure 4B). *D. piger* consumed sulphate rapidly and sulphate was completely depleted after 24 h in all of the cultures that contained *D. piger*. Concomitantly, sulphide was produced rapidly in the first 24 h, after which no more sulphide was produced. In the

monoculture of *D. piger*, the BD and DM cocultures and the BDM triculture, no difference in sulphate reduction was observed, indicating that *B. hydrogenotrophica* and *M. smithii* did not affect sulphate reduction by *D. piger*.
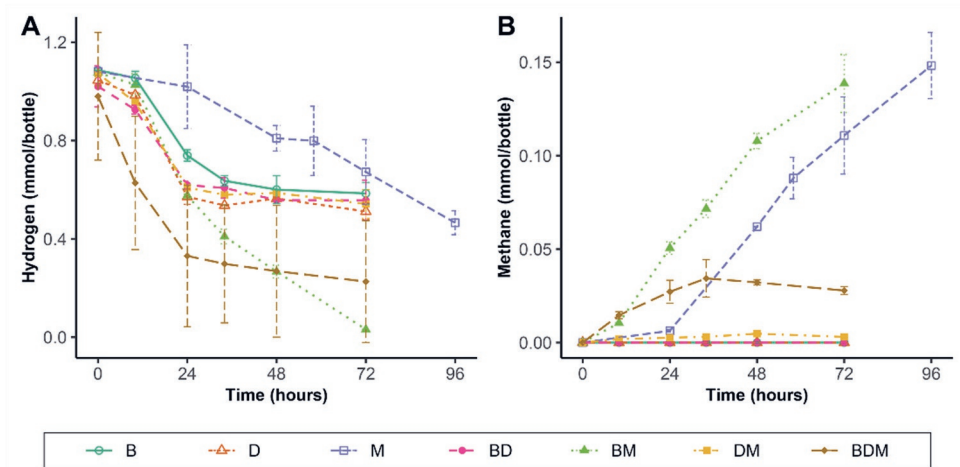
Figure 3: Consumption of hydrogen *(A)* and production of methane *(B)* in monocultures, binary cocultures and a triculture. Hydrogen and methane are given as absolute amounts (mmol/bottle). B: *B. hydrogenotrophica* monoculture; D: *D. piger* monoculture; M: *M. smithii* monoculture; BD: *B. hydrogenotrophica* and *D. piger* coculture; BM: *B. hydrogenotrophica* and *M. smithii* coculture; DM: *D. piger* and *M. smithii* coculture; BDM: *B. hydrogenotrophica*, *D. piger* and *M. smithii* triculture. Results are shown as averages for duplicate cultures with error bars indicating standard deviation.

Figure 4: Sulphate reduction *(A)* and sulphide production *(B)* in monocultures, binary cocultures and a triculture. Sulphate and sulphide are shown as concentrations (mM). B: *B. hydrogenotrophica* monoculture; D: *D. piger* monoculture; M: *M. smithii* monoculture; BD: *B. hydrogenotrophica* and *D. piger* coculture; BM: *B. hydrogenotrophica* and *M. smithii* coculture; DM: *D. piger* and *M. smithii* coculture; BDM: *B. hydrogenotrophica*, *D. piger* and *M. smithii* triculture. Results are shown as averages for duplicate cultures with error bars indicating standard deviation.
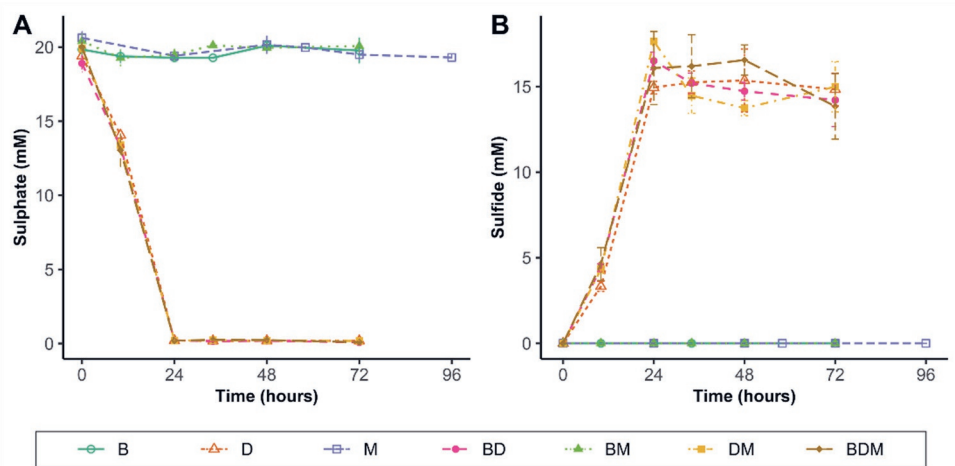
B. hydrogenotrophica produces less acetate in the presence of *D. piger* and *M. smithii*

Formate was only detected in cultures containing *B. hydrogenotrophica* or *D. piger* (Figure 5A). For monocultures, *B. hydrogenotrophica* had fast formate production with the highest formate concentrations at 34 h. Afterwards, formate started to decrease. In contrast, the formate in the monoculture of *D. piger* increased during the whole 72 h of incubation. Interestingly, in the binary BM coculture, the highest formate concentration was observed at 24 h, after which it started to decrease until depletion at 72 h. In contrast, the formate in the BD and DM cocultures followed a similar trend as the monoculture of *D. piger*. The BDM triculture had fast formate production in the first 24 h, after which its concentration remained stable over time.

Acetate production was only observed in the groups with the presence of *B. hydrogenotrophica* (Figure 5B). The monoculture of *B. hydrogenotrophica* showed fast acetate production with the highest amount of acetate produced after 72 h compared to the other incubations. The BM coculture showed rapid acetate production as well but ended up with a lower concentration at the end of the incubation compared to the monoculture of *B. hydrogenotrophica*. The BD coculture and BDM triculture had a small amount of acetate (~ 5 mM) produced in

the first 10 h, but not anymore afterwards, indicating that the presence of *D. piger* inhibited acetate production.
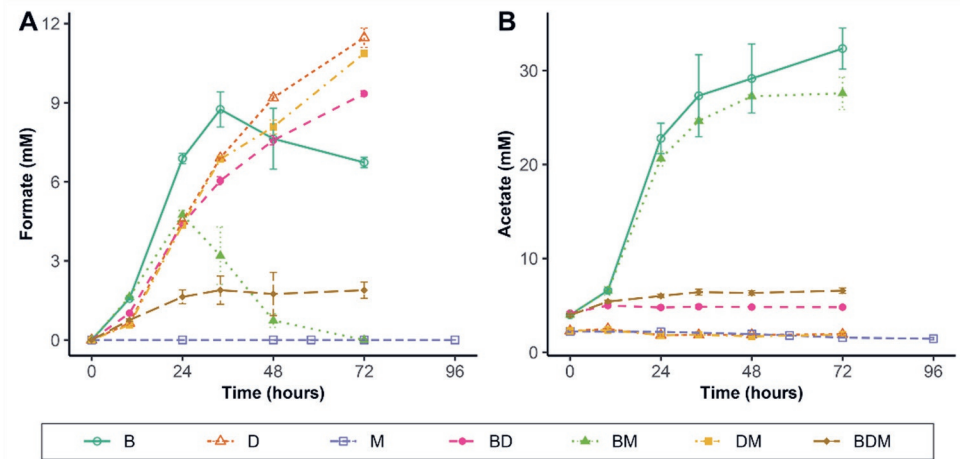

Figure 5: Formate *(A)* and acetate *(B)* production in monocultures, binary cocultures and a triculture. Formate and acetate are shown as concentrations (mM). B: *B. hydrogenotrophica* monoculture; D: *D. piger* monoculture; M: *M. smithii* monoculture; BD: *B. hydrogenotrophica* and *D. piger* coculture; BM: *B. hydrogenotrophica* and *M. smithii* coculture; DM: *D. piger* and *M. smithii* coculture; BDM: *B. hydrogenotrophica*, *D. piger* and *M. smithii* triculture. Results are shown as averages for duplicate cultures with error bars indicating standard deviation.

Cocultivation with *B. hydrogenotrophica* or *D. piger* shortens the lag phase of *M. smithii*

In monocultures, *B. hydrogenotrophica* and *D. piger* grew rapidly and their 16S rRNA gene copy numbers peaked at 24 h. In contrast, *M. smithii* showed no growth in the first 24 h but started its growth afterwards (Figure 6). Growth of *B. hydrogenotrophica* was comparable in monocultures as well as in binary coculture with *M. smithii* (Figure 6A). However, in the binary coculture with *D. piger*, the 16S rRNA gene copy numbers of *B. hydrogenotrophica* started to decrease after 34 h. Coculturing with *B. hydrogenotrophica* or *M. smithii*, *D. piger* showed similar growth compared to its monoculture, indicating that neither *B. hydrogenotrophica* nor *M. smithii* affects the growth of *D. piger* (Figure 6B). Remarkably, in cocultures with *B. hydrogenotrophica* or with *D. piger*, *M. smithii* had a much shorter lag phase compared to its monoculture in the first 10 h (Figure 6C). Afterwards, *M. smithii* stopped growing when cocultured with *D. piger*, while it continued to grow in

coculture with *B. hydrogenotrophica* with a similar trend as in its monoculture after 48 h. In the BDM triculture, the three species showed fast growth in the first 10 h, but afterwards, no further increase in 16S rRNA gene copy numbers was observed for *B. hydrogenotrophica* and *M. smithii*, whereas 16S rRNA gene copy numbers of *D. piger* declined from 24 h. The 16S rRNA gene copy numbers of *B. hydrogenotrophica* and *M. smithii* in BD coculture and DM coculture were lower than those in respective their monocultures in the late phase of incubation, indicating that the presence of *D. piger* inhibits the growth of *B. hydrogenotrophica* and *M. smithii* in the late phase of incubation.

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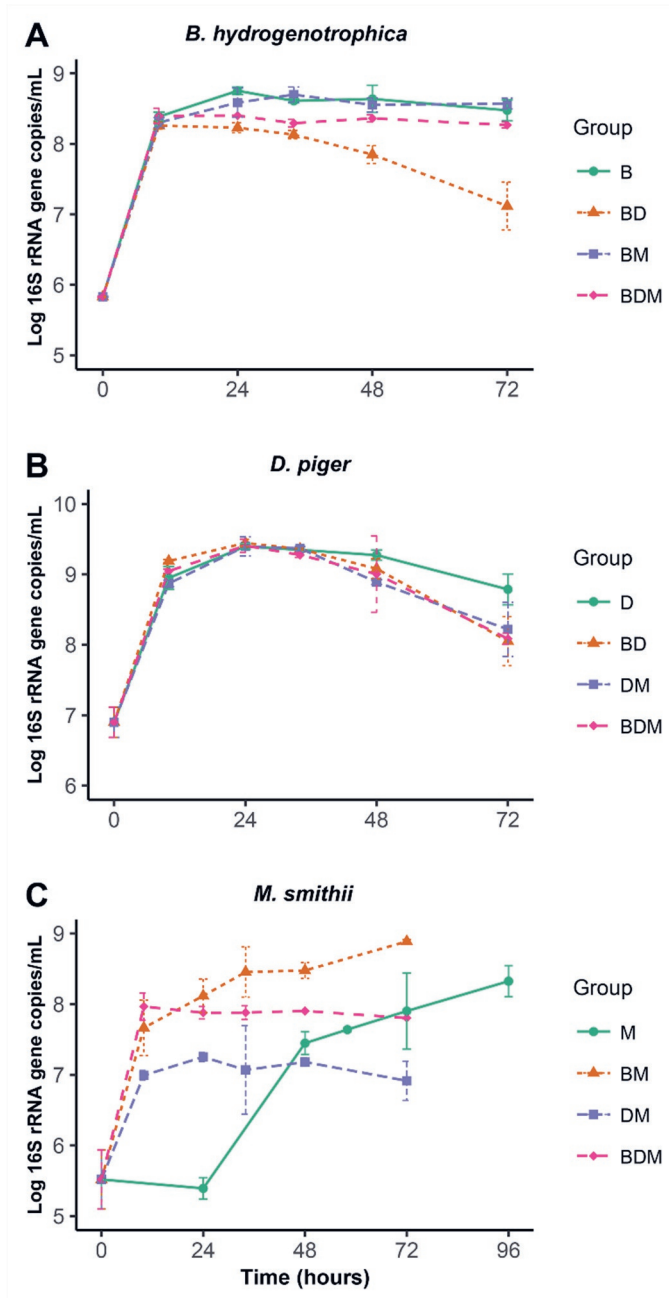
Figure 6: Log 16S rRNA gene copies per mL of *B. hydrogenotrophica (A)*, *D. piger (B)* and *M. smithii (C)* in monocultures, binary cocultures and a triculture. B: *B. hydrogenotrophica* monoculture; D: *D. piger* monoculture; M: *M. smithii* monoculture; BD: *B. hydrogenotrophica* and *D. piger* coculture; BM: *B. hydrogenotrophica* and *M. smithii* coculture; DM: *D. piger* and *M. smithii* coculture; BDM: *B. hydrogenotrophica*, *D. piger* and *M. smithii* triculture. Results are shown as averages for duplicate cultures with error bars indicating standard deviation.
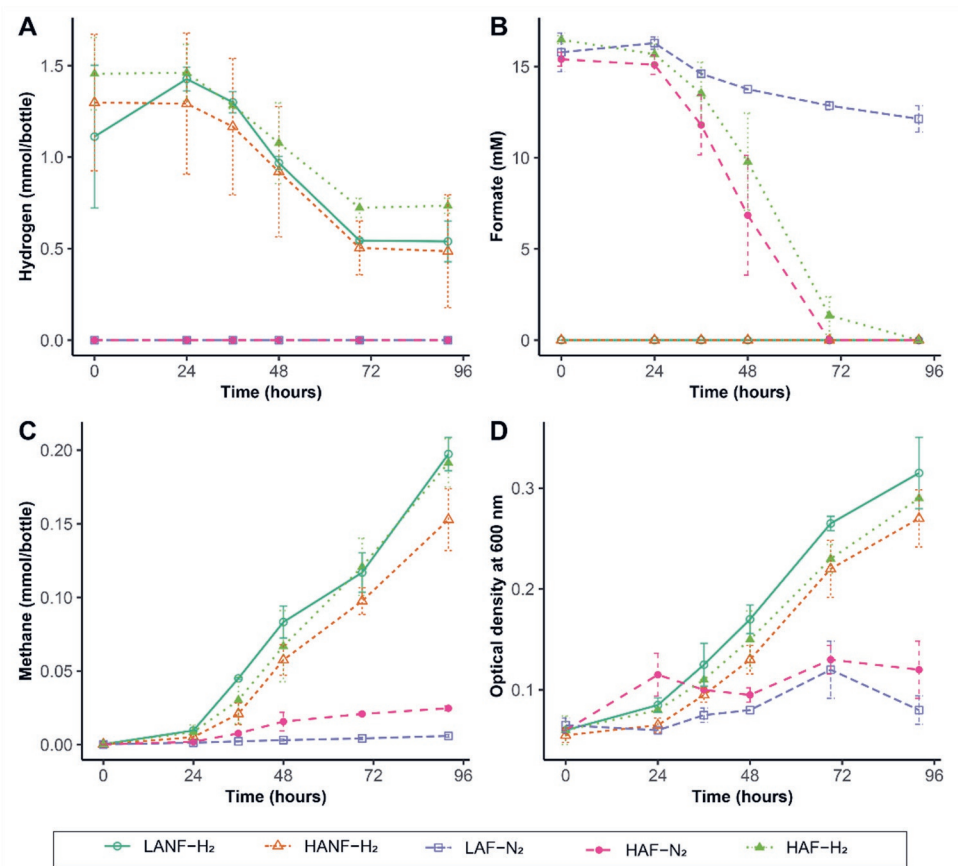
Figure 7: Hydrogen *(A)*, formate *(B)*, methane *(C)* and microbial density of *M. smithii (D)* during the incubation. LANF-H$_2$*:* 2 mM acetate, 0 mM formate, H$_2$-CO$_2$ headspace; HANF-H$_2$*:* 20 mM acetate, 0 mM formate, H$_2$-CO$_2$ headspace; LAF-N$_2$*:* 2 mM acetate, 15 mM formate, N$_2$-CO$_2$ headspace; HAF-N$_2$*:* 20 mM acetate, 15 mM formate, N$_2$-CO$_2$ headspace; HAF-H$_2$*:* 20 mM acetate, 15 mM formate, H$_2$-CO$_2$ headspace.

A high acetate concentration contributes to the usage of formate by *M. smithii*

Since the lag phase of *M. smithii* was abolished by the presence of *B. hydrogenotrophica* and *D. piger*, we determined if this could be explained by the production of acetate and formate in these cocultures. We observed that both hydrogen and formate were consumed, concomitantly with an increase of methane and culture density (Figure 7). Compared to LANF-H$_2$, LAF-H$_2$ showed less

hydrogen consumption after 92 h (Figure 7A) with the same amount of methane produced (Figure 7C), indicating that formate was used to replace hydrogen as the electron donor for methanogenesis. Compared to HANF-H_2, LANF-H_2 showed higher methane production after 92 h indicating that a lower acetate concentration was more favourable for hydrogenotrophic methanogenesis. However, using formate as an electron donor, HAF-N_2 showed increased formate consumption (Figure 7B) with increased methane production (Figure 7C) compared to LAF-N_2 after 92 h, indicating that a higher acetate concentration was more favourable for formate-dependent methanogenesis. Despite the fact that we observed differences in metabolic activities when the concentrations of formate and acetate were modified, a shortening of the lag phase was not observed, suggesting that a yet unidentified mechanism in the cocultures is responsible for this.
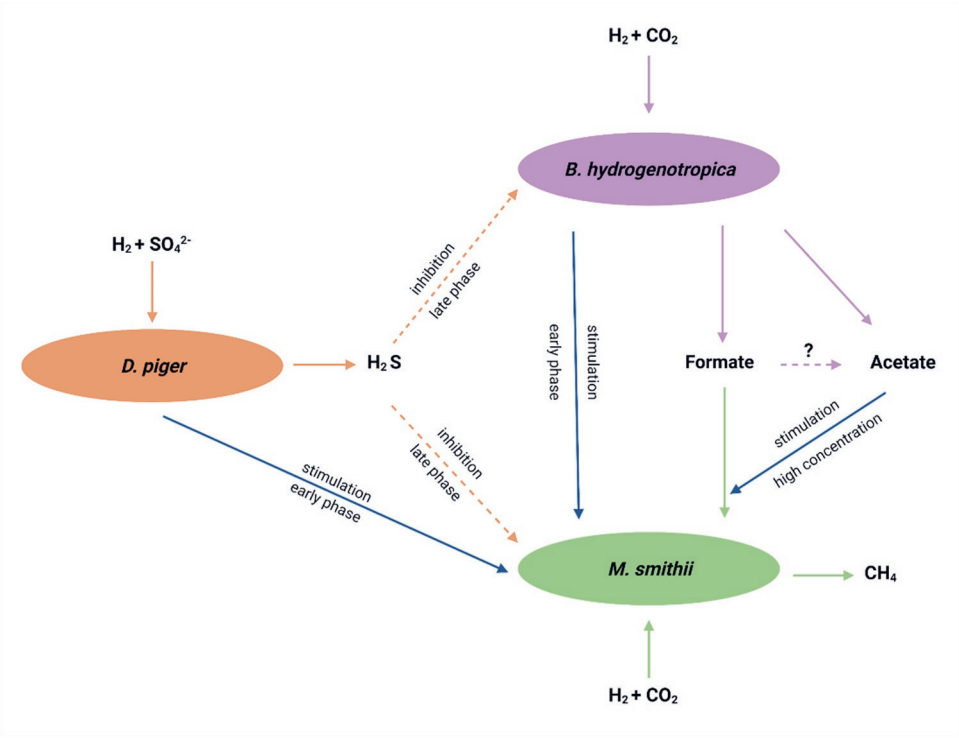

Figure 8: Summary of the interactions between hydrogenotrophic species under hydrogenotrophic conditions. The solid line indicates what we observed in this study, and the dashed line indicates what we speculate based on the findings in this study.

Discussion

In this study, *B. hydrogenotrophica*, *D. piger* and *M. smithii* were cultured singly, or together in binary and cocultures and a triculture to investigate the interactions between these three microbes under hydrogenotrophic conditions. Furthermore, the impact of acetate and formate concentrations on *M. smithii* was investigated. The main findings (Figure 8) indicated that the three hydrogenotrophic species coexisted and did not compete for hydrogen. Coculturing with *B. hydrogenotrophica* and *D. piger* shortened the lag phase of *M. smithii*, concomitantly resulting in faster methane production. Under these conditions *B. hydrogenotrophica* showed a lower acetate production compared to its monoculture. The presence of *D. piger* inhibited the growth of *B. hydrogenotrophica* and *M. smithii* and their metabolite production, and it is tempting to speculate that this is due to the formation of hydrogen sulphide. In addition, a higher acetate concentration stimulated the usage of formate by *M. smithii*, but this could not explain the shortening of the lag phase observed in its cocultures.

It has been considered that the three hydrogenotrophic functional groups may compete because all of them use hydrogen as an energy source in gut ecosystems [4, 5]. Our results showed that *B. hydrogenotrophica*, *D. piger* and *M. smithii* coexisted without competition for hydrogen. This seems to contrast with some earlier suggestions, however, it probably has to be ascribed to the fact that hydrogen was not the limiting factor in this *in vitro* study. It is evident that hydrogen concentrations in gut ecosystems vary over time and are associated with the amount and types of carbohydrate intake [31, 32], which implies that the interactions between the three hydrogenotrophic functional groups may vary according to the hydrogen production *in vivo*. Nevertheless, our study demonstrated that the three microbes can coexist with each other and thereby confirming our earlier *in vivo* observations in IBS patients and healthy subjects (**Chapter 4**). Considering the complexity of the gut ecosystem including its nutrient supply, the variable environmental conditions throughout the gut, as well as the metabolic flexibility of some hydrogenotrophic microbes, the interactions between hydrogenotrophic microbes in the gut are potentially complex and certainly environment-dependent [33, 34].

Coculturing the three hydrogenotrophic species revealed that the presence of *B. hydrogenotrophica* and *M. smithii* did not affect the growth and metabolite production of *D. piger*. The depletion of sulphate by *D. piger* within 24 h may indicate that this was the limiting factor for growth of *D. piger* during all incubations. Remarkably, after 10 h of incubation in the presence of *D. piger*, *B. hydrogenotrophica* and *M. smithii* stopped growing and their metabolite production

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stopped, which is likely due to the hydrogen sulphide produced by *D. piger*. Hydrogen sulphide is highly reactive and toxic to microbes since it can diffuse across the cell membrane and is involved in protein denaturation and enzyme inhibition [35, 36]. Moreover, this toxicity has been associated with a > 4 mM hydrogen sulphide concentration [36]. This is consistent with our findings as the concentrations of sulphide were below ~4 mM during the first 10 h in all incubations, but reached concentrations of ~15 mM after 24 h. In gut ecosystems, it has been reported that the mean total sulphide content in wet faeces from 14 subjects was 0.66 mM/kg [37] and remaining at sub-toxic levels in subjects consuming diets rich in sulphur-containing components [38], suggesting that sulphate is likely not inhibiting the growth of methanogens and reductive acetogens *in vivo*.

Remarkably, the lag phase that was consistently observed for *M. smithii* in monoculture was shortened by both *B. hydrogenotrophica* and *D. piger*. Formate and acetate can be used by *M. smithii* [21], and due to their lower oxidation status compared to carbon dioxide, we speculated that their production by *B. hydrogenotrophica* and *D. piger* in the cocultures may explain the shortening of the growth lag phase of *M. smithii*. Therefore, we investigated the potential effect of increasing formate and acetate concentrations in the medium in *M. smithii* monocultures. Nevertheless, although this did result in differences in metabolism, it did not shorten the lag phase, which suggests that the stimulating effect by *B. hydrogenotrophica* and *D. piger* remains unknown and needs further studies to unravel the underlying mechanisms.

Although the higher acetate and formate concentrations did not explain what shortened the lag phase of *M. smithii*, it demonstrated that formate was rapidly converted to methane only in the presence of a high concentration of acetate. Formate is a metabolite produced by *B. hydrogenotrophica* and is also an intermediate in the Wood-Ljungdahl pathway of reductive acetogenesis [4]. Moreover, it serves as a substrate for *M. smithii* for methane production [21]. Coculturing *B. hydrogenotrophica* with *M. smithii* showed that formate was depleted, while it remained at higher concentration in the monoculture of *B. hydrogenotrophica*. Moreover, significantly lower acetate production was observed in the coculture with *M. smithii* compared to the *B. hydrogenotrophica* monoculture. The lower acetate production could thus be due to the usage of the formate by *M. smithii* at a high acetate concentration in the late phase of incubation.

In conclusion, this *in vitro* coculture study gives a detailed overview of interactions between three different hydrogenotrophic microbes occurring in the human gut under hydrogenotrophic conditions. Our study revealed that these three microbes

coexist, do not compete for hydrogen under hydrogenotrophic conditions and may even stimulate each other's growth, although their relationships may vary throughout the gut due to the variable environmental conditions *in vivo* [33, 34]. Interestingly, the usage of formate by *M. smithii* was associated with a high acetate concentration. Although the underlying mechanisms are unknown, this indicates that the metabolism of the hydrogenotrophic species is flexible and environment-dependent, which demonstrates that the interactions between hydrogenotrophic microbes are complex and thus not easy to extrapolate to the *in vivo* situations in the gut.

Acknowledgements

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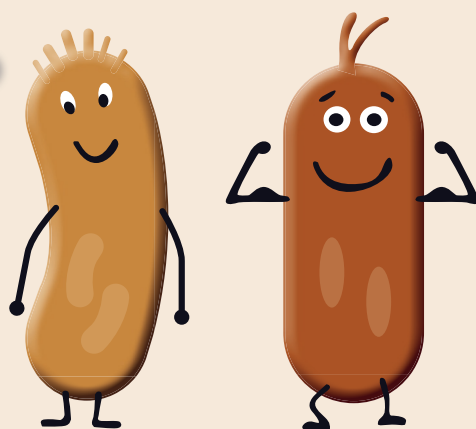
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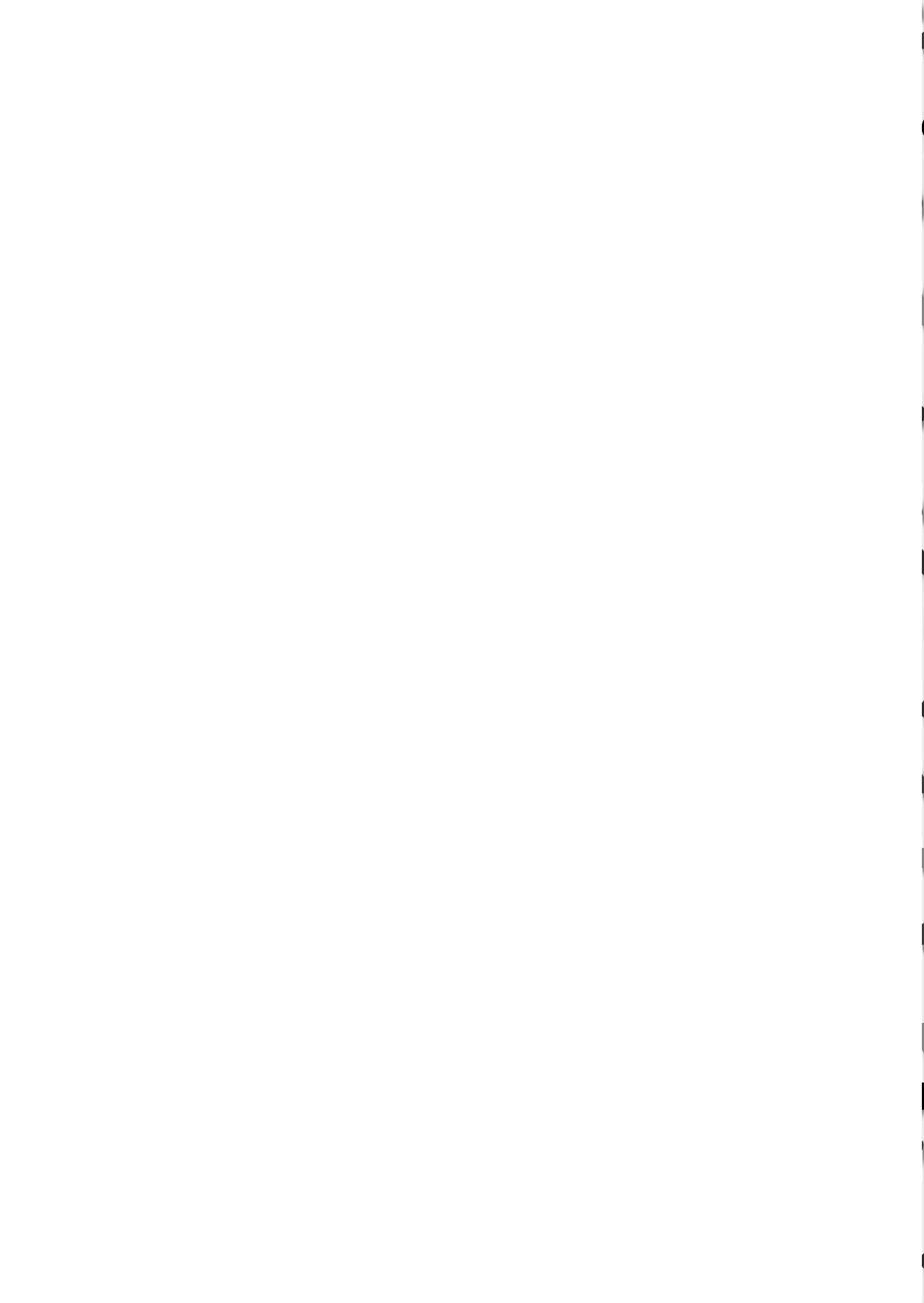
CHAPTER 6

In vitro modulation of hydrogenotrophic activities reveals that the gut microbiota is resilient towards these changes in which methanogenesis is an optional niche for hydrogen consumption

Taojun Wang, Hauke Smidt, Erwin G. Zoetendal

Abstract

Hydrogen metabolism plays a central role in microbial fermentation. However, how hydrogenotrophic microbes impact microbiota composition and metabolites in gut ecosystems remains largely unknown. Hence, this study investigates the impact of altering two of the key hydrogenotrophic activities, namely methanogenesis and sulphate reduction, on human gut microbiota composition and metabolite production. Faecal slurries from three methane excretors (MEs) and three non-methane excretors (NMEs) were inoculated into basal medium with 1% (w/v) pectin or a carbohydrate mixture (pectin, potato starch , inulin, xylan and arabinogalactan; 0.2% each) as substrates. 2-bromoethanesulfonate (BES) was added to inhibit methane production in MEs, and *Methanobrevibacter smithii* was added to stimulate methane production in NMEs. Sodium sulphate was added to stimulate sulphate reduction in both MEs and NMEs. Based on analysis of prokaryotic 16S rRNA gene amplicon sequence data and metabolites, microbial richness and composition, propionate and methane production significantly differed between MEs and NMEs. Lower hydrogen concentrations were observed in MEs compared to NMEs in the incubations with pectin, but not in the incubations with the carbohydrate mixture. Remarkably, sulphate was not consumed in either ME or NME incubations. Adding *M. smithii* to the NME inocula resulted in its persistence in the community and methane production during incubations. The addition of BES inhibited methane production in the ME incubations, accompanied with a lower relative abundance of methanogens when pectin was used as substrate. However, altering methanogenesis did not significantly change overall microbiota composition and metabolite production in MEs and NMEs. These findings suggest that methanogens can occupy a niche in a microbiota that originally lacks methanogens, but that modulating methanogenesis has a minor effect on overall microbiota composition and activity.

Keywords: methane excretors; non-methane excretors; methanogenesis; sulphate reduction; microbiota modulation

Introduction

The human gut resembles a bioreactor that inhabits a dense community of approximately 3.9×10^{13} microorganisms which is termed the gut microbiota [1, 2]. The importance of the gut microbiota is widely acknowledged as its composition and metabolites play an important role in human health. Many factors have been associated with gut microbiota composition, such as genetics, living environment, health status, use of antibiotics, medication and diet [3-5]. Although these factors play an important role in determining microbiota composition, they only explain less than 20% of the variation in microbiota composition between subjects [6, 7], implying that other factors, such as the interactions among different gut microbes are fundamental in shaping gut microbiota composition [8]. However, our knowledge on these internal factors is limited.

Interspecies hydrogen transfer is a key microbial interaction in anaerobic ecosystems, including gut ecosystems, as it plays a central role in microbial fermentation [9, 10]. The gut microbiota ferments the non-digestible and unabsorbed dietary components and thereby produces hydrogen [11, 12]. Hydrogen accumulation leads to a high partial pressure that would inhibit the regeneration of the coenzyme NAD^+ from NADH, and thermodynamically restrict further microbial fermentation and growth [9, 13]. However, hydrogen-consuming (hydrogenotrophic) microbes including methanogens, sulphate-reducing bacteria (SRB) and reductive acetogens use hydrogen as an energy source and thereby decrease the hydrogen partial pressure, which positively contributes to overall microbial fermentation [10, 14]. It has been reported that a high NAD^+/NADH ratio was maintained with the presence of methanogens and reductive acetogens in mice [15, 16]. As such, hydrogenotrophic microbes play an important role in overall microbial fermentation and therefore may, in turn, also impact overall microbiota composition.

The number and composition of hydrogenotrophic microbes vary between individuals. The difference between individuals with high or low numbers of methanogens is reflected in the presence of methane in exhaled breath. In the Western world, approximately one-third of individuals have detectable levels of methane (>1 ppm, above atmospheric methane levels) in their breath [17], while this is approximately 80% in Africans [18]. These so-called methane excretors (MEs) have a significantly higher number of methanogens in the gut with approximately 10^9 CFU/g in stool, whereas individuals who are non-methane excretors (NMEs) harbour less than 10^4 CFU/g [17, 19]. However, whether this difference in methanogen levels between MEs and NMEs impacts the overall microbiota composition and fermentation remains largely unknown.

6

Therefore, we hypothesise that the kinetics of hydrogen consumption by hydrogenotrophic microbes is an important driver of gut microbiota composition and metabolite production. Hence, we aim to investigate how overall gut microbiota composition and fermentation are affected by modulating the hydrogenotrophic metabolism within the community.

Materials and Methods

Study setup

The overview of the study is presented in Figure 1. This *in vitro* study included three healthy MEs and NMEs, determined via a breath test, who did not use antibiotics or probiotics at least during the four weeks prior to the study (Table 1). Faecal slurries were prepared from freshly collected faeces of the six participants and subsequently used as inoculum. Batch incubations were done using basal medium [20] to which 1% (w/v) pectin (10g/L) or a mixture of non-digestible carbohydrates (2g/L for each carbohydrate, apple pectin, Sigma-Aldrich, St. Louis, USA; potato starch, Sigma-Aldrich; inulin, Sensus B.V., Roosendaal, the Netherlands; beechwood xylan, Carl Roth, Karlsruhe, Germany; arabinogalactan, Sigma-Aldrich) was added as substrates for fermentation. Incubations inoculated only with faeces (1%, w/v) from MEs and NMEs were treated as negative controls. To investigate the impact of methanogenesis on gut microbiota composition and metabolite production, 10 mM 2-bromoethanesulfonate (BES; inhibits the production of methane) was added to incubations with faecal slurries from MEs (BES group), while *Methanobrevibacter smithii* (see below for details) was added together with the faecal slurries from NMEs as inoculum (*M. smithii* group). To control for potential side effects of BES on the faecal microbiota communities, 10 mM BES was added to the incubations with faecal slurries from NMEs. To investigate the impact of sulphate reduction on gut microbiota composition and metabolite production, 20 mM sodium sulphate was added to incubations with faecal slurries from MEs and NMEs to stimulate dissimilatory sulphate reduction (Sulphate group). The Medical Ethics Reviewing Committee of Wageningen University has decided that the study was exempt from obtaining ethics approval as it does not fall within the remit of the "Dutch Medical Research Involving Human Subjects" Act.

Table 1: Characteristics of the participants included in this study

Participant type	Gender	Age
	Female	24
Methane excretor (n=3)	Male	32
	Male	25
	Female	24
Non-methane excretor (n=3)	Female	25
	Male	29
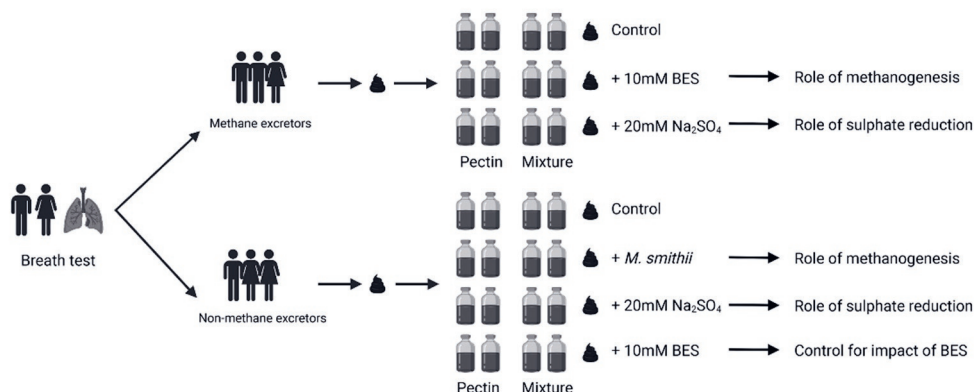
Figure 1: Schematic overview of the study design. Mixture: carbohydrate mixture consisting of equal amounts of apple pectin, potato starch, inulin, beechwood xylan, and arabinogalactan. BES: 2-bromoethanesulfonate.

Breath test for screening of methane excretors and non-methane excretors

MEs and NMEs were identified via the breath test as described as follows: A 0.6 L Tedlar® Push-Lock Valve Gas Sampling Bag (DuPont, Wilmington, USA) with Thermogreen® LB-2 Septa (Sigma-Aldrich, St. Louis, USA) was used to collect the exhaled breath based on the manufacturer's instructions. Afterwards, the methane content was measured in 1 mL of the collected breath using CompactGC gas chromatography (Global Analyser Solutions, Breda, the Netherlands) with a sensitive Pulsed Discharge Detector (PDD). Subjects with breath methane > 1 ppm

(above atmospheric methane levels) were classified as MEs, whereas subjects with methane levels < 1ppm were classified as NMEs.

Faecal sample collection and storage

Faecal samples were collected as described previously [21]. Faeces was collected using a stool collector (Excretas Medical BV, Enschede, the Netherlands). After defecation, an Anaerocult® A mini (Merk KGaA, Darmstadt, Germany) was activated by adding 10 mL sterile water and placed in the collector to create an anoxic atmosphere. Afterwards, the stool collector was placed into an anaerobic box (AnaeroPack™ 7.0 L Rectangular Jar, Thermo Fisher Scientific, Waltham, MA, USA) with two open bags of AnaeroGen (AnaeroGen™ 3.5 L, Thermo Fisher Scientific). Subsequently, the anaerobic box was transferred immediately into the anaerobic chamber (MK3 Workstation, Don Whitley, United Kingdom). For each participant, 17.5 g faeces, 7.5 g dialysate (Tritium Microbiologie, Eindhoven, the Netherlands), 35.7 g nuclease-free water (Promega, Madison, WI, USA), and 9.8 g sterile glycerol were mixed thoroughly. These faecal slurries were subsequently transferred into 120 mL serum bottles and stored at -80 °C until further use.

Medium preparation and inoculation

The basal medium was prepared as described previously [20]. Briefly, the basal medium contained per litre the following components: 0.408 g KH_2PO_4, 0.534 g $Na_2HPO_4 \cdot 2H_2O$, 0.3 g NH_4Cl, 0.3 g $NaCl$, 0.1 g $MgCl_2 \cdot 6H_2O$, 0.5 mg resazurin, 4 g $NaHCO_3$, 0.11 g $CaCl_2$, 0.2402 g $Na_2S \cdot 9H_2O$, trace elements in acid (1.8 mg HCl, 0.0618 mg H_3BO_3, 0.06125 mg $MnCl_2 \cdot 4H_2O$, 0.9435 mg $FeCl_2 \cdot 4H_2O$, 0.0645 mg $CoCl_2$, 0.01286 mg $NiCl_2$, 0.0677 mg $ZnCl_2$, and 0.01335 mg $CuCl_2 \cdot 2H_2O$), trace elements in alkaline (0.4 mg $NaOH$, 0.0173 mg Na_2SeO_3, 0.0294 mg Na_2WO_4, and 0.0205 mg Na_2MoO_4), vitamins (0.020 mg biotin, 0.200 mg nicotinamide, 0.100 mg p-aminobenzoic acid, 0.200 mg thiamin (vitamin B1), 0.100 mg panthotenic acid, 0.500 mg pyridoxamine, 0.100 mg cyanocobalamin (vitamin B12), and 0.100 mg riboflavin), and 10 g carbohydrates (pectin or carbohydrate mixture) as described above. Some modifications were made for each group: for the BES group, 10 mM BES was added to inhibit the methane production [22]; for the sulphate group, 20 mM sodium sulphate was added to stimulate dissimilatory sulphate reduction; and for the *M. smithii* group, 0.1 mL *M. smithii* culture (OD_{600} = 0.20) was added to introduce methanogens to the community. Each 30 mL serum bottle contained a 10 mL basal medium with a 20 mL headspace flushed with a mixture of N_2-CO_2 (80:20, v/v; 1.5 atm). For inoculation, the faecal slurry was added to a final concentration of 1% faeces (w/v) for all incubations. All bottles were incubated at 37°C with 150 rpm for 48 h. *Methanobrevibacter smithii* DSM-

11795 that was used as co-inoculum in the experiments, was cultivated at 37°C with 150 rpm in a 30 mL serum bottle with basal medium supplemented with 2 mM sodium acetate and 0.5 g/L yeast extract (OXOID). The bottle headspace was flushed with a mixture of H_2-CO_2 (80:20, v/v; 1.7 atm).

Sample collection

Samples were taken at 0 h, 6 h, 12 h, 24 h and 48 h. At each sampling timepoint, 0.2 mL gas from the headspace was sampled using a 1 mL syringe to determine hydrogen and methane levels. For liquid samples, 1 mL culture was taken directly and centrifuged at 4°C at maximum speed (21,130 × g) for 10 min to separate the supernatant and microbial biomass. The microbial biomass was used to determine the microbial composition, and the supernatant was used to determine microbial metabolites. Moreover, 1 mL culture was mixed with 100 μL methanol thoroughly and subsequently centrifuged using the same conditions as indicated above to obtain the supernatant that was then used for sulphate analysis. In addition, 1 mL culture was taken and mixed with 250 μL of $ZnCl_2$ solution ($ZnCl_2$: 50 g/L; 0.2 mL/L acetic acids) for hydrogen sulphide analysis. After collection, all of the samples were stored at -80 °C for further analysis.

Gas, SCFA and sulphate analysis

A Compact gas chromatograph (GC) 4.0 (Global Analyser Solutions) equipped with a molsieve 5A column was used to measure hydrogen and methane levels. This column was operated at 100 °C coupled to a Carboxen 1010 pre-column. Argon was used as carrier gas with a flow rate of 5 mL/min and pressure of 325 kPa. Detection was done via a Thermal Conductivity Detector. The software Chromeleon (Version 7.2, Thermo Fisher Scientific) was used for data processing.

High-Pressure Liquid Chromatography (HPLC) equipped with a Shodex SH1821 column (Showa Denko K.K., Tokyo, Japan) was used to determine short chain fatty acid (SCFA; acetate, propionate and butyrate) concentrations. Preparation of the samples was done by mixing 0.4 mL supernatant with 0.6 mL 10 mM DMSO as the internal standard in 0.1 N H_2SO_4 solution. The column was operated at 45 °C with an eluent (0.01 N H_2SO_4) flow rate of 1 mL/min. A refractive index detector was used for detection. Data processing was done using Chromeleon software (Version 7.2, Thermo Fisher Scientific).

Ion Chromatography (IC, ICS-2100, Thermo Fisher Scientific) equipped with a Dionex IonPac AS16 column was used to measure the sulphate concentration. Preparation of the samples was done by mixing 30 μL supernatant with 0.5 mM sodium iodide as the internal standard in 970 μL ultra-pure water with 5% methanol

(50 mL methanol in 950 mL ultra-pure water). The column was operated at 30 °C with an eluent (5% methanol) flow rate of 0.1 mL/min. An electrochemical IC detector was used for the detection. The data processing was done using Chromeleon software (Version 7.2, Thermo Fisher Scientific).

Hydrogen sulphide was measured via the methylene-blue method as described previously [23].

Microbiota composition analysis

DNA extraction was performed via a repeated beat-beating method as described previously [24] with a small adaptation of the buffer volume. Briefly, 300 µL of Stool Transport and Recovery (STAR) buffer (Roche Diagnostics, Almere, the Netherlands) was mixed with each pellet for the first beat-beating. After centrifugation and collecting of the supernatant, 200 µL of the STAR buffer was added for the second round of bead-beating. Subsequently, DNA was purified using the Maxwell® 16 Total RNA system (Promega) with the 16 Tissue LEV Total RNA purification Kit Cartridge (XAS1220). DNA concentration and purity were determined by a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). Afterwards, DNA concentrations were adjusted to 20 ng/µL with nuclease-free water. Amplification of the 16S ribosomal RNA (rRNA) gene of bacteria and archaea was performed in duplicate with uniquely barcoded primers [25] 515F (5'-GTGYCAGCMGCCGCGGTAA-3') [26] and 806R (5'-GGACTACNVGGGTWTCTAAT-3') [27]. Reaction conditions and library preparation were performed as described previously [25]. Afterwards, the libraries were purified with the CleanPCR kit (CleanNA, Waddinxveen, the Netherlands), and sent to Eurofins Genomics Germany GmbH (Konstanz, Germany) for sequencing (Illumina Hiseq2500, 150bp paired end). NG-Tax 2.0 was used to process the raw sequencing data for Amplicon Sequencing Variant (ASV) picking with default settings and for taxonomic assignments using the SILVA database (version 128) [28, 29].

Statistical analyses

Microbiota and metabolite data were analysed in R version 4.0.0 [30]. Alpha diversity (within-sample diversity) and beta diversity (between-sample diversity) were calculated at the ASV level using phyloseq [31]. Alpha diversity metrics ASV richness and Shannon diversity were calculated. To visualise beta diversity, Principle Coordinate Analysis (PCoA) based on unweighted Unifrac distance, weighted Unifrac distance and Bray-Curtis dissimilarity was performed. Permutational Multivariate Analysis of Variance (PERMANOVA) was performed

based on unweighted Unifrac distance, weighted Unifrac distance and Bray-Curtis dissimilarity to assess the factors associated with microbiota variation. To assess the impact of the participant type (MEs and NMEs), carbohydrate usage, and the treatment groups on the microbial metabolites (hydrogen, methane, acetate, propionate and butyrate) and alpha diversity metrics, a two-way mixed ANOVA was used with participant type (MEs and NMEs) as between-subject factor and time as within-subject factor.

The p-values for multiple pairwise tests were corrected using Benjamini-Hochberg false-discovery rate (FDR). A (corrected) p-value ≤ 0.05 was considered statistically significant, and 0.05 ≤ p-value (or corrected) <0.1 was considered a trend.

Results

Microbiota composition differs between methane excretors and non-methane excretors

PCoA analysis based on unweighted Unifrac distance, weighted Unifrac distance and Bray-Curtis dissimilarity showed distinct clusters of microbiota composition in all samples associated with the six different participants (Figure 2). PERMANOVA based on unweighted Unifrac distance, weighted Unifrac distance, and Bray-Curtis dissimilarity showed that the factors participant identity, type of carbohydrates (pectin and the mixture), and type of participants (MEs and NMEs) had a significant association with the total microbiota variation with all of the incubation samples. Independent of the algorithm used, variation partitioning between samples at all timepoints indicated that most of the microbiota variation was explained by participant identity, followed by participant type at all sampling points. Although the effect of these two factors slightly decreased during the incubations, it is evident that these microbiota-specific characteristics overruled the incubation conditions. Interestingly, microbiota variation explained by participant type was much larger when unweighted Unifrac distances were used. This can likely be explained by the differential detection of methanogens since phylogeny is an important factor in calculating Unifrac distances. As expected, the microbiota variation explained by the type of carbohydrates increased with the progress of incubation. However, it remained smaller than the variation explained by participant identity and participant type throughout the incubation process.

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In the following, we analysed the contribution of inhibiting the methanogenesis in MEs and adding methanogens in NMEs to microbiota composition, however, these modulations did not cause a significant effect on microbiota composition (Supplementary Figure 1). In addition, adding BES to the microbiota of NMEs did not cause a significant effect on microbiota composition indicating that BES only inhibited methane production, but did not affect the microbiota composition itself (Supplementary Figure 2). In line with the fact that sulphate was not used during the incubation, no effect was observed for sulphate on microbiota composition as well (Supplementary Figure 3). Moreover, these treatments did not affect relative abundances of the top 10 family-level taxa throughout the incubation (Supplementary Figure 4). Overall, these observations indicate that the microbiota of each subject was relatively resilient towards the variations introduced by the different incubation conditions.
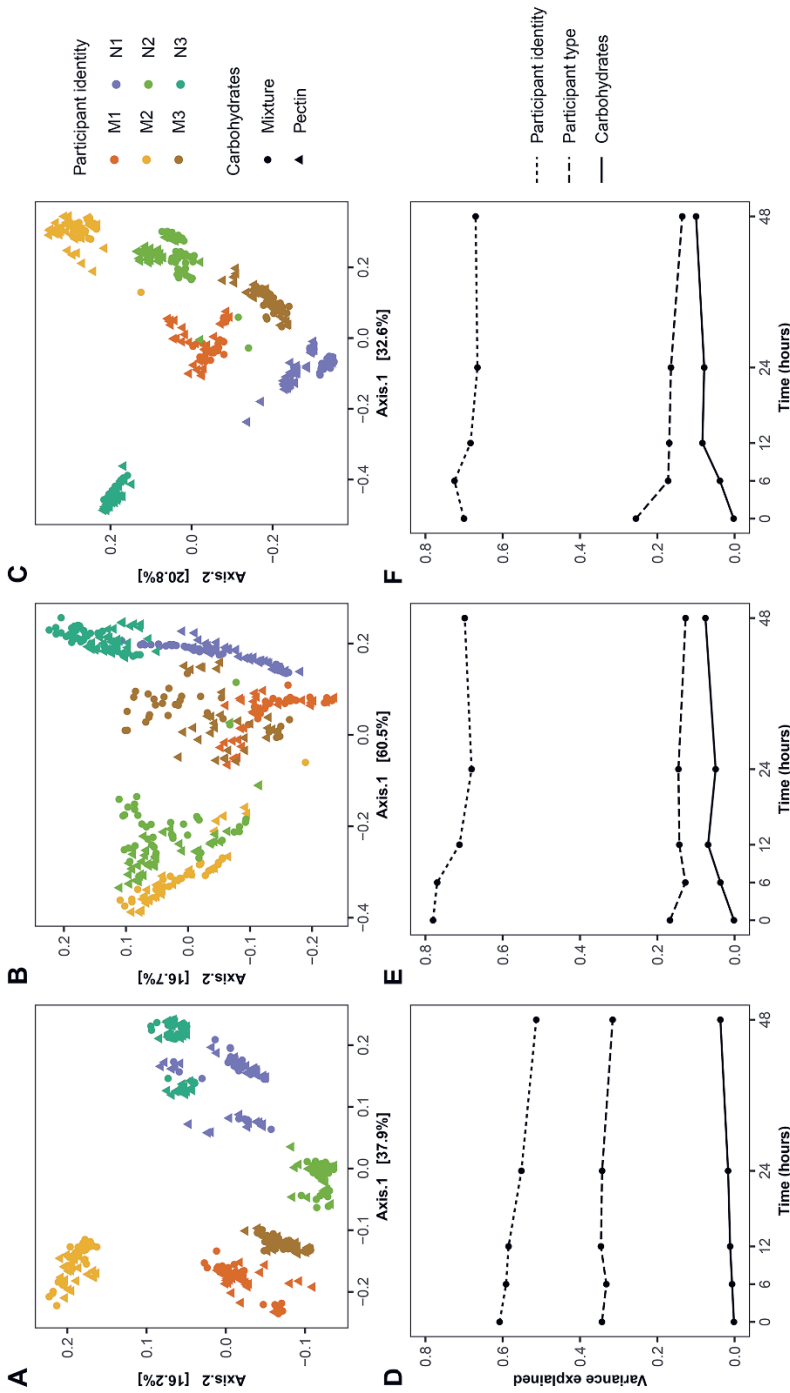
Figure 2: Microbiota variation in the dataset. PCoA based on **A** unweighted Unifrac distance, **B** weighted Unifrac distance and **C** Bray-Curtis dissimilarity matrices of all incubation samples. Variation in microbiota composition that can be explained by carbohydrates (pectin or the mixture carbohydrates: pectin from apple, starch from potato, inulin, xylan, arabinogalactan), participant, and the participant type (methane excretors and non-methane excretors) at each sampling timepoint based on **D** unweighted Unifrac distance, **E** weighted Unifrac distance and **F** Bray-Curtis dissimilarity matrices of all incubation samples. M: methane excretors; N: non-methane excretors.

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A higher microbial richness in incubations with methane excretor faeces compared to those of non-methane excretors

Since a significant part of the microbiota variation was explained by the distinction between MEs and NMEs, details in the microbial alpha diversity were analysed as well (Table 2). After inoculation, microbiota alpha diversity metrics ASV richness (the presence of the taxa) and Shannon diversity (the presence and relative abundance of the taxa) dramatically decreased in MEs and NMEs due to the selective force of the medium on microbiota development. We observed that MEs had a significantly higher ASV richness at the start of the incubation as well as throughout the incubations with both pectin and the mixture of carbohydrates compared to NMEs. In contrast, a higher Shannon diversity in MEs was only observed at 24 h ($p < 0.05$) in the incubations with pectin, suggesting that the observed differences in microbial richness were largely due to the presence of subdominant taxa.

Adding BES, *M. smithii* or sulphate to the cultures did not change ASV richness or Shannon diversity throughout the incubation compared to control groups, indicating that microbial alpha diversity was not affected by these treatments.

Higher propionate production in incubations with methane excretor faeces compared to non-methane excretors

Since we observed distinct microbiota signatures for MEs and NMEs, we next determined whether this was associated with a distinct metabolite profile. SCFAs, namely acetate, propionate and butyrate, were produced during the incubations (Table 3). Although acetate concentration did not significantly differ between MEs and NMEs throughout the incubation with either pectin or the carbohydrate mixture, a faster acetate production ($p < 0.05$) in NMEs was observed during the first 6 h incubation compared to that in MEs in the control incubations with pectin. However, this was not observed with the carbohydrate mixture. Interestingly, the propionate concentration was significantly higher in incubations with faecal slurries of MEs compared to NMEs during the incubations with both types of carbohydrates, with consistently more propionate production in the incubations with the carbohydrate mixture than those with pectin ($p < 0.01$), indicating both the type of microbiota and carbohydrate affect the propionate production. As for butyrate, no difference was observed between MEs and NMEs in the incubations using either pectin or the carbohydrate mixture. Furthermore, adding BES, *M. smithii* or sulphate did not change the SCFA production during any of the incubations, suggesting that modulating the hydrogenotrophic activities did not distinctly affect the metabolic profile.

Sulphate is not used during *in vitro* incubations

Although the general effect of the different treatments was minor on the incubations with faecal slurries of both MEs and NMEs, we wondered whether some specific impacts were detectable. To our surprise, sulphate was not used in any of the incubations, indicating that in none of the incubations dissimilatory sulphate reduction was stimulated (Supplementary Figure 5). In line with this, the sulphate did not have any effect on the gut microbiota and metabolite production in any of the incubations. Remarkably, the genus *Desulfovibrio* was detected in two of the three inocula in both MEs and NMEs (Figure 3). Although its relative abundance decreased during the incubations, it remained at least detectable until 24 h of incubation.

Methane production is stimulated by adding exogenous methanogens to the faecal inocula of non-methane excretors

Hydrogen production was observed in all incubations. Hydrogen levels were significantly lower in MEs compared to NMEs ($p < 0.01$) in the incubations with pectin, in contrast, this was not observed in the incubations with the carbohydrate mixture. In addition, hydrogen levels showed a higher trend ($0.05 < p < 0.1$) in the incubations with the carbohydrate mixture compared to pectin only in the incubations with the faecal inocula from NMEs, indicating that both the type of carbohydrate and participant affected the hydrogen production (Table 4).

As expected, methane was produced in the incubations with the faecal inocula from MEs, whereas no methane was produced by using the faecal slurries from NMEs, confirming that the breath test is a reliable way to predict methanogenesis capacity in human gut microbiomes. Significantly higher methane levels were observed in the incubations with the carbohydrate mixture ($p < 0.05$) compared to pectin, indicating that methane production is dependent on the way different carbohydrates are fermented.

To determine the effect of methanogenesis in gut ecosystems, it was modulated by adding BES in ME incubations as well as by adding *M. smithii* as co-inoculum in NME incubations. As expected, no methane was detected when BES was added in MEs, confirming that this compound inhibited methane production. Although adding BES did not statistically impact the overall hydrogen production, the clear decline in hydrogen levels after 12 h with the carbohydrate mixture as substrate did not occur when BES was added in the incubations of the MEs. As expected, no effect of BES on hydrogen production was observed in the NMEs. In contrast to BES, adding *M. smithii* as co-inoculum in NMEs resulted in a similar amount of methane produced

to that using the faecal slurries of MEs, indicating that *M. smithii* could occupy a niche in the gut microbiota of NMEs during the fermentation process.

Dynamics of hydrogenotrophic genera over time

To determine whether the introduction or inhibition of methanogenesis impacted different hydrogenotrophic microbes, we studied the dynamics of the genera *Methanobrevibacter*, *Desulfovibrio* and *Blautia* as most abundant representatives of methanogens, SRB and reductive acetogens, respectively, throughout the incubations (Figure 3). *Methanobrevibacter* was detected in control incubations in MEs, but not in NMEs. Adding *M. smithii* in NMEs increased the relative abundance of *Methanobrevibacter* throughout the whole incubation process, confirming that *M. smithii* survived and occupied a niche in the faecal communities of NMEs. Although BES inhibited methane production throughout the incubation, no difference in the relative abundance of *Methanobrevibacter* was observed between the control and BES group in MEs in the incubations with the carbohydrate mixture. However, a lower relative abundance of *Methanobrevibacter* was found in the pectin incubations when BES was added compared to controls ($p < 0.001$). These results indicated that the impact of BES on the relative abundance of *Methanobrevibacter* depended on the type of carbohydrates that were used as substrate.

As indicated before, the genus *Desulfovibrio* was detected in two of the three incubations with a decrease in relative abundance during the incubation in both MEs and NMEs. No differences in *Desulfovibrio* relative abundance were observed between MEs and NMEs, and inhibiting the methane production by BES in MEs or stimulating the methane production in NMEs did not impact the relative abundance of *Desulfovibrio,* reinforcing that *Desulfovibrio* did not compete with *Methanobrevibacter* for hydrogen in our experiments, in line with the fact that sulphate concentrations did not change as mentioned above.

Significantly higher relative abundances of *Blautia* were observed in the ME incubations with the carbohydrate mixture ($p < 0.05$) compared to NMEs during the incubations. In addition, using the carbohydrate mixture led to much higher relative abundances of *Blautia* in both MEs and NMEs than when using pectin, which indicated that the type of carbohydrates played an important role in the relative abundance of *Blautia*. Similar to the observations for *Desulfovibrio*, the inhibition or stimulation of methanogenesis did also not affect *Blautia* relative abundances.
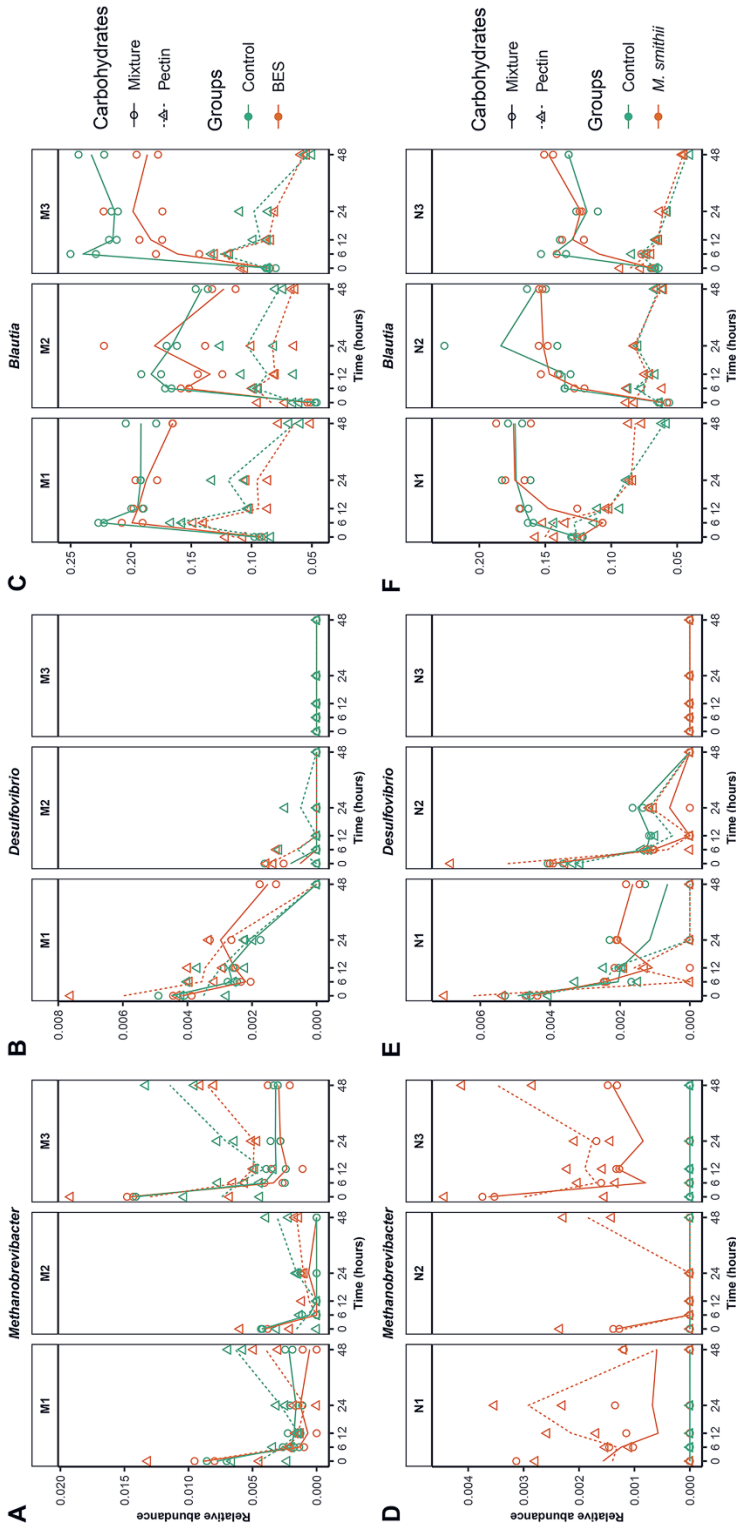
Figure 3: Relative abundance of genera *Methanobrevibacter*, *Desulfovibro*, and *Blautia* throughout the incubation with pectin or the carbohydrate mixture. *Methanobrevibacter* (**A**), *Desulfovibro* (**B**), and *Blautia* (**C**) in control and BES groups for MEs; *Methanobrevibacter* (**D**), *Desulfovibro* (**E**), and *Blautia* (**F**) in control and *M. smithii* groups for NMEs. Lines represent the average of the duplicate incubations at each timepoint. MEs: methane excretors; NMEs: non-methane excretors; BES: 2-bromoethanesulfonate.

6

Table 2 Microbiota alpha diversity metrics (ASV richness and Shannon diversity) during incubations over time.

			Methane excretors (n=3)					Non-methane excretors (n=3)					p values (participant type × time)
			0h	6h	12h	24h	48h	0h	6h	12h	24h	48h	
Pectin	ASV richness	Control	127.00 ± 19.70*	127.50 ± 7.05*	105.67 ± 3.79*	108.00 ± 14.73*	111.00 ± 14.73*	87.50 ± 8.05	91.67 ± 5.30	79.67 ± 6.90	71.50 ± 9.99	67.83 ± 15.46	0.444
		BES	138.50 ± 11.27*	113.33 ± 6.66*	111.67 ± 4.75*	103.50 ± 5.22*	100.67 ± 4.25*	87.17 ± 3.33	83.17 ± 10.49	77.50 ± 4.09	82.00 ± 4.27	69.17 ± 9.65	0.007
		M. smithii						97.67 ± 6.90	77.67 ± 4.54	83.00 ± 4.09	77.83 ± 8.31	76.67 ± 10.79	-
		Sulphate	125.00 ± 19.22*	111.33 ± 12.50*	108.33 ± 6.35*	110.00 ± 7.57*	105.83 ± 12.35*	85.00 ± 8.67	76.50 ± 5.77	81.17 ± 2.31	72.83 ± 8.50	65.67 ± 11.64	0.485
	Shannon	Control	3.97 ± 0.32	3.91 ± 0.23	3.52 ± 0.18	3.61 ± 0.09	3.70 ± 0.24	3.46 ± 0.22	3.48 ± 0.17	3.25 ± 0.24	3.02 ± 0.19	2.81 ± 0.33	0.170
		BES	4.14 ± 0.23	3.71 ± 0.29	3.61 ± 0.19	3.48 ± 0.19	3.51 ± 0.12	3.44 ± 0.23	3.24 ± 0.33	3.19 ± 0.33	3.19 ± 0.38	2.88 ± 0.34	0.331
		M. smithii						3.60 ± 0.16	3.14 ± 0.12	3.26 ± 0.23	3.10 ± 0.13	3.00 ± 0.35	-
		Sulphate	3.96 ± 0.34	3.65 ± 0.40	3.53 ± 0.21	3.51 ± 0.20	3.45 ± 0.29	3.46 ± 0.22	3.18 ± 0.33	3.25 ± 0.24	3.04 ± 0.15	2.86 ± 0.24	0.582
Mixture	ASV richness	Control	136.33 ± 15.12*	99.83 ± 3.51*	109.67 ± 7.29	101.67 ± 5.92*	87.00 ± 4.77*	95.00 ± 9.00	79.33 ± 4.16	78.00 ± 6.50	91.83 ± 10.41	73.17 ± 9.00	0.000
		BES	126.83 ± 17.10*	98.67 ± 8.08*	98.83 ± 5.86*	121.00 ± 7.40*	95.67 ± 4.54*	88.33 ± 6.83	77.00 ± 6.24	72.50 ± 3.91	71.67 ± 7.15	67.33 ± 9.29	0.005
		M. smithii						88.17 ± 10.00	75.67 ± 3.40	74.83 ± 5.86	76.83 ± 8.50	68.50 ± 7.81	-
		Sulphate	126.17 ± 20.43*	106.33 ± 8.81*	95.17 ± 3.51*	98.83 ± 5.53*	87.83 ± 4.54*	85.33 ± 9.93	81.50 ± 6.08	80.83 ± 6.01	73.83 ± 7.09	67.00 ± 8.67	0.028
	Shannon	Control	4.07 ± 0.24	3.51 ± 0.13	3.54 ± 0.14	3.41 ± 0.18	3.20 ± 0.17	3.59 ± 0.17	3.13 ± 0.25	3.10 ± 0.23	3.32 ± 0.22	3.02 ± 0.25	0.004
		BES	3.97 ± 0.29	3.50 ± 0.21	3.44 ± 0.17	3.68 ± 0.12	3.32 ± 0.21	3.41 ± 0.24	3.07 ± 0.32	2.98 ± 0.22	2.99 ± 0.19	2.88 ± 0.25	0.390
		M. smithii						3.52 ± 0.19	3.21 ± 0.35	3.01 ± 0.23	3.08 ± 0.16	2.93 ± 0.28	-
		Sulphate	3.97 ± 0.33	3.54 ± 0.18	3.39 ± 0.16	3.42 ± 0.19	3.23 ± 0.19	3.50 ± 0.20	3.17 ± 0.21	3.17 ± 0.21	3.09 ± 0.18	2.91 ± 0.28	0.195

Data are presented as mean ± SD. Differences in ASV richness and Shannon diversity between methane excretors and non-methane excretors over time were analysed using two-way mixed ANOVA. p values were corrected for multiple pairwise comparisons using Benjamini-Hochberg. SD: standard deviation.

*Significant differences between methane excretors and non-methane excretors at corresponding timepoint.

Table 3: Concentration of SCFAs (mM) during incubations over time.

			Methane excretors (n=3)					Non-methane excretors (n=3)					p values (participant type × time)
			0h	6h	12h	24h	48h	0h	6h	12h	24h	48h	
Pectin	Acetate	Control	0.53 ± 0.23	**12.20 ± 1.01***	32.19 ± 3.00	35.46 ± 3.29	38.26 ± 3.50	1.00 ± 0.28	**21.42 ± 3.35**	32.01 ± 6.79	36.82 ± 7.85	37.12 ± 9.27	0.089
		BES	0.51 ± 0.36	12.67 ± 1.64	32.66 ± 5.32	33.74 ± 4.58	37.93 ± 1.85	1.42 ± 0.15	22.68 ± 6.74	31.52 ± 10.21	33.68 ± 11.14	32.76 ± 10.88	0.057
		M. smithii						1.14 ± 0.31	25.34 ± 2.63	33.53 ± 8.64	36.44 ± 9.71	35.80 ± 9.01	-
		Sulphate	0.56 ± 0.34	13.18 ± 1.55	34.77 ± 3.81	38.20 ± 4.98	38.84 ± 3.67	0.90 ± 0.33	23.66 ± 6.02	33.24 ± 9.47	34.47 ± 10.77	35.38 ± 11.19	0.122
	Propionate	Control	0.08 ± 0.08	1.77 ± 0.73	4.12 ± 1.15	4.68 ± 1.68	5.04 ± 1.60	0.45 ± 0.01	2.24 ± 0.31	3.00 ± 0.84	3.26 ± 0.89	3.29 ± 0.90	**0.011**
		BES	0.02 ± 0.04	1.74 ± 0.63	4.48 ± 1.60	4.77 ± 2.18	5.10 ± 1.80	0.45 ± 0.07	2.16 ± 0.38	3.00 ± 0.46	3.24 ± 1.01	3.24 ± 0.90	0.121
		M. smithii						0.43 ± 0.05	2.15 ± 0.38	2.85 ± 0.67	3.23 ± 0.99	3.41 ± 1.02	-
		Sulphate	0.03 ± 0.04	1.91 ± 0.80	4.41 ± 1.47	5.04 ± 2.39	5.22 ± 2.48	0.46 ± 0.09	2.27 ± 0.51	3.04 ± 0.75	3.35 ± 0.84	3.52 ± 1.18	0.178
	Butyrate	Control	0.02 ± 0.03	3.25 ± 0.86	6.73 ± 1.32	7.95 ± 0.97	8.60 ± 1.65	0.44 ± 0.18	2.82 ± 0.70	5.23 ± 1.43	6.99 ± 3.68	7.32 ± 4.80	0.853
		BES	0.00 ± 0.00	3.32 ± 1.04	7.17 ± 1.62	7.87 ± 0.67	8.68 ± 1.74	0.43 ± 0.15	3.02 ± 0.93	5.13 ± 1.49	7.22 ± 5.23	7.91 ± 6.66	0.911
		M. smithii						0.46 ± 0.19	3.38 ± 1.40	5.64 ± 1.96	7.35 ± 4.63	8.64 ± 6.91	-
		Sulphate	0.00 ± 0.00	3.54 ± 1.42	7.43 ± 1.30	8.57 ± 1.10	8.66 ± 1.17	0.41 ± 0.23	2.94 ± 0.76	5.09 ± 1.24	6.53 ± 4.04	7.91 ± 5.86	0.521
Mixture	Acetate	Control	0.77 ± 0.15	19.49 ± 3.45	30.87 ± 4.84	39.10 ± 5.33	41.72 ± 6.65	1.08 ± 0.20	25.48 ± 2.91	33.44 ± 1.16	40.75 ± 2.70	40.53 ± 2.33	0.181
		BES	0.46 ± 0.19	18.78 ± 2.53	29.66 ± 5.25	38.00 ± 4.75	38.00 ± 5.68	1.39 ± 0.51	25.31 ± 2.83	33.12 ± 2.60	39.49 ± 2.89	39.35 ± 1.64	0.259
		M. smithii						1.16 ± 0.28	23.15 ± 4.30	33.77 ± 1.41	40.21 ± 2.30	40.73 ± 3.83	-
		Sulphate	0.57 ± 0.14	19.39 ± 3.47	31.25 ± 4.33	41.83 ± 2.56	40.33 ± 3.91	1.21 ± 0.07	25.30 ± 2.45	32.88 ± 2.19	40.78 ± 1.80	41.06 ± 0.43	0.052
	Propionate	Control	0.07 ± 0.11	2.30 ± 0.11	**4.92 ± 0.53***	8.39 ± 2.00	12.36 ± 4.71	0.42 ± 0.04	2.20 ± 0.33	**2.97 ± 0.33**	4.72 ± 2.04	4.85 ± 2.04	**0.005**
		BES	0.06 ± 0.10	2.40 ± 0.30	**4.93 ± 0.68***	8.93 ± 2.39	12.44 ± 3.50	0.44 ± 0.03	2.44 ± 0.15	**3.08 ± 0.22**	4.45 ± 1.77	4.85 ± 1.80	**0.034**
		M. smithii						0.42 ± 0.07	2.29 ± 0.40	2.93 ± 0.38	4.59 ± 1.84	4.81 ± 1.93	-
		Sulphate	0.06 ± 0.10	2.41 ± 0.29	**5.18 ± 0.79***	9.67 ± 1.73*	12.96 ± 4.39	0.41 ± 0.09	2.36 ± 0.09	**3.12 ± 0.18**	**4.64 ± 1.80**	4.90 ± 2.01	**0.040**
	Butyrate	Control	0.00 ± 0.00	4.61 ± 1.22	9.00 ± 3.54	12.78 ± 6.62	14.26 ± 8.36	0.27 ± 0.15	3.58 ± 1.87	5.65 ± 2.74	10.53 ± 2.42	13.97 ± 1.14	0.579
		BES	0.00 ± 0.00	4.44 ± 1.26	8.75 ± 2.79	12.76 ± 4.85	14.94 ± 7.76	0.30 ± 0.15	3.41 ± 1.78	5.67 ± 2.98	8.99 ± 2.12	12.17 ± 2.29	0.525
		M. smithii						0.31 ± 0.19	3.05 ± 1.30	5.87 ± 3.07	10.64 ± 1.85	12.49 ± 2.25	-
		Sulphate	0.00 ± 0.00	4.74 ± 1.14	8.70 ± 2.03	14.01 ± 5.58	14.83 ± 4.51	0.54 ± 0.38	3.40 ± 1.84	5.57 ± 2.77	9.34 ± 2.03	11.81 ± 2.27	0.320

Data are presented as mean ± SD. Differences in concentrations of acetate, propionate and butyrate between methane excretors and non-methane excretors over time were analysed using two-way mixed ANOVA. p values were corrected for multiple pairwise comparisons using Benjamini-Hochberg. SD: standard deviation.

*Significant differences between methane excretors and non-methane excretors at corresponding timepoint.

6

158

Table 4: Concentration of hydrogen and methane (mM) during incubations over time.

			Methane excretors (n=3)					Non-methane excretors (n=3)					p values (participant type × time)
			0h	6h	12h	24h	48h	0h	6h	12h	24h	48h	
Pectin	Hydrogen	Control	0.00 ± 0.00	1.18 ± 0.53	1.02 ± 0.22	1.00 ± 0.41	0.79 ± 0.45	0.00 ± 0.00	1.16 ± 0.44	2.74 ± 0.74	2.42 ± 0.80	2.27 ± 1.14	**0.003**
		BES	0.00 ± 0.00	1.42 ± 0.68	**1.22 ± 0.40***	**1.15 ± 0.63***	**1.04 ± 0.70***	0.00 ± 0.00	1.50 ± 0.69	**2.82 ± 0.40**	**3.12 ± 0.22**	**3.21 ± 0.57**	**0.000**
		M. smithii						0.00 ± 0.00	0.95 ± 0.53	2.07 ± 0.53	1.72 ± 0.59	1.25 ± 0.86	-
		Sulphate	0.00 ± 0.00	1.20 ± 0.44	1.14 ± 0.25	1.21 ± 0.54	0.95 ± 0.59	0.00 ± 0.00	1.42 ± 0.62	2.72 ± 0.76	2.70 ± 0.49	2.47 ± 0.69	**0.002**
	Methane	Control	0.00 ± 0.00	**0.08 ± 0.02***	**0.13 ± 0.05***	**0.13 ± 0.05***	**0.11 ± 0.04***	0.00 ± 0.00	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.000**
		BES	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
		M. smithii						0.00 ± 0.00	0.17 ± 0.09	0.21 ± 0.04	0.26 ± 0.03	0.28 ± 0.06	-
		Sulphate	0.00 ± 0.00	**0.07 ± 0.02***	**0.12 ± 0.04***	**0.13 ± 0.05***	**0.12 ± 0.05***	0.00 ± 0.00	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.000**
Mixture	Hydrogen	Control	0.00 ± 0.00	4.16 ± 2.67	5.16 ± 4.21	4.06 ± 3.97	3.80 ± 4.31	0.00 ± 0.00	4.28 ± 2.47	4.98 ± 2.94	5.38 ± 2.52	6.25 ± 3.26	0.602
		BES	0.00 ± 0.00	4.52 ± 2.00	5.68 ± 3.86	5.80 ± 5.01	6.07 ± 5.32	0.00 ± 0.00	4.55 ± 2.89	5.77 ± 3.58	7.30 ± 4.16	7.88 ± 3.71	0.679
		M. smithii						0.00 ± 0.00	3.57 ± 2.52	4.20 ± 3.17	4.26 ± 3.56	4.61 ± 3.29	-
		Sulphate	0.00 ± 0.00	3.90 ± 2.30	4.91 ± 3.72	3.10 ± 3.27	2.67 ± 3.66	0.00 ± 0.00	4.34 ± 2.68	5.25 ± 3.38	6.30 ± 3.23	7.23 ± 3.47	0.173
	Methane	Control	0.00 ± 0.00	**0.10 ± 0.05***	**0.19 ± 0.04***	**0.44 ± 0.16***	**0.63 ± 0.31***	0.00 ± 0.00	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.000**
		BES	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
		M. smithii						0.00 ± 0.00	0.22 ± 0.12	0.32 ± 0.12	0.44 ± 0.07	0.45 ± 0.08	-
		Sulphate	0.00 ± 0.00	**0.09 ± 0.03***	**0.21 ± 0.05***	**0.46 ± 0.24***	**0.73 ± 0.20***	0.00 ± 0.00	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.000**

Data are presented as mean ± SD. Differences in concentrations of hydrogen and methane between methane excretors and non-methane excretors over time were analysed using two-way mixed ANOVA. p values were corrected for multiple pairwise comparisons using Benjamini-Hochberg. SD: standard deviation.

*Significant differences between methane excretors and non-methane excretors at corresponding timepoint.

Discussion

In this study, we compared the *in vitro* dynamics of faecal microbiota composition and metabolite production between MEs and NMEs during incubations with different carbohydrates. In addition, we studied the impact of modulating potential hydrogen consumption by the microbiota via altering methanogenesis and sulphate reduction during incubations. We found that classifying subjects into MEs and NMEs via measuring methane in their breath is a reliable method that matched the methanogenesis capacity in their microbiome. A significant proportion of microbiota variation in our data could be explained by this classification. Moreover, we observed that the faecal microbiota of MEs had a higher ASV richness and a different microbiota composition compared to NMEs. We furthermore found that metabolite production was not only determined by the type of microbiota (MEs vs. NMEs) but also the type of carbohydrates that were used as carbon and energy source. Although methanogenesis was inhibited in MEs by adding BES and stimulated in NMEs by adding *Methanobrevibacter smithii* as co-inoculum, altering this activity did not change overall microbiota composition and metabolite production.

Remarkably, sulphate was not consumed in either MEs or NMEs throughout the incubations. Although SRB have the greatest affinity for hydrogen and dissimilatory sulphate reduction is thermodynamically more favourable than methanogenesis and reductive acetogenesis [32, 33], our *in vitro* study showed that adding sulphate did not stimulate SRB to use hydrogen as an energy source during the incubations, indicating that sulphate may be not the limiting factor for SRB under the conditions used in this study. It has been reported that adding sulphate into basal (low sulphate) diets increased SRB counts and hydrogen sulphide production with a concomitant decrease in breath methane levels [34], which indicated that dietary sulphate can be directly consumed *in vivo* and regulate the methanogenic activity. However, this was observed only in half of the six subjects, suggesting that SRB were either absent or performing other metabolic activities in the gut of these "non-responders". It has been reported that SRB are versatile in their metabolism and are able to perform fermentations [35, 36]. We speculate that this was also the case for our incubations, although we cannot exclude that *in vitro* conditions in our study may be less suitable for the SRB to adapt to.

The breath test was used to identify MEs and NMEs in this study, and the methanogenic activities based on this were in line with the incubation results, thereby confirming earlier reports that the breath test is an effective way to reflect methanogenic activity in gut ecosystems [17]. The abundance of the genus *Methanobrevibacter* in the human gut shows a bimodal distribution, which means

that this genus is either very abundant or nearly absent [37]. It has been reported that MEs have approximately 10^9 CFU/g of methanogens in stool, whereas individuals who are NMEs harbour less than 10^4 CFU/g [17, 19]. In line with this, we also found a higher relative abundance of methanogens in MEs than in NMEs. Interestingly, adding *M. smithii* into the faecal inoculum of NMEs resulted in its persistence in the community and concomitant methane production, indicating that *M. smithii* could integrate into the community by occupying the niche for using hydrogen as an energy source. Earlier studies have shown that approximately one-third of individuals in the Western world and 80% of Africans are MEs [17, 18]. Although diet, host genetics, and microbial composition have been suggested to play an important role in harbouring gut methanogens, a clear mechanistic explanation has not been provided. Our study demonstrated that methanogenesis can be introduced into NME microbiomes *in vitro* and thereby suggests that a potential niche for methanogens exists within the NME microbiome.

A higher ASV richness was observed in MEs compared to NMEs. Although the reason for this difference is still unclear, we speculate that removal of the hydrogen by methanogens creates an environment that is accessible for different microbial populations to grow as hydrogen accumulation may lead to inhibition of fermentation. This is supported by a humanized gnotobiotic mouse model colonised with *Bacteroides thetaiotaomicron* and *M. smithii*, which revealed that co-colonisation with these two microbial strains led to higher population densities than when singly inoculated, and that *M. smithii* enhanced *B. thetaiotaomicron* to degrade polyfructose-containing glycans [15]. However, during the incubations in this study, inhibiting the methane production in MEs or adding methanogens into the microbiota communities of NMEs did not significantly alter overall microbiota composition and diversity compared to the control incubations. We observed that the overall development of the microbiota over time was nearly identical in all incubations (with and without treatments), suggesting that it was resilient towards these perturbations, which is a property of the gut microbiota to remain relatively stable over time and to maintain a homeostatic equilibrium state [8, 38, 39]. Considering this property of gut microbiota, the driving force of altering methanogenic activity may be not powerful enough to significantly change the total microbiota composition *in vitro* within a short period. We observed that the introduction of sulphate into the medium did not result in hydrogen usage by SRB and that the introduction of BES into the ME incubations led to the accumulation of hydrogen without further affecting the community. Therefore, an alternative explanation for our observations is that there is a niche for methanogenesis in both ME and NME, but that this niche is not crucial for the overall functioning of the gut microbiota under the conditions applied. This deserves further attention in future studies.

It has been reported that gas production is dependent on the types of carbohydrates that are fermented by the microbiota [40]. Our study found that hydrogen and methane production varied between individuals and differed based on the type of carbohydrates that were used as carbon and energy source during the incubations, thereby confirming previous observations [21]. In addition, we observed that although BES consistently inhibited methanogenesis, a lower relative abundance of *Methanobrevibacter* in the BES incubations was only found when pectin was used as the substrate, which indicates that the effect of BES on *Methanobrevibacter* population dynamics also depends on the type of carbohydrates that are fermented. Although it remains speculative which mechanisms underly these observations, it is evident that diet needs to be taken into consideration when trying to modulate the microbiota via altering the microbial activities.

In conclusion, we conducted well-controlled *in vitro* batch incubations with faeces of MEs and NMEs as inocula, which enabled us to study the impact of modifying conditions for hydrogenotrophy in microbial communities with different levels of methanogens. We observed that microbial richness, microbiota composition, propionate and methane production differed between MEs and NMEs and that dissimilatory sulphate reduction was not taking place when sulphate was added to the medium. Although we observed that the microbiota was resilient towards modulation of the hydrogenotrophic activities, we demonstrated that methanogenesis is a specific but optional niche for hydrogen consumption as methanogens can easily be introduced or inhibited without affecting the overall microbiota composition and metabolite production. We realise that our *in vitro* study, like any *in vitro* study, cannot mimic completely the complexity of the gut environment *in vivo* and that further *in vivo* research is needed to evaluate our findings. Nevertheless, our study provided mechanistic insights into the complex world of hydrogenotrophic microbes which may ultimately help us to improve our ecological understanding of the human gut.

Specific author contributions: TW: study design, laboratory work, statistical analysis and interpretation of data, drafting this manuscript. HS: study design, contributed to study supervision and critically revised this manuscript. EZ: study concept and design, assisted in interpretation of data, lead the study supervision and critically revised this manuscript.

**Potential competing interest:** the authors report no conflict of interest.
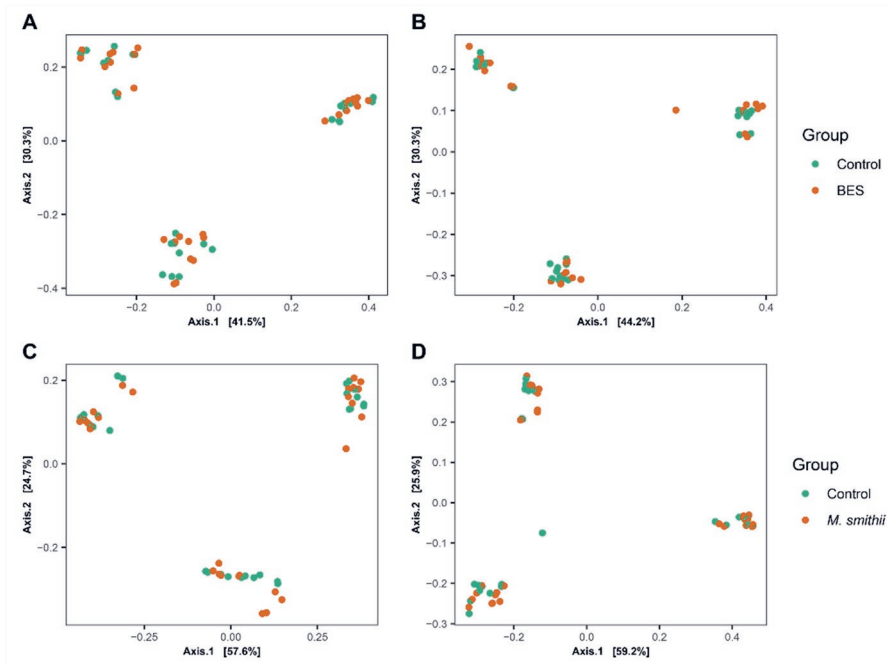
Supplementary Figure 1: Inhibiting methane production by BES in MEs and adding *Methanobrevibacter smithii* in NMEs did not change the overall microbiota composition. PCoA analysis based on Bray-Curtis dissimilarity using the faecal slurries of MEs in the incubations with pectin (**A**) and the carbohydrate mixture (**B**) and of NMEs in the incubations with pectin (**C**) and the carbohydrate mixture (**D**). MEs: methane excretors; NMEs: non-methane excretors; BES: 2-bromoethanesulfonate.
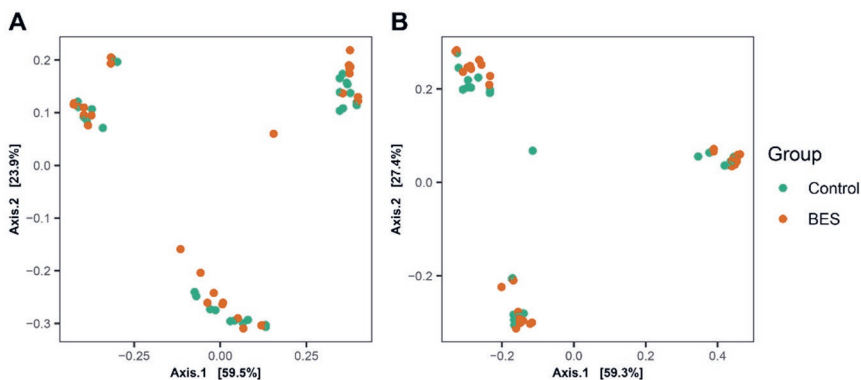

Supplementary Figure 2: Adding BES in NMEs did not change the overall microbiota composition. PCoA analysis based on Bray-Curtis dissimilarity using the faecal slurries of NMEs in the incubations with pectin (**A**) and the carbohydrate mixture (**B**) NMEs: non-methane excretors; BES: 2-bromoethanesulfonate.
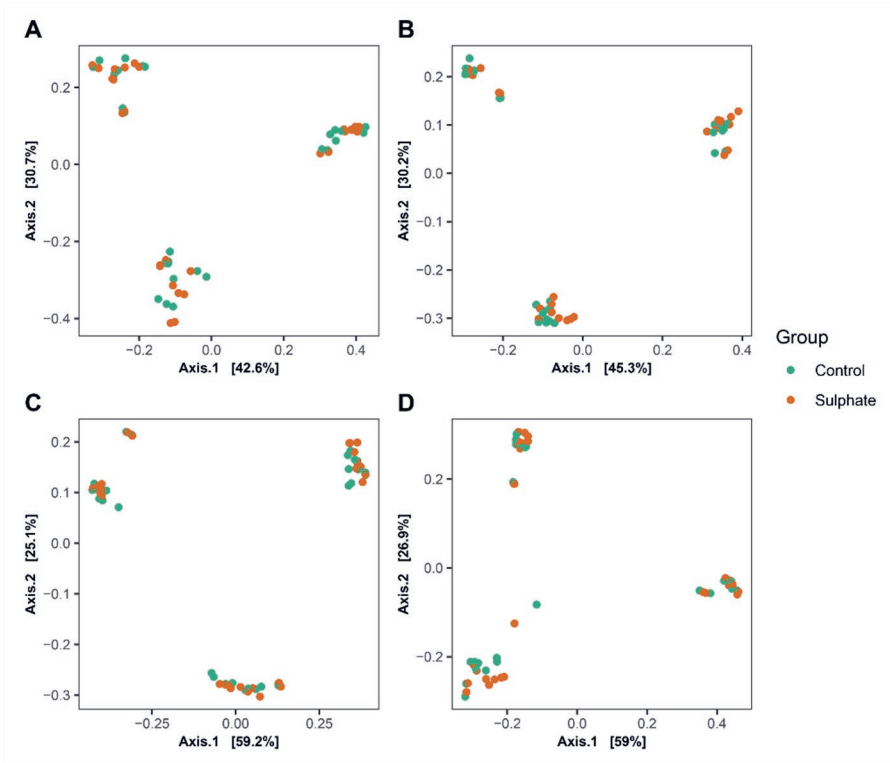
Supplementary Figure 3: Adding sulphate did not change the overall microbiota composition. PCoA analysis based on Bray-Curtis dissimilarity using the faecal slurries of MEs in the incubations with pectin (**A**) and the carbohydrate mixture (**B**) and of NMEs in the incubations with pectin (**C**) and the carbohydrate mixture (**D**). MEs: methane excretors; NMEs: non-methane excretors; BES: 2-bromoethanesulfonate.

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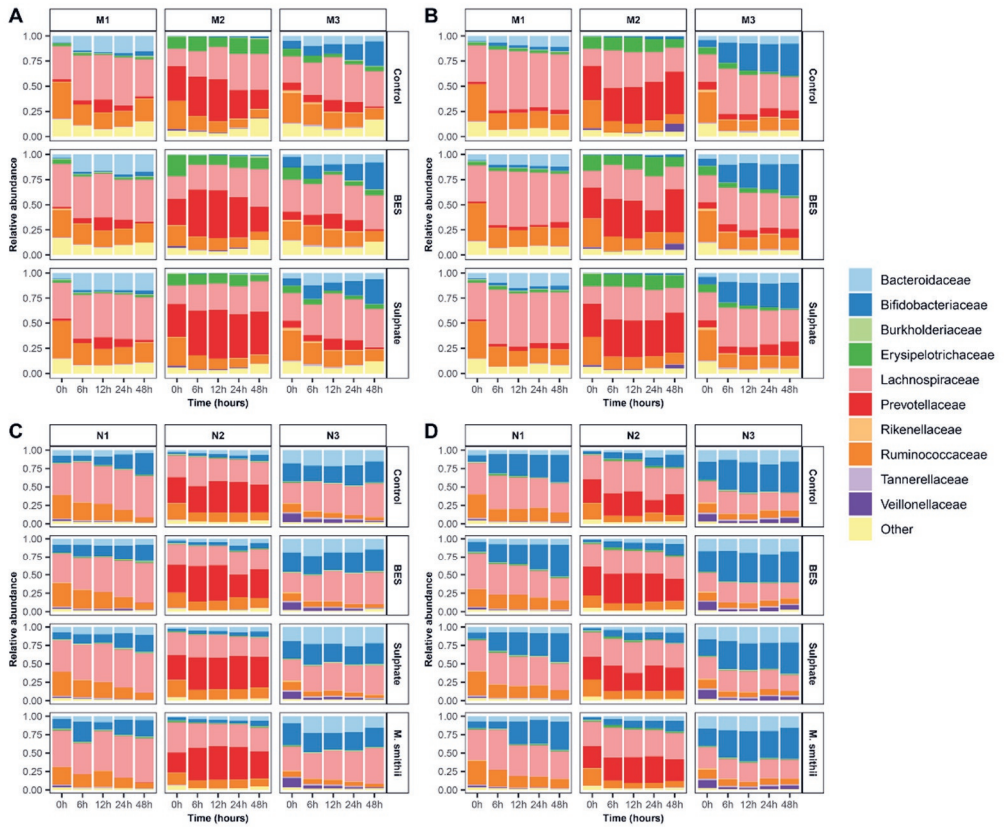
Supplementary Figure 4: Top 10 family level taxa ranked based on the average relative abundance across all of the incubation samples using the faecal slurries of methane excretors with pectin (A) and the carbohydrate mixture (B) and of non-methane excretors in the incubations with pectin (C) and the carbohydrate mixture (D). M, methane excretor; N, non-methane excretor. BES: 2-bromoethanesulfonate.
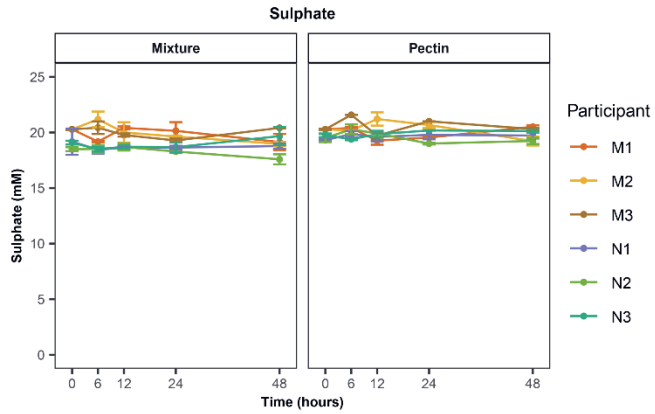
Supplementary Figure 5: Overview of the sulphate concentrations measured during the different incubations. M, methane excretor; N, non-methane excretor.

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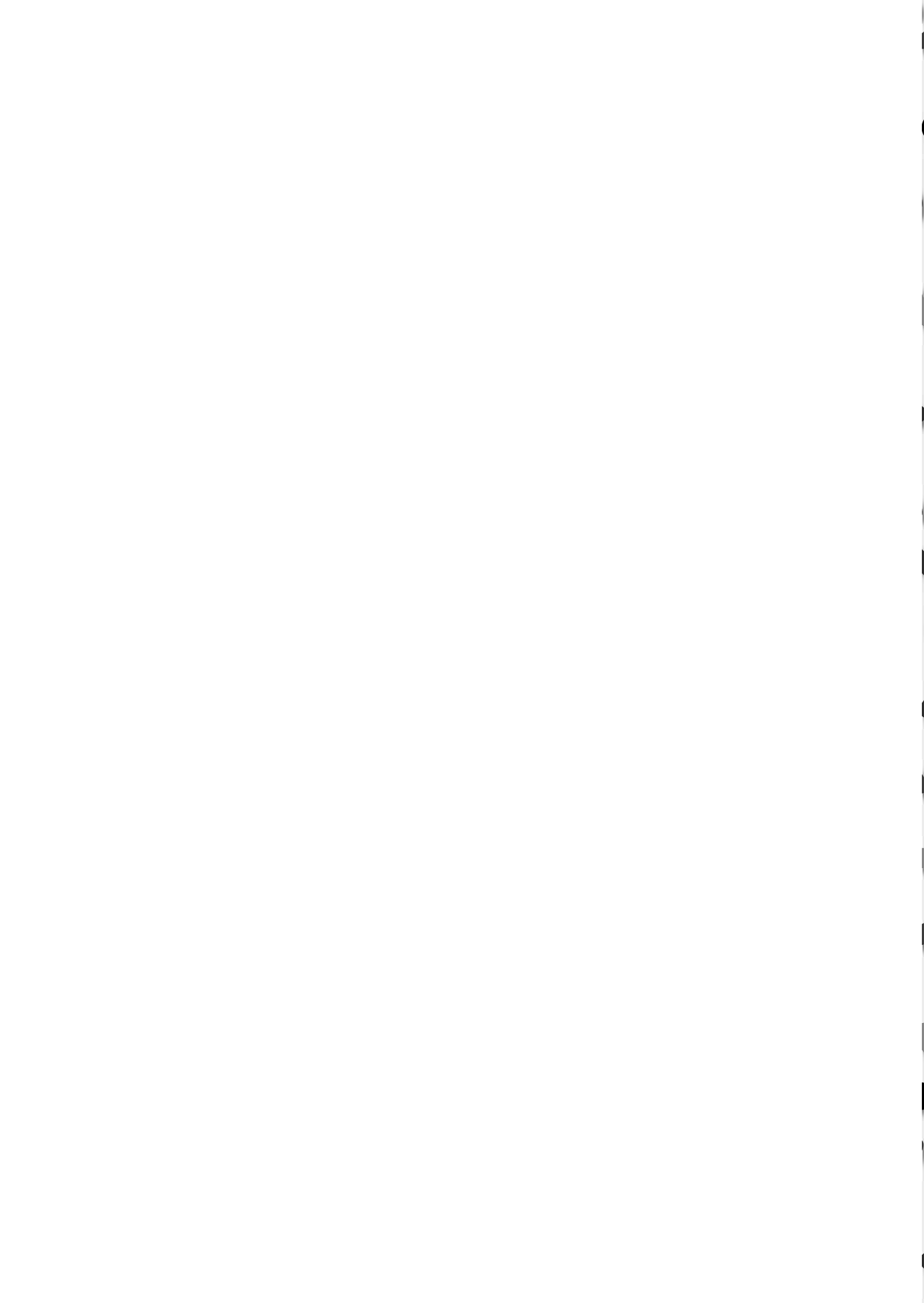
CHAPTER 7

General discussion
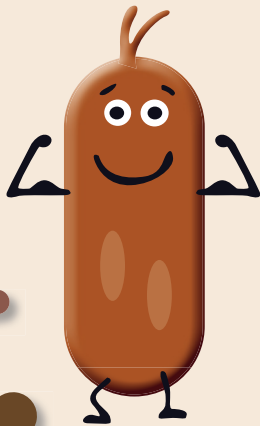
Introduction

The human gut is home to a large number of microbial species, collectively termed gut microbiota, that play a crucial role in the human body by executing important functions, such as fermenting indigestible carbohydrates and modulating immune response [1, 2]. The gut microbiota remains relatively stable over time and maintains a homeostatic equilibrium state due to its resistance against and resilience towards perturbations [3-5]. Considering this property, maintaining a stable state of the gut microbiota in healthy subjects can protect against microbiota related diseases, through e.g. limiting the number and abundance of pathobionts. In turn, maintaining a stable unhealthy state can be detrimental as this may contribute to the onset and maintenance of chronic intestinal disorders, such as inflammatory bowel disease (IBD) and metabolic syndrome [3, 5]. Hence studying the mechanisms that underly maintaining a stable microbiota composition and function is fundamental in order to understand the concept of healthy gut microbiota and to discover health or disease associated biomarkers for microbiome-based diagnostics and therapeutics [6].

External drivers (environmental factors), such as diet (**Chapter 2**) and health status (e.g. healthy, IBS; **Chapter 3**) have a clear association with microbiota composition and function, and have been widely studied. In contrast, the role of internal drivers, which are coming from within the microbiota, remains largely unknown. Hydrogen metabolism plays a central role in the overall microbial fermentation within anaerobic ecosystems such as the human gut and is hypothesised to be one of the important internal drivers in shaping microbiota composition and function [7, 8]. In this thesis, I studied various aspects of the hydrogenotrophic microbiota that primarily consists of microbes that use hydrogen as an energy source including methanogens, reductive acetogens and sulphate reducing bacteria (SRB). **Chapter 4** focused on the association of the hydrogenotrophic microbiota with overall microbiota composition and the correlations between methanogens, reductive acetogens and SRB in a human cohort study, whereas **Chapter 5** and **Chapter 6** employed *in vitro* studies to address the interactions between methanogens, reductive acetogens and SRB, and whether and how altering hydrogenotrophic activities modifies gut microbiota composition and function.

This chapter will discuss the key findings described in this thesis (Figure 1) and provide future perspectives.
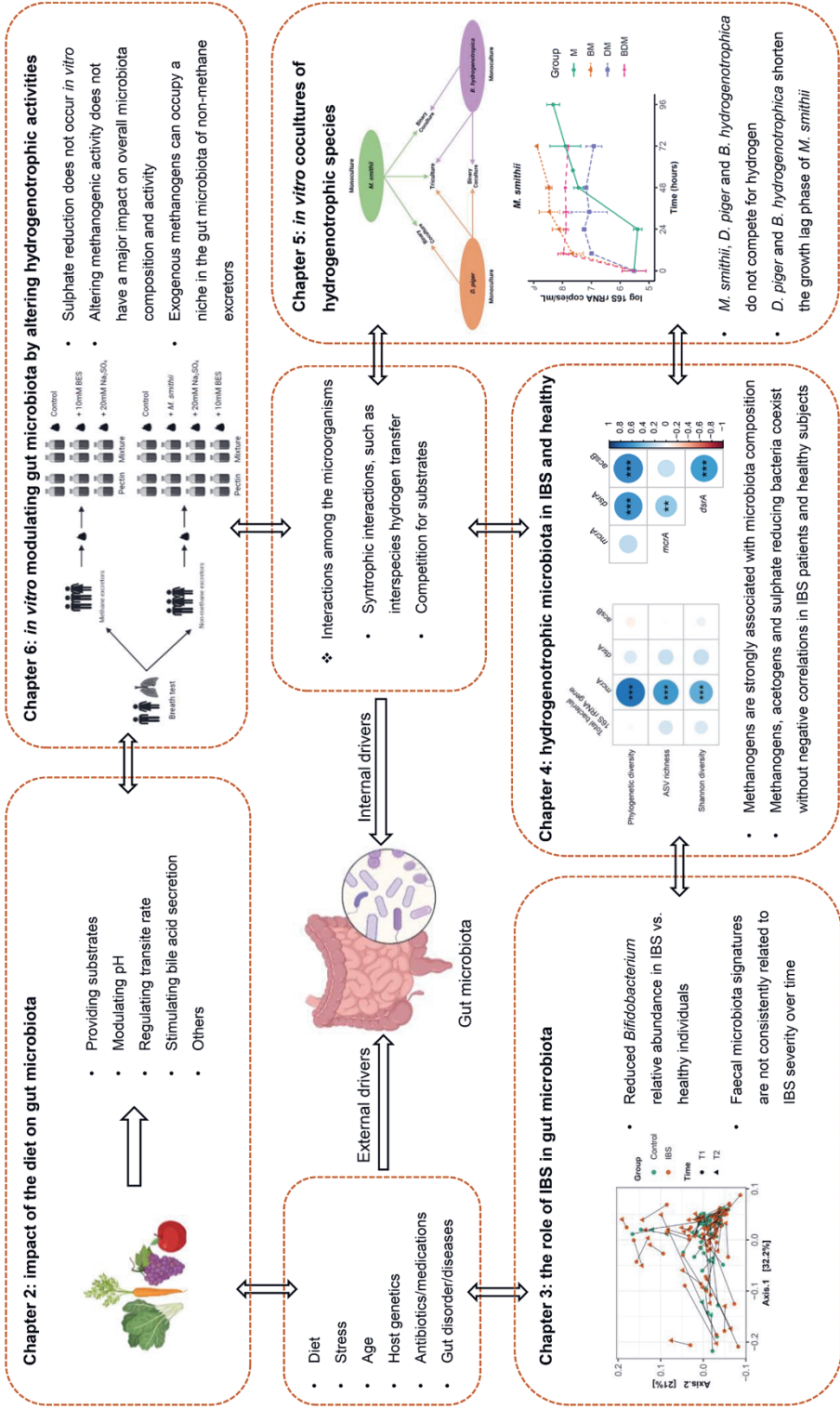
Figure 1: Graphical overview of the topics and main findings described in this thesis.

Hydrogen metabolism is related to dietary components in gut ecosystems

Dietary fibres and other complex substrates that escape digestion and absorption in the upper intestinal tract are fermented by gut microbiota resulting in the production of metabolites including gases, such as carbon dioxide and hydrogen [9-11]. In addition, hydrogenotrophic microbiota can consume the hydrogen that is produced via fermentation [12]. The combination of hydrogen production and consumption determines the level of hydrogen in the gut and is related to both the type of microbiota and dietary components [13]. As shown in **Chapter 6**, when faecal slurries from methane excretors (MEs) and non-methane excretors (NMEs) were inoculated in a basal medium [14] with pectin or a mixture of carbohydrates, methane was only produced in the incubations with the faecal slurries of MEs, and hydrogen levels were significantly lower in MEs compared to NMEs in the incubations with pectin.

Hydrogen production is positively associated with the amount of carbohydrates that are fermented as has been reported in previous studies [15, 16]. In addition, the amount of hydrogen produced differs between the types of carbohydrates that are fermented [13]. Studies in a mouse model using a non-invasive continuous real-time analysis revealed that the exposure to a lowly-digestible starch diet (rich in amylose) produced more hydrogen than a highly-digestible starch diet (rich in amylopectin) [17]. In the research described in **Chapter 6,** we also observed higher hydrogen levels in incubations with a complex carbohydrate mixture as compared to pectin. In addition, incubations with one of the MEs faecal slurries using lactose, fructose, xylan and inulin as substrates showed that hydrogen production varied in the incubations with different carbohydrates as substrates (Figure 2). These observations are in line with previously reported *in vitro* incubations with faecal inocula from elderly and younger adults in a medium simulating ileal effluent [18]. Aside from hydrogen production, methane production also varied between types of carbohydrates. In **Chapter 6** we observed higher methane in the incubations with the carbohydrate mixture as compared to pectin using the faecal slurries from MEs. In addition, as shown in Figure 2, the incubations with inulin had more methane produced than those with other carbohydrates in the late phase of incubations.

Altogether, these data show that hydrogen production and consumption are highly associated with dietary components consumed as well as with the type of microbiota. Accordingly, these hydrogenotrophic activities are important to take into consideration in dietary intervention studies, especially when symptoms related to gas production, such as bloating and distension, are investigated.
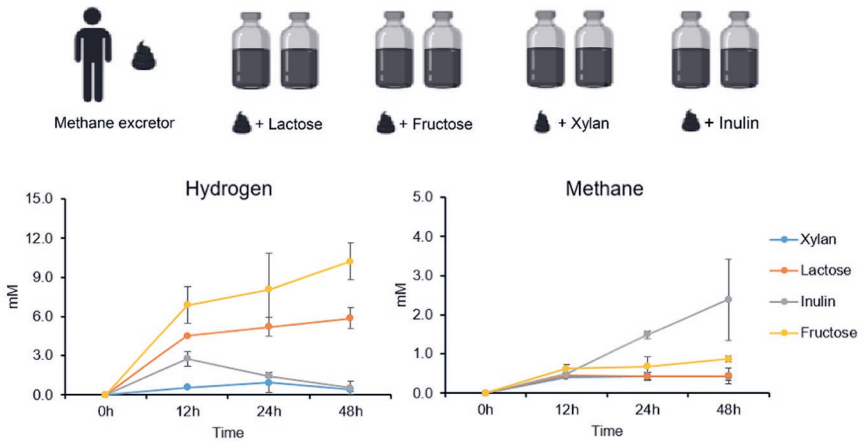
Figure 2: Hydrogen and methane production vary *in vitro* using lactose, fructose, xylan and inulin as substrates. The faecal slurry from one methane excretor was used as inoculum. Gas samples were collected to determine hydrogen and methane levels as described in **Chapter 6**.

Stratification of methanogen levels in gut ecosystems

Previous studies have shown that the number of methanogens, as well as methanogenic activity, varies between individuals [19, 20]. Using a breath test to determine the presence of methane aligns well with the methanogenic activity in the gut and is, therefore, an easy non-invasive method to classify subjects as MEs or NMEs as we have demonstrated in **Chapter 6**. It has been reported that in Africans, approximately 80% of the population is ME [21], which is much higher than the around 33% of MEs reported for the Western world [20]. Remarkably, African Americans and Caucasian Americans who consumed a typical Western diet do not show a difference in the percentage of MEs and NMEs, whereas native Africans who consumed a diet that had a lower protein and fat than the Western diet exhaled more methane than African Americans and Caucasian Americans, implying that diet is related to methane excretion [22]. In addition, it has also been reported that treatment of healthy subjects with a cocktail of antibiotics resulted in the disappearance of *M. smithii*, the dominant and most prevalent methanogen in the human gut and that it did not recover after treatment [23]. Considering the fact that the usage of antibiotics in European countries is higher than in Africa [24], antibiotic usage could play a role in the explanation for the lower prevalence of MEs in the Western world. However, a longitudinal study found that the methanogenic activity remained remarkably stable over 35 years despite the use of antibiotics and change of diet in individuals [20], which makes the mechanism that

drives the large difference in amounts of gut methanogens between MEs and NMEs remain elusive.

Interestingly, our *in vitro* observations described in **Chapter 6** demonstrated that methanogenesis can be introduced into the microbiome of NMEs, and it seems that this trait can be considered a specific niche that is optional. In addition, I mixed the faecal slurries from one ME and one NME as inoculum for incubations and observed that methane was produced during the incubation, providing further evidence that the gut microbiota in NMEs does not affect methanogenesis upon addition of methanogens (data not shown). Nevertheless, I cannot rule out the impact of the selective conditions applied during the batch incubations and it is evident that these observations need to be further confirmed *in vivo*.

Association of hydrogenotrophic microbiota with overall microbiota composition and function

Interestingly, **Chapter 4** showed that methanogens were positively correlated with higher microbial alpha diversity and that subjects with a high-level of methanogens had a significantly different microbiota composition compared to those with low-levels of methanogens. In line with this, **Chapter 6** indicated that MEs had higher microbial richness and significantly different microbiota composition compared to NMEs. The level of methanogens has thus a strong association with overall microbiota composition. It has been reported that the accumulation of hydrogen leads to a higher hydrogen partial pressure that would inhibit the regeneration of coenzyme NAD^+ from NADH, which then thermodynamically restricts further microbial fermentation and growth [7, 25]. Hence, the consumption of hydrogen by methanogens reduces the hydrogen partial pressure and facilitates fermentation, which may contribute to microbial growth and, in turn, affect the microbial composition. This theory is supported by experiments using a humanised gnotobiotic mouse model, which showed that the addition of *M. smithii* increased the population size of *Bacteroides thetaiotaomicron*, and maintained a high ratio of NAD^+/NADH [26]. In addition, it has also been reported that cellulose-degrading bacterial communities differed between individuals according to the presence or absence of methanogens [27]. It can thus be hypothesised that these beneficial conditions provided by the methanogens sustain multiple niches for different microbes, which results in a higher diversity.

It has been reported that methanogens do not only affect the microbial composition but that they also shift the metabolic output of microbial fermentation. As shown in the humanised gnotobiotic mouse model mentioned above, cocolonisation with *M. smithii* resulted in higher production of acetate by *B. thetaiotaomicron*, less

propionate and butyrate, and simultaneously enhanced degradation of polyfructose-containing glycans and energy harvest from the diet by the host compared to the corresponding mono-colonisations [26]. In addition, coculturing members of the bacterial family *Christensenellaceae* and *M. smithii* indicated a syntrophic relationship between *Christensenella* and *Methanobrevibacter* via interspecies hydrogen transfer resulting in higher acetate but lower butyrate production compared to monocultures of *Christensenellaceae* strains [28]. In line with this, **Chapter 4** described that the family *Christensenellaceae* was consistently associated with a high level of methanogens in both healthy adults and IBS patients. However, changing the methanogenic activities by inhibiting methane production in MEs or adding *M. smithii* as coinoculum into the faecal slurries of NMEs did not significantly alter the overall microbiota composition and short chain fatty acid production compared to the control incubations (**Chapter 6)**. The fact that it was observed that all hydrogenotrophy-modifying treatments did not induce significant changes in the microbiota development during incubations, suggests that gut microbiota is resilient towards such perturbations, which is a characteristic of the human gut microbiome to maintain a homeostatic equilibrium state [3-5]. As indicated above, it seems that methanogenesis is a specialised but optional niche of the microbiota under the conditions applied in the *in vitro* incubations, and it remains to be determined if this is consistent under other conditions or *in vivo*.

The research described in **Chapter 4** indicated that methanogens are strongly correlated with microbiota alpha diversity, but this was not the case for SRB and acetogens. This could be due to the fact that hydrogen is only essential to methanogens, whereas SRB and acetogens are more metabolically flexible. Without sulphate, SRB switch to use other compounds as an energy source, like lactate, and thereby become even a hydrogen-producer [29, 30]. Reductive acetogens such as *Blautia hydrogenotrophica* are also able to ferment carbohydrates [31]. The study in the humanised gnotobiotic mouse model mentioned above revealed that unlike *M. smithii*, *Desulfovibrio piger* has no significant impact on the metabolism of *B. thetaiotaomicron* [26]. Colonising gnotobiotic mice with an eight-member synthetic community alone or plus *D. piger* and feeding animals with a chondroitin sulphate-supplemented diet revealed that the introduction of *D. piger* increased the abundance of hydrogen-producing bacteria and production of hydrogen sulphide [32]. It has to be mentioned that the presence of *D. piger* did not increase the concentration of fermentation products, mainly short chain fatty acids, but decreased the propionate concentration [32]. The *in vitro* study described in **Chapter 6** showed that sulphate was not consumed by SRB during the incubations, despite the fact that SRB have a higher affinity for hydrogen than other hydrogenotrophic microbes and that sulphate reduction is thermodynamically more favourable compared to methanogenesis and reductive

7

acetogenesis [12, 33]. Altogether this suggests that SRB do not play an important role in regulating the hydrogen metabolism under all conditions. As for reductive acetogens, it is estimated that around one third of acetate is derived from reductive acetogenesis, implying the important role of reductive acetogens in the disposal of hydrogen in the gut [34]. In **Chapter 6**, we did not specifically monitor reductive acetogenesis, but observed that the genus *Blautia* was likely involved in fermentation rather than reductive acetogenesis, especially when a mixture of carbohydrates was used as substrate. In a study with gnotobiotic mice cocolonised with *B. thetaiotaomicron* and acetogens, it was observed that the presence of *B. hydrogenotrophica* contributed to maintaining a high $NAD^+/NADH$ ratio and increasing the efficiency of fermentation [35]. In addition, it has been revealed that acetogenesis is more abundant than methanogenesis and sulphate reduction via analysing human metagenomes obtained from six monozygotic twins and their mothers, although not all acetogenesis occurs via the use of hydrogen and carbon dioxide [35]. Rather, most acetate in the gut is produced by fermentation via acetyl-CoA derived from pyruvate directly [36]. Nevertheless, compared to methanogens and SRB, the role of reductive acetogens in the gut is less well addressed, and thus their potential role in modulating microbial composition and function still remains to be further studied.

Coexistence of hydrogenotrophic functional groups in gut ecosystems

The main hydrogenotrophic functional groups, methanogens, SRB and reductive acetogens, have been typically thought to compete with each other for hydrogen as an energy source in the gut [8, 25]. However, **Chapter 4** demonstrated that these hydrogenotrophic functional groups did not show any anti–occurrences but rather cooccurrences in the microbiota of IBS patients and healthy adults, suggesting their coexistence. In **Chapter 5**, representatives of each hydrogenotrophic functional group (*B. hydrogenotrophica*, *D. piger,* and *M. smithii*) were cocultured with each other under hydrogenotrophic conditions and confirmed that these three hydrogenotrophic functional groups can coexist under the conditions used for these experiments. Moreover, the lag phase of *M. smithii* grown in monoculture was drastically reduced when cocultured with the other hydrogenotrophic species, suggesting that its growth was stimulated by them. Furthermore, the research described in **Chapter 6** showed that the genera *Desulfovibro, Methanobrevibacter,* and *Blautia* were detected together in the inoculations in which *Methanobrevibacter* was using the produced hydrogen. Altogether, this thesis highlights that the three hydrogenotrophic functional groups coexist rather than being mutually exclusive.

It has been reported that the availability of sulphate regulates the advantage of SRB using hydrogen in gut ecosystems [37]. Using sulphated mucin that serves as

the sulphate source for dissimilatory sulphate reduction in an *in vitro* continuous culture incubation with polysaccharides as carbon source inoculated with faeces showed that sulphate reduction predominated, and methanogenesis was completely inhibited [38]. In **Chapter 5** competition for hydrogen was not observed, which may be due to the fact that hydrogen was not the limiting factor in the incubations, whereas the inhibition of methanogenesis seemed to be associated with a high hydrogen sulphide concentration. Hydrogen sulphide is highly reactive and toxic to microbes as it can diffuse across the cell membrane and is involved in protein denaturation and enzyme inhibition [39, 40]. In addition, a > 4 mM hydrogen sulphide concentration is suggested to be toxic in gut ecosystems [40]. **Chapter 5** showed that the growth of *B. hydrogenotrophica* and *M. smithii* as well as their metabolism stopped when hydrogen sulphide reached a high concentration (~15 mM). In addition, the inhibition of methanogenesis in the study by Gibson et al. [38] as mentioned above was also accompanied with a high hydrogen sulphide concentration (~10 mM), which is in line with the hypothesis that it is the high hydrogen sulphide concentration rather than the competition for hydrogen that inhibits methanogenesis in gut ecosystems. It has been reported that the mean total sulphide content in wet faeces from seven MEs and seven NMEs was 0.66 mM/kg [41]. However, a study in which individuals consumed a high-meat diet (600 g/day) that was rich in sulphur-containing amino acids, showed a much higher faecal sulphide content reaching levels of 3.38 mmol/kg [42]. Together, these studies suggest that toxic levels of hydrogen sulphide probably are not reached in the gut and thereby do not inhibit the growth of methanogens and reductive acetogens. This also supports the previous statements that under certain conditions the different hydrogenotrophic microbes can in principle co-exist in the same ecosystem, both *in vitro* and *in vivo*.

Coculturing *M. smithii* and *B. hydrogenotrophica* under hydrogenotrophic conditions *in vitro* only resulted in a slightly lower acetate production compared to the monoculture of the reductive acetogen (**Chapter 5**). In addition, methane production was higher when *M. smithii* was cultivated together with *B. hydrogenotrophica* due to the reduction of the lag phase during the growth of *M. smithii*. However, this contrasted with another *in vitro* study, which showed that lower acetogenesis was found in the incubations with the faecal slurries from MEs than those from NMEs, and that inhibition of methanogenesis concomitantly lead to a threefold greater acetate production in the incubations with faecal slurries from MEs [43]. These conflicting findings could be due to the availability of hydrogen as this *in vitro* study did not keep a high hydrogen concentration throughout the incubations.

Overall, a consistent observation throughout this thesis was that the three different hydrogenotrophic functional groups can coexist in the gut. It has to be realized that the interactions between hydrogenotrophic microbes depend on the gut conditions, which fluctuate throughout the gut, such as pH [37]. Indeed, it has been shown that SRB were more abundant than acetogens in mucosal samples taken from the right colon, whereas this was the opposite in the left colon and rectum [44]. This thesis provided insights into the ecology of hydrogenotrophic microbes in the gut and showed that the interplay between different hydrogenotrophic functional groups largely depends on the environmental conditions in which they are present. The findings described in my thesis certainly open new avenues for future studies as outlined in the next section.

Future perspectives

Findings described in this thesis (**Chapter 4, Chapter 5, Chapter 6**) revealed the important role of hydrogenotrophic microbiota in gut ecosystems, especially methanogens that are positively associated with microbiota diversity. Although the underlying causalities remain unknown, it has been revealed that *M. smithii* contributed to the growth of the cocolonisers in a humanised gnotobiotic mouse model [26]. Hence, I hypothesize that the presence of methanogens contributes to the colonisation of other microbes which results in higher microbiota diversity. Currently, faecal microbiota transplantation (FMT) is considered a treatment to reestablish gut microbiota homeostasis in which the improvement of microbial diversity is considered a mechanism underlying the success of FMT [45, 46]. In this light, FMT with faecal samples from MEs with the ambition to increase gut microbiota diversity could be an interesting study to confirm or reject my hypothesis.

It is widely accepted that 16S ribosomal rRNA (rRNA) gene profiling to study microbial composition is heavily influenced by primer choice due to incomplete coverage of the primer pairs of known microbial lineages, which is especially the case for archaeal diversity [47, 48]. For example, members of the *Methanomassiliicoccales* in the gut are able to use hydrogen to reduce trimethylamine (TMA) for methanogenesis [49], which were not detected in this thesis by sequencing the V4 region of the 16S rRNA gene with the commonly used primers [50, 51]. Remarkably, based on a human gut metagenomic dataset, Chibani et al [52] recovered 1,167 archaeal genomes from existing human gut samples across 24 countries with rural and urban populations, highlighting that the archaeal diversity remains largely unexplored. Hence, I argue that more input

should be put in isolation of novel species, which includes those involved in hydrogen metabolism and other interesting metabolic features.

Investigating hydrogen metabolism in the gut commonly involves determining gas composition in the flatus or the breath as collecting these samples is non-invasive. However, these samples can only in part reflect hydrogen production and consumption, and thus do not systematically show the hydrogenotrophic activities along the intestine. Recently, an electronic capsule that can sense hydrogen and carbon dioxide was developed, which allows monitoring the hydrogen metabolism throughout the intestine [53, 54]. Hence, adopting these and other new tools to give insights into the hydrogenotrophic activities in the human gut *in situ* has the potential to unravel the complex relationships between hydrogenotrophic functional groups and may ultimately serve as the way forward for microbiome-based therapeutics related to hydrogen metabolism.

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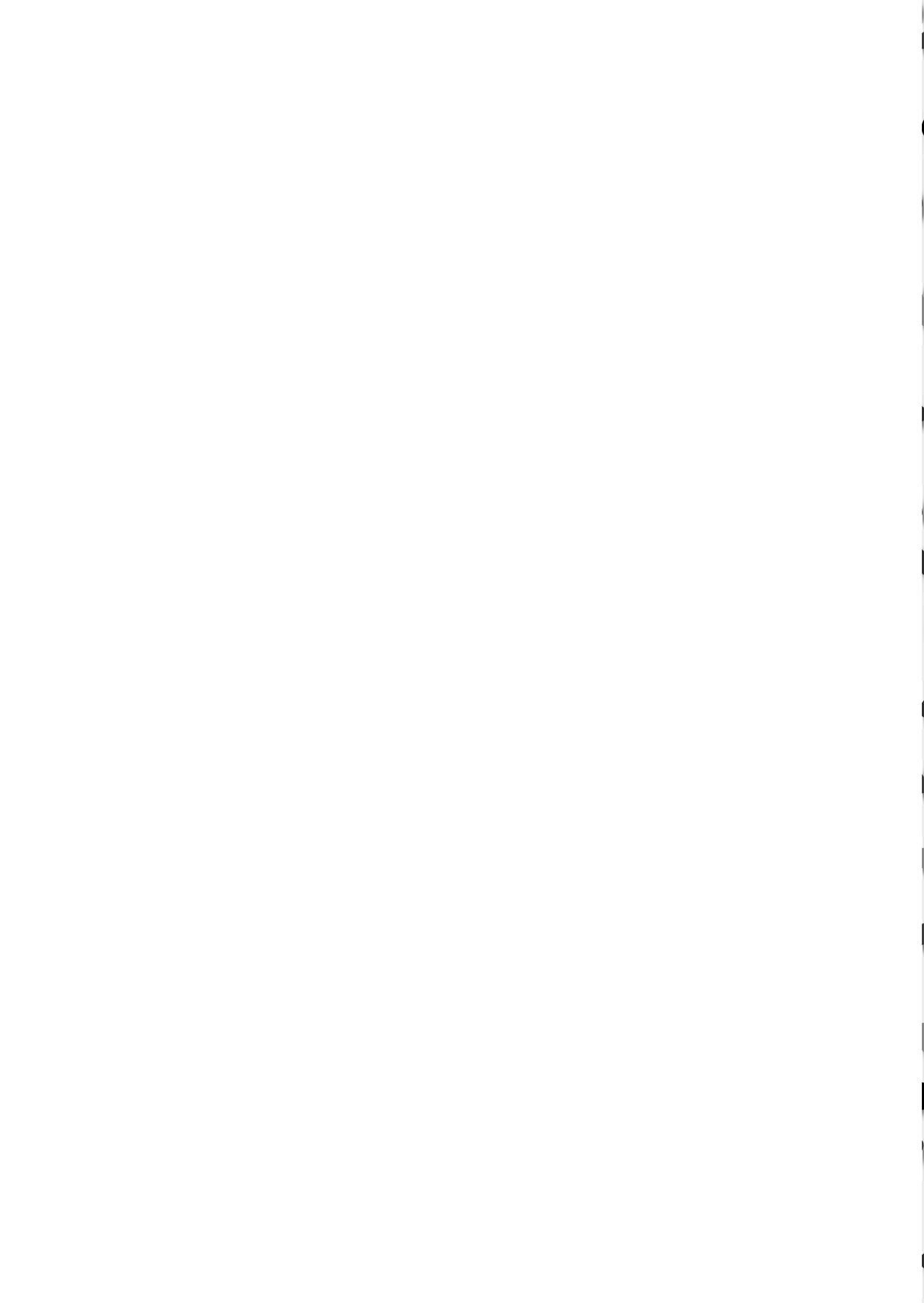
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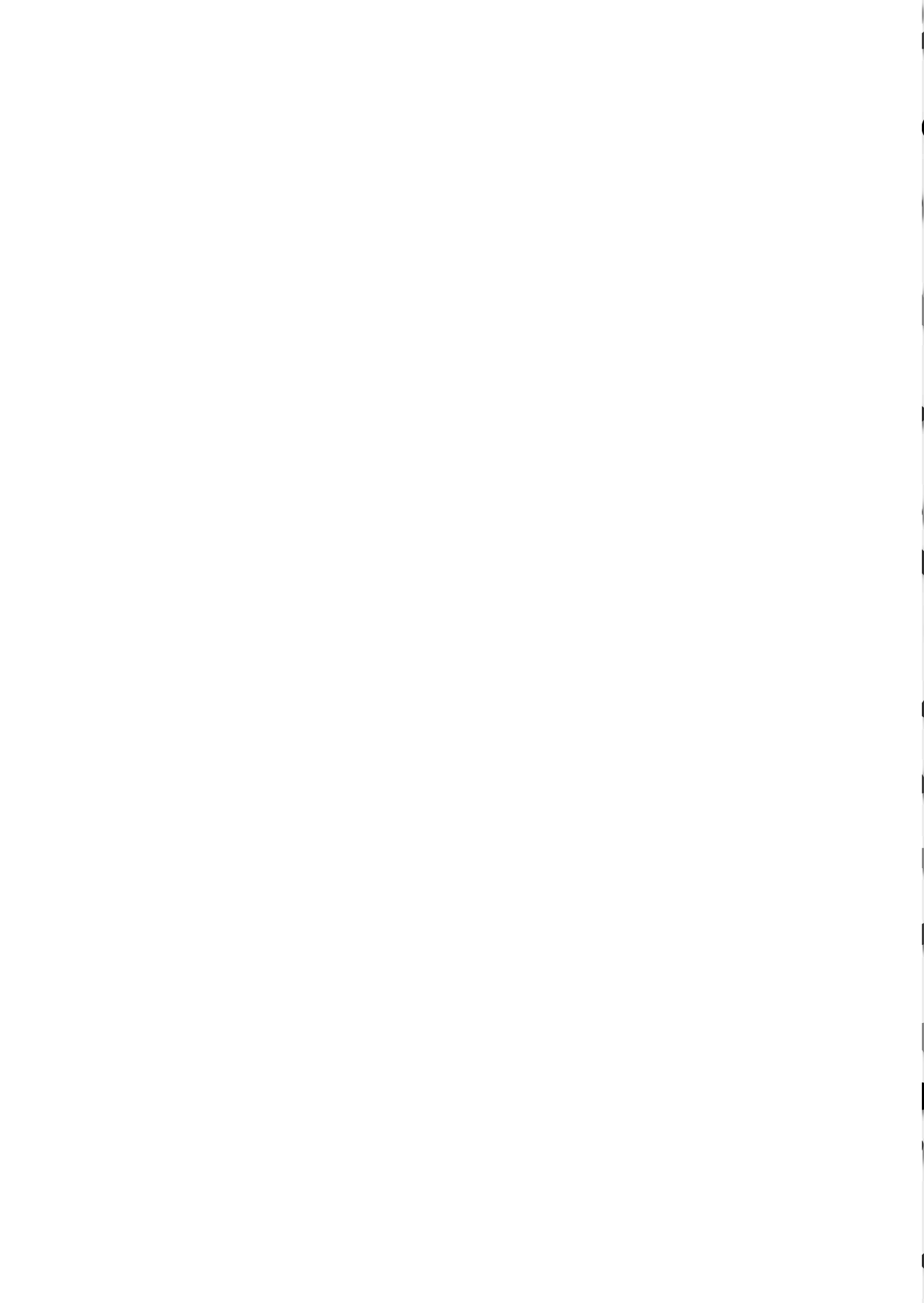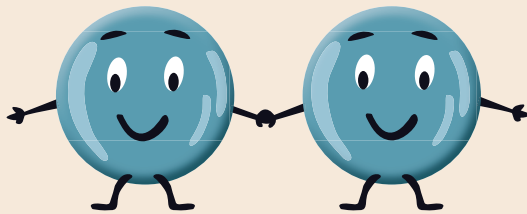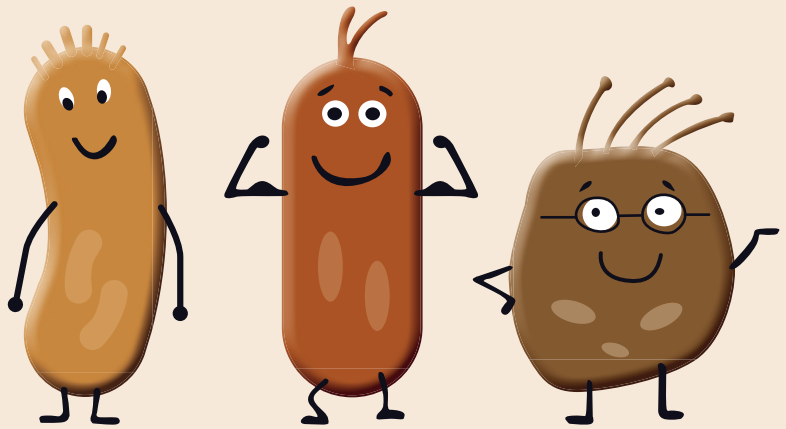
SUMMARY

Summary

Hundreds to thousands of microbial species residing in the gut, collectively termed as gut microbiota, have been widely acknowledged to play an important role in human health. Maintaining a healthy gut microbiota protects us from microbiota related diseases. In contrast, a distorted gut microbiota contributes to chronic intestinal disorders. Therefore, understanding the mechanisms underlying microbiota composition and function is fundamental to maintaining a healthy gut microbiota and to assist in the development of microbiome-based therapeutics for gut disorders/diseases. External drivers of microbiota composition and function, such as diet, host physiology and genotype, have been widely studied, whereas the internal ones, namely the interactions among the microorganisms, on microbiota composition and function remain largely unknown. Hydrogen metabolism plays a central role in regulating overall microbial fermentation in anaerobic ecosystems and is hypothesised to serve as one of the important internal drivers in shaping gut microbiota composition and function, in which hydrogenotrophic microbiota plays an essential role by disposing of the accumulated hydrogen in gut ecosystems. The work described in this thesis aimed to unravel the role of hydrogenotrophic microbiota in overall gut microbiota composition and function as well as the interactions between different hydrogenotrophic microbes.

In **Chapter 1**, I gave a brief overview of the current knowledge regarding the gut microbiota with specific focus on the hydrogenotrophic microbiota, and highlighted the aims and motivations for conducting the studies described in this thesis. Diet is considered one of the most important external drivers affecting the gut microbiota, and **Chapter 2** summarised and discussed our insights into the ways how diet impacts the gut microbiota and its metabolite production. Health status is another factor associated with the gut microbiota, and **Chapter 3** described the longitudinal dynamics of faecal microbiota signatures and short chain fatty acids (SCFAs) in irritable bowel syndrome (IBS) patients and healthy subjects, and their association with IBS symptoms, such as symptom severity. Although the overall microbiota composition did not differ between IBS patients and healthy subjects, IBS patients had consistently lower relative abundance of *Bifidobacterium* but higher relative abundance of *Terrisporobacter* and *Turicibacter* compared to healthy subjects. In addition, this study highlighted the dynamics in stool patterns, symptom severity, and their associations with microbiota composition over time, and argued that longitudinal studies are crucial to understanding the links between microbiota and host physiology and health in IBS.

To investigate the role of the hydrogenotrophic microbiota in the human gut, **Chapter 4** further studied the subjects included in **Chapter 3** to assess the distribution and stability of the three hydrogenotrophic functional groups (methanogens, sulphate-reducing bacteria and acetogens) in both healthy adults and IBS patients over time, as a distorted hydrogen metabolism is suggested to be associated with IBS. Higher alpha diversity was observed in subjects with a high level of methanogens, and a subset of subjects with high methanogen levels showed a distinct microbiota composition. In addition, of note was that the three hydrogenotrophic functional groups showed cooccurrences rather than anti-occurrences in IBS patients and healthy adults, suggesting that these microbes coexist in the human gut. To further investigate what could be the mechanisms underlying these cooccurrences, the interactions between representative isolates of each hydrogenotrophic functional group (*Methanobrevibacter smithii* for methanogens; *Desulfovibro piger* for sulphate-reducing bacteria; *Blautia hydrogenotrophica* for reductive acetogens) were studied in *in vitro* coculture incubations in **Chapter 5**. We observed that the three hydrogenotrophic species not only coexisted with each other under hydrogenotrophic conditions when hydrogen was used as the sole energy source, but also that *D. piger* and *B. hydrogenotrophica* shortened the growth lag phase of *M. smithii*. Moreover, we noticed that a high hydrogen sulfide concentration, produced by *D. piger* likely inhibited the growth and metabolite production of *B. hydrogenotrophica* and *M. smithii*.

To obtain insights into the importance of hydrogenotrophic microbiota for overall microbiota composition and function, the study described in **Chapter 6** aimed to modulate gut microbiota via altering hydrogenotrophic activities *in vitro* in which faecal inocula of subjects characterised as either methane excretors (MEs) with a high-level of methanogens or non-methane excretors (NMEs) with a low level of methanogens were used. In this study, methanogenesis was inhibited by adding 2-bromoethanesulfonate to the incubations in MEs, or stimulated by adding *M. smithii* as coinoculum to incubations in NMEs, and sulphate was added to the incubations in both MEs and NMEs to stimulate sulphate reduction. We observed that sulphate was not consumed in any of the incubations while the genus *Desulfovibrio* was detected in four of the six inocula, suggesting that this genus did not use hydrogen for its metabolism under the conditions used for these experiments. Although altering the methanogenic activities did not change overall microbiota composition and SCFA profiles, *M. smithii* was able to sustain and produce methane in a faecal community of NMEs.

In **Chapter 7**, I summarized and discussed all of the research findings described in this thesis and provided novel insights that can be further investigated in future studies.
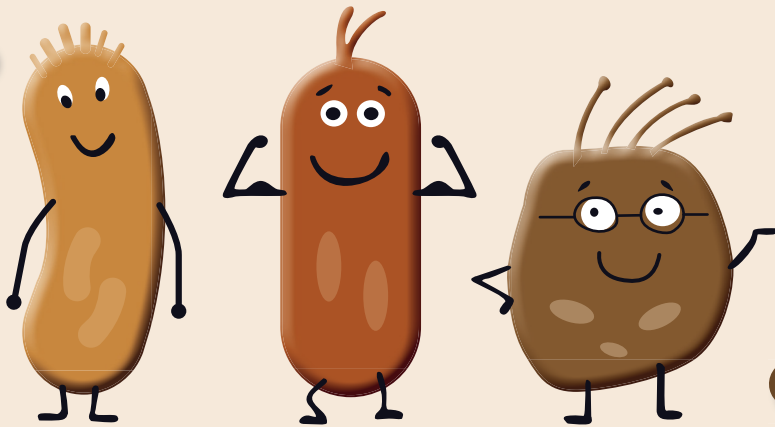
APPENDICES

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List of publications
Overview of completed training activities

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Taojun, May 2022, in Wageningen

About the author

Taojun Wang was born in April 1991 in Handan, China. After obtaining his bachelor degree from Hebei Agricultural University in Baoding in 2014, he continued his study at China Agricultural University, Beijing, specialising in food microbiology under the supervision of Dr Huiyuan Guo and Prof. Fazheng Ren. In 2017, he moved to Wageningen University & Research and started his PhD under the supervision of Dr Erwin Zoetendal and Prof. Hauke Smidt at the Laboratory of Microbiology supported by the China Scholarship Council (CSC). During his PhD, he mainly focused on the hydrogenotrophic microbes in the human gut, and the results of his research are presented in this thesis.

List of publications

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Taojun Wang, Leander van Dijk, Iris Rijnaarts, Gerben D.A. Hermes, Nicole M. de Roos, Ben J.M. Witteman, Nicole J.W. de Wit, Coen Govers, Hauke Smidt, Erwin G. Zoetendal. Methanogen levels significantly associate with fecal microbiota composition and alpha diversity in healthy adults and irritable bowel syndrome patients (*Submitted to Microbiology Spectrum*).

Taojun Wang, Nils Leibrock, Caroline M. Plugge, Hauke Smidt, Erwin G. Zoetendal. Coculturing with *Blautia hydrogenotrophica* or *Desulfovibro piger* shortens the lag phase of *Methanobrevibacter smithii* under hydrogenotrophic conditions (*Manuscript in preparation)*.

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Overview of completed training activities

Discipline specific activities	Organizing institute (s)	Year
Microbiology centennial symposium	Lab of Microbiology	2017
Gut day	University college Roosevelt	2017
KNVW microbial ecology fall symposium	Lab of Microbiology	2017
Gut day	Lab of Microbiology	2018
Open & reproducible microbiome data analysis	Lab of Microbiology	2018
Summer school on microbial community modelling	KU Leuven	2019
Data exploration and analysis in gut microbiome profiling studies	The Developmental Psychobiology Lab	2019
Intestinal microbiome of humans and animals	Lab of Microbiology	2019
In vitro studies of the human intestinal microbiota	Lab of Microbiology	2020
The virtual microbiome summit	Lucy Mailing, PhD	2020
Bioinformatics with Linux and Python	WIMEK	2021
Metagenomics, metatranscriptomics, and multi'omics for microbial community studies	Physalia Courses	2021
World microbe forum	ASM & FEMS	2021

General courses	Organizing institute (s)	Year
VLAG PhD week	VLAG	2018
Illustrator for scientists	Lab of Microbiology	2018
Introduction to R	VLAG	2018
Applied statistics	VLAG	2018
WGS PhD workshop carousel	WGS	2018
Project and time management	WGS	2018
Scientific writing	WGS	2020

Other activities	Organizing institute (s)	Year
Preparation of research proposal	Lab of Microbiology	2017
PhD trip	Lab of Microbiology	2019
AIO/Postdoc meeting/PhD meeting	Lab of Microbiology	2017-2021
Molecular ecology group meeting	Lab of Microbiology	2017-2021
Journal club	Lab of Microbiology	2017-2019