Towards Gut Microbiome-Based Stratification of Dietary Intervention Strategies

ZHUANG LIU
Propositions

1. Inter-individual gut microbiome variation challenges existing intervention strategies and opens up new opportunities for personalised nutrition.
   (this thesis)

2. Simple faecal batch culturing continues to hold an indispensable position in dietary fibre supplement selection.
   (this thesis)

3. The rise in severe weather events is a sign that the global climate system has already crossed a tipping point.

4. The global Covid-19 pandemic shifted focus towards virology, diverting attention from other fields of microbiology.

5. Studying oneself as the primary subject is often the most effective approach to nutrition research.

6. The risk of artificial intelligence resulting in job displacement necessitates careful consideration by policymakers before wide implementation.

7. Team sports outperform group outings in fostering interpersonal relationships.

8. Fine food nourishes not only human bodies but also fuels their social interactions.

Propositions belonging to the thesis, entitled

“Towards Gut Microbiome-Based Stratification of Dietary Intervention Strategies”

Zhuang Liu

Wageningen, 14 September 2023
Towards Gut Microbiome-Based
Stratification of Dietary Intervention Strategies

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Chapter 1

General Introduction and Thesis Outline
The gut microbiome – a hidden world of microbes in our intestines

Who are they? What do they do?

Our intestines are home to a diverse community of trillions of microbes, including bacteria, archaea, eukaryotes such as fungi and protozoa, and viruses, collectively known as the gut microbiota (1). The term microbiome is used to refer to the collection of microbes occupying a given habitat, and also includes their activities that contribute to shaping this niche (2). The gut microbiota is primarily composed of bacteria (3), and in fact, microbial cells make up about half of the total cells in the human body (4). The gut microbiota is highly diverse among individuals, with many factors, such as extrinsic host factors (e.g., diet, medication use, lifestyle) and intrinsic host factors (e.g., host genetic background, age), contributing to this variation (5). Despite the inherent variability, the gut microbiota consistently exhibits two predominant phyla: Firmicutes (50-
80%) and Bacteroidetes (20-30%), as evidenced by 16S ribosomal RNA (rRNA) gene sequencing (3, 6, 7).

The gut microbiome is involved in many aspects of human health. The genomes of members of the gut microbiota encode a substantively larger number of genes compared to the host genome, enabling the microbiota to perform a variety of functions that humans are unable to do (Fig. 1). The bacteria in the gut microbiome are able to break down nondigestible, complex carbohydrates (e.g. dietary fibre (DF)) that our body cannot digest on its own, resulting in the production of short-chain fatty acids (SCFAs) including acetate, propionate and butyrate (9). These microbial metabolites exhibit a range of physiologic functions. For instance, butyrate provides a source of energy for colonic epithelial cells and can also have anti-inflammatory effects (10). Certain bacteria are also capable of synthesising vitamins such as vitamin K and B or carrying out biotransformation of bile acids (11). The dialogue between host and microorganisms at the mucosal interface is known to play a crucial role in the development and maintenance of the immune system. Furthermore, many intestinal bacteria can help to prevent infections by producing antimicrobial compounds or occupying ecological niches and thereby making it difficult for exogenous bacteria to access and colonise these niches (9). This so-called colonisation resistance also contributes to the prevention of outgrowth of opportunistic pathogens, which after outgrowth can cause infection or illness in individuals with a weakened immune system or compromised health conditions.

**Healthy vs aberrant gut microbiome**

Characterising the gut microbiome in healthy individuals is an important initial step in understanding its contribution to health and disease. The general assumption is that a healthy gut microbiome is diverse and balanced, with a homeostatic equilibrium among a wide variety of microorganisms (12). Within this intricate gut ecosystem, these microorganisms engage in diverse interactions that sustain microbiome stability and functionality. These interactions include mutualistic partnerships, cooperative behaviours and cross-feeding, but also competition, amensalism and even predation, all working together to uphold a balanced, robust, and resilient ecosystem (13, 14). The understanding of a healthy gut microbiome is closely intertwined with the elucidation of features of aberrant gut microbiomes and the associations of such aberrations with disease. From an ecological perspective,
community stability can be thought of as an important hallmark of a healthy community. Stability in a community refers to its capacity to maintain a consistent composition and function over time, whereas resilience describes its ability to restore its equilibrium state after undergoing stress-related perturbations (15). These attributes of stability and resilience are fundamental components of healthy as well as aberrant microbiomes (14). Aberrant gut microbiomes may have different compositional and functional attributes in different diseases. An aberrant gut microbiome is linked with the onset and progression of a variety of gastrointestinal (GI) disorders or diseases such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (16, 17). In many diseases, the gut microbiome is characterised by (1) low microbial richness and/or diversity, (2) depletion of SCFA-producing bacteria, and (3) lack of compositional stability over time (18).

The gut microbiome – a unique, stable, and resilient ecosystem

Uniqueness between individuals

The gut microbiome exhibits significant inter-individual variation both in healthy individuals and those with aberrant microbiomes. In healthy individuals, inter-individuality is characterised by variation in taxonomic composition and functional potential, with certain core microbial species shared among individuals and variable components specific to each person (19, 20). Besides composition, the response of the gut microbiome to diet is also individualised as revealed e.g. by a longitudinal study in healthy individuals (21). When considering aberrant gut microbiomes associated with diseases or disorders, inter-individuality remains a prominent feature. Disease-specific alterations in microbial composition and functionality exhibit substantial variation between affected individuals (22, 23). This variability poses challenges in identifying consistent microbial signatures associated with specific diseases or aberrant states. Inter-individual differences in gut microbiota composition have been associated with disease risk, susceptibility, and response to therapeutic interventions. Understanding the implications of inter-individual variation in both healthy and aberrant gut microbiomes is crucial for personalised interventions such as personalised nutrition. Leveraging this knowledge
can facilitate the development of personalised approaches for disease prevention, diagnosis, and treatment.

**Stability over time**

As explained above, stability is a fundamental attribute of the gut microbiome, referring to its ability to maintain a consistent composition and function over time. In healthy individuals, the gut microbiome demonstrates remarkable stability, with relatively minor fluctuations in microbial composition and functionality (24). The stability of the gut microbiome has been previously identified in a daily pattern (25) or weekly pattern (26), which is crucial for the maintenance of host health and optimal physiological functioning. In fact, the gut microbiome in healthy individuals can be stable in terms of composition spanning one year (27) and even a decade (28). In a healthy gut microbiome, stability is achieved through intricate interactions between microbial species, host factors, and environmental influences. The presence of a diverse microbial community, with a balance of symbiotic species, contributes to stability by preventing the overgrowth of potentially harmful microorganisms. Additionally, the gut microbiome exhibits functional redundancy, meaning that multiple microbial species have the capacity to perform similar functions, ensuring that essential processes are maintained even if certain species decline. In contrast, aberrant gut microbiomes associated with diseases or disorders often exhibit reduced stability, characterised by reduced diversity and an increase in facultative pathogens (29). Imbalances in microbial composition and functionality disrupt the stability and functional redundancy of the gut microbiome.

**Resilience within individuals**

Resilience is another key characteristic of the gut microbiome, referring to its ability to maintain stability and functional integrity in the face of perturbations. The degree of resilience of the gut microbiome influences both health and disease. In healthy individuals, the gut microbiome demonstrates remarkable resilience, allowing it to recover from disturbances and restore its composition and functionality (30). In a healthy gut microbiome, the microbial community possesses a diverse repertoire of species and functional pathways, which contribute to its resilience (15). This diversity provides redundancy and functional complementarity, ensuring that the ecosystem can withstand disturbances and maintain its overall stability. Aberrant
gut microbiomes can also exhibit resilience in certain conditions (14). A highly resilient microbiota has the ability to recover and restore a healthy state. However, in cases where an aberrant microbiome demonstrates high resilience, it can impede treatment efforts (30). Therefore, identifying a less resilient state within an aberrant microbiome presents an advantageous opportunity for effective interventions aimed at reverting the aberrant microbiome to a healthy state.

**Gut microbiome stratification and manipulation**

**Stratification**

Gut microbiome stratification involves classifying individuals into different groups or categories based on particular, conserved signatures in their gut microbiome profiles (31). This approach aims to identify patterns, clusters, or subgroups within a population that may have distinct microbial profiles associated with certain health outcomes or disease risks. One early stratification approach proposed the existence of distinct enterotypes or clusters of healthy individuals with different dominant bacterial taxa in their gut microbiome. The original study by Arumugam et al. (32) suggested the presence of three enterotypes characterised by a network of co- and anti-occurring genera and dominance of *Bacteroides*, *Prevotella*, or *Ruminococcus* species, respectively. Another stratification approach focuses on the relative abundance of *Prevotella* and *Bacteroides*, two dominant bacterial genera in the gut microbiome (33). *Prevotella*-rich and *Bacteroides*-rich gut microbiome profiles have been associated with different dietary patterns and host health characteristics. Additionally, researchers have discovered a robust bimodal distribution of *Prevotella* and a specific species within the *Bacteroides* genus (34). This discovery suggests the existence of two stable and resilient states within the gut microbiome, with the potential for interchanging between them. This cross-transition phenomenon could carry significant health implications and could potentially serve as a therapeutic tool. In the context of aberrant gut microbiomes, their stratification has also been explored. For example, two microbiome subtypes with distinct response to modified diets have been identified in IBS patients (35), with the pathogenic-like subtype being enriched in *Firmicutes* and genes for amino acid and carbohydrate metabolism, whereas the health-like subtype was found to be similar to that of healthy controls. Similarly, four alternative intestinal ecosystem states have been
described in paediatric ulcerative colitis (UC, one type of IBD) patients, with improvement of inflammation accompanied with a shift among these states (36).

As described above, the gut microbiome is a versatile ecosystem. Ecological systems typically comprise of alternative resilient states that can withstand minor perturbations (34). Transitioning from one stable state to another requires a significant perturbation, crossing a tipping point (Fig. 2). Tipping points refer to the shift between contrasting system states that occur when external conditions reach certain thresholds that trigger an accelerating transition from one system state to a contrasting new state (37, 38). To illustrate this concept, one can consider deserts and rainforests as resilient states. Only a drastic perturbation, such as deforestation, can push a rainforest past its tipping point, transforming it into a desert. Similarly, transitioning from a desert back into a rainforest would necessitate substantial effort, involving the crossing of another tipping point. As such, comparable ecological principles were speculated to govern the dynamics of the gut microbiome as well (14).

**Dietary manipulation**

The role of diet in shaping the gut microbiome has been recognised as a significant factor. A study conducted on approximately 100 healthy individuals found that their long-term dietary habits influenced the composition of their gut
microbiome, resulting in the formation of specific above-mentioned enterotypes (39). Specifically, the Bacteroides enterotype was associated with a diet rich in protein and animal fats, while the Prevotella enterotype was linked to a diet high in carbohydrates. Moreover, it was observed that even short-term and drastic changes in dietary patterns can influence the human gut microbiome in a diet-specific manner. Specifically, the consumption of animal- or plant-based products for a short period resulted in alterations in the structure of the microbial community, surpassing the inherent inter-individual variation (40). However, long-term (12 months) consumption of either a healthy low-carbohydrate or a healthy low-fat diet revealed resilience of the gut microbiome to perturbations (41). Nevertheless, habitual dietary intake provides a consistent supply of nutrients to bacteria, creating an environment that continuously shapes the gut microbiome (42).

Targeting the gut microbiome, dietary modification such as increasing dietary fibre (DF) intake has shown positive effects on both healthy and health-compromised individuals, and DF could reach the colon where the majority of bacteria reside (43). However, a given dietary manipulation strategy does not necessarily fit all individuals due to differences in gut microbiome features between individuals. As a result, response to an intervention differs drastically between subjects, with some subjects showing desired and beneficial outcomes such as symptom relief after intervention (responders), while others showing none or even adverse reactions or outcomes such as symptom worsening (non-responders) (44). The reason(s) for this inconsistent response between subjects remain speculative but may be the result of differences in their gut microbiome, given its intermediate role between diet and host (45). In fact, gut microbiome variation was found to modulate the effects of DF on host metabolism (46). Different species of bacteria have varying capacities to ferment DF, and their preferences for specific DF types also influence the extent of metabolism (43). Moreover, emerging evidence has shown that the host response to dietary interventions is highly individual-specific, and that this heterogeneity stems from unique microbiome signatures (47, 48). Therefore, the need for developing interventions that target an individual or a defined population is suggested. Identifying ‘responders’ and ‘non-responders’ prior to intervention is the most challenging part given the fact that it requires a substantial understanding of the complexity of the microbiome-host interaction.
Research aims and thesis outline

Unravelling the gut microbiome signatures that determine the response of subjects to specific dietary interventions is fundamental to developing more effective dietary intervention strategies. Therefore, I hypothesise that gut microbiome stratification could aid the current research and clinical/nutritional practices to achieve higher efficacy. By combining both *in vivo* and *in vitro* approaches with faecal samples taken from healthy individuals, IBS patients and UC patients, I aim to demonstrate the feasibility of gut microbiome-based stratification of dietary intervention strategies.

Following this introductory Chapter 1, this thesis contains six additional chapters, including one literature review (Chapter 2), three research chapters (Chapters 3-5), one study protocol (Chapter 6), and a general discussion (Chapter 7) (Fig. 3).

- **Chapter 2** gives an overview of current gut microbiome-based stratification strategies, including 16S rRNA gene-, metagenome-, and metabolite-based stratification strategies. This chapter also provides specific gut microbiome features associated with various clinical conditions (e.g., IBS, UC), and proposes a

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**Figure 3 Schematic overview of the research hypothesis, approach and group.** Numbers indicate thesis chapters in which each topic is covered. Abbreviations: IBS: irritable bowel syndrome, UC: ulcerative colitis.
Chapter 1

5-step strategy to apply microbiome-based stratification to obtain improvements in dietary intervention efficacy.

- **Chapter 3** identifies the microbiota signatures of IBS patients who achieved a clinical improvement in an intervention cohort and an observation cohort. Facilitated by the similar set-up and sampling procedures of these two cohorts, I perform cross-cohort prediction of clinical improvement with baseline metadata and microbiota composition data as input to machine learning modelling that can be used to predict the outcome for new patients and ultimately improve intervention efficacy.

- **Chapter 4** describes an *in vitro* batch incubation study with faecal inocula from healthy individuals with the aim to investigate the impact of donor individuality, faeces collection date, and culture medium type on microbiota composition and metabolic activity with the fibre polydextrose as carbon source. In addition to revealing the level of contribution of each of these factors to microbial succession and metabolic activity, I also provide recommendations with respect to planning and interpretation for future *in vitro* studies.

- **Chapter 5** applies the same *in vitro* approach as described in chapter 4 with the main objective to select butyrate-boosting DF mixtures to stimulate bacterial butyrate production by the gut microbiome in UC patients. In addition to selecting the DFs that exert the highest stimulation of butyrate production, research described in this chapter also investigates the impact of DF mixture composition and faecal *Bacteroidetes* levels on butyrate production.

- **Chapter 6** describes a study protocol for a clinical trial (PREDUCTOME) in mild UC patients based on observations from the previous chapters. With the main objective to predict dietary intervention efficacy in mild UC patients based on faecal microbiome signatures, this four-arm randomised double-blind placebo-controlled parallel trial aims to validate the prediction that eight weeks of prebiotic intervention with a DF mixture induces a higher response in adult, mild UC patients with low faecal *Bacteroidetes* levels (predicted responders), but not in those with high faecal *Bacteroidetes* levels (predicted non-responders).

- **Chapter 7** summarises and discusses all findings from the thesis. Furthermore, I point out the remaining challenges in gut microbiome-based stratification and provide future perspectives.
References


Chapter 1


Chapter 1


Chapter 2

Microbiome-Based Stratification to Guide Dietary Interventions to Improve Human Health

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Abstract

Diverse evidence has suggested that the gut microbiome is closely associated with overall human health. Modulation of the gut microbiome through nutritional intervention is recognised as a robust and attainable strategy to prevent disorders/diseases and improve human health. However, universal dietary recommendations demonstrated to have different, sometimes even opposite, effects due to the considerable inter-individual variability between subjects, especially in the gut microbiome. Hence, implementation of personalised nutrition or other treatment strategies have been suggested to tackle the individuality problem. A first step into this direction includes the stratification of subjects into specific groups based on their gut microbiome. The gut microbiome could serve as a pool of potential biomarkers for distinguishing “responders” and “non-responders” to specific treatments, which subsequently can be used to classify subjects with ambition to increase treatment efficacy. In this review, we explain the need for human gut microbiome stratification, introduce the concepts and show with specific examples potential options of microbiome-based stratifications. Finally, we propose a strategy for how microbiome-based stratification can be introduced to obtain improvements in dietary efficacy that can be implemented in real-life settings.

Keywords: diet, health, gut, microbiome, stratification, personalised nutrition
Introduction

The human gastrointestinal (GI) tract harbours trillions of microbes, commonly referred to as the “gut microbiota”, that is dominated by bacteria, but also contains archaea, eukaryotes and viruses. The term microbiome has been used to refer to the collection of microbes and all the genes and functionalities they encode, and it has been found that the collective microbial metagenome outnumbers our own eukaryote genome by more than 100 times (1). Several factors including age, dietary habits, genetics, mode of birth delivery, and medication use have been found to affect the composition and functionality of the gut microbiota. Among them, diet has been identified as one of the main drivers in the modulation of the gut microbiome (2). Moreover, numerous studies have demonstrated that the human gut microbiome plays a vital role in host health, and the onset and progression of a variety of gut-associated as well as more systemic disorders or diseases such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), obesity and diabetes (3, 4). Indeed, there is a plethora of studies describing differences in microbiota composition between healthy and compromised subjects. However, these comparisons, which are mostly cross-sectional, do not provide consensus observations, such as a microbial taxon that is specific for a certain disease or disorder. Even at phylum level, observations from different studies are sometimes contradicting as has been observed for the *Bacteroidetes* to *Firmicutes* ratio in relation to body mass index (BMI) (5, 6). Reasons for these inconsistencies could include technical aspects, such as differing laboratory protocols, recruitment strategies and cross-sectional study designs, but also biological aspects, such as intrinsic differences in microbiota composition, functional redundancy and microbial flexibility towards different environmental conditions within an ecosystem (e.g. availability of substrates, pH variation) (7). Furthermore, changes in activity are not always reflected in corresponding changes in microbiota composition, and a recent study has indeed shown that for example switching traditional African and Western diets for two weeks had a limited impact on microbiota composition, but large impact on metabolite production, notably short chain fatty acids (SCFAs) (8). Even with more drastic measures, such as faecal microbiota transplantation (FMT), it has been demonstrated that only a subset of subjects shows positive responsiveness, even in the control group (9). Although the
variation in outcomes between different studies might at least in part be explained by variations in study set-up, recruitment criteria and laboratory protocols as indicated previously, it is evident that a significant part of the inconsistency is due to the considerable inter-individual variability in microbiota composition. Therefore, just continuing randomly selecting individuals for intervention studies only based on clinical and/or demographic parameters will likely not further improve our understanding of the mechanisms underlying response efficacy of an intervention. Instead, we should consider to stratify participants beforehand based on microbiome characteristics (e.g. composition, metabolic capacity, metabolite production) in order to better predict efficacy of an intervention that ultimately will lead, for example, to personalised dietary recommendations. Assuming that the occurrence of a certain disease/disorder is found to be related with a certain microbiota composition profile, patients can be stratified according to this profile prior to the envisaged intervention. Ultimately, personalised stratification-based diagnostics and therapeutics would be employed to improve efficacy.

Therefore, the aim of this review is to explore how the current knowledge on the gut microbiome and microbial signatures associated with human health parameters can be used to stratify subjects for intervention studies, with focus on the bacterial part of the microbiota. In addition, we will highlight some examples of potential gut microbiome targets that can be used for stratification of subjects in order to predict the effect of certain dietary components in an intervention and thereby improving the success rate of the intervention.

**Overview of gut microbiome-based stratification strategies**

To ensure that primary end-points of clinical studies are reached, target group selection is important. Detailed selection of prospective subjects before starting an intervention is very common and starts with specifying in- and exclusion criteria. These criteria may include stratification based on age, gender as well as on specific measurable health biomarkers, such as insulin resistance or inflammation scores. In addition, selection of subjects can also be based on questionnaire-derived criteria such as the Rome criteria to classify IBS into different subtypes. Sometimes the selection of subjects can be straightforward to obtain high efficacy of a given
intervention when it concerns for example a disease with known underlying mechanism and mode of action of the respective medication that is evaluated. However, this is not the case when diseases are multifactorial, have an unknown underlying mechanism, and/or include a role of the microbiome. In those cases, the efficacy of interventions is difficult to predict and as a result the selection of subjects with respect to the target of the intervention is often random. Typical examples are interventions targeting the microbiome, such as dietary interventions based on fibre or other non-digestible dietary components where subject selections are random from a microbiome point of view (Fig. 1). Although the efficacy of such dietary interventions may sometimes be disappointing, these studies are very relevant as they may provide associations between subject-specific response to the diet and microbiome features that can be used to define or refine microbiome-based

Figure 1 Overview of different intervention strategies with microbiome-based stratification as an intermediate step between random and personalised dietary intervention. (A) random selection without prior knowledge on microbiome. (B) stratification of subjects in groups based on microbiome features with predicted efficacy. (C) personalised approach based on individual microbiome-disease characteristics.
stratification of subjects in a subsequent intervention and thereby increasing efficacy with the ultimate goal of reaching personalised dietary recommendations.

The first step to move towards such a microbiome-based personalised recommendation is stratification of subjects in subgroups based on specific microbiome features (Fig. 1). This microbiome-based stratification can in principle be based on any microbial feature of which 16S ribosomal RNA (rRNA) gene data, metagenome data and metabolite data are currently most practical (Table 1). The potential merits and drawbacks of these stratifications will be discussed in this section.

Table 1 Overview of the human microbiome-based stratification possibilities based on different microbial characteristics.

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Microbiome-Based Stratification to Guide Dietary Interventions to Improve Human Health

16S rRNA gene-based stratification strategies

Direct sequencing of the bacterial 16S rRNA gene has become the most widely adopted method to obtain information with respect to microbiota composition of any given ecosystem, including that of the human gut. This type of microbiota profiling has provided a phylogenetic framework of the gut microbiota. Typical ecosystem features that can be obtained from such analyses include microbial community typing, determining microbial diversity as well as identifying microbes that are differently abundant between groups of subjects, all of which can be used as targets for microbiome-based classification (Table 1). Microbial community typing based on 16S rRNA genes includes identifying the presence of community types and the existence of alternative stable compositional states (13). Although originally based on metagenome data, the study by Arumugam et al. (16) laid the foundation for microbial community typing of the gut microbiome. This study proposed that individuals could be divided into three groups based on their gut microbiota composition. The three groups were defined based on robust clusters which are each dominated by different genera, namely Bacteroides, Prevotella and Ruminococcus, and were found to be independent of gender, nationality, ethnicity, health status, age and BMI. However, with more studies focusing on the stratification of the human gut microbiome based on enterotypes, it has been recognised that the number and the category of alternative states vary between studies, with the level of Prevotella and Bacteroides being the most common drivers for identification of alternative compositional states (10, 11, 24-27). Although the number of alternative states and the way to identify them is still a point of ongoing discussion, the concept of identifying different alternative states might be used as a target for stratifying people based on their microbiota at the start of an intervention as has been suggested earlier (14).

Besides the stratification of individuals based on different alternative steady states of microbiota composition, differentiating microbiota based on 16S rRNA gene diversity opens another avenue for microbiome-based stratification. Diversity of a microbiota includes the number of different taxa (richness) as well as their (relative) abundance distribution (evenness) within an ecosystem. A high microbial diversity is considered to be beneficial as it is suggested to contribute to resilience after disturbance of the microbiome (28). Indeed, the microbiota diversity is generally higher in healthy subjects than compromised subjects (29). It is interesting to note
that the average diversity of the microbiota declines during aging, with high subject-to-subject variation (12). Since this reduced diversity is hypothesized to be associated with a decline in health status, stratification of elderly based on diversity could be an interesting approach for intervention studies.

Apart from the above-mentioned approaches, the presence or absence of specific microbes or their differential abundance between groups of subjects could also be regarded as a characteristic for stratification. For example, individuals who had a higher abundance of combinations of taxa, i.e., the genera *Lactobacillus*, *Leuconostoc* and *Pediococcus*, at baseline lost less weight and rapidly regained weight (30). Other examples include higher baseline levels of *Akkermansia muciniphila* associated with a greater improvement in insulin sensitivity markers after a low-calorie diet (15), and low initial numbers of *Bifidobacterium* that were associated with the biggest increase in the abundance of this genus after a bifidogenic (partially hydrolysed guar gum and fructo-oligosaccharides in the biscuit) intervention (31).

**Metagenome-based stratification strategies**

Besides stratification based on 16S rRNA genes, also the collection of microbial genomes (commonly referred to as the metagenome) can be used as targets for microbiome-based stratification (*Table 1*). Metagenomics enhances resolution of identifying and characterising microbial strains as compared to 16S rRNA gene sequencing and provides potentially important information about the capabilities of the organisms in the community. Besides 16S rRNA gene-data, metagenomic data can also be used for stratification. As indicated before, the discovery of enterotypes is based on metagenome data (16). Similarly, microbial diversity can also be determined based on metagenomic datasets. A hallmark example is that obese subjects with a low-diversity (richness) microbiome were found to be more prone to weight gain and developing insulin resistance as compared to obese subjects with a high-diversity microbiome (17).

The most promising feature of metagenome-based microbiome stratification is the fact that differences in functional capacities between groups of subjects can be identified. For example, a comprehensive meta-analysis by Armour and colleagues (19) revealed a variety of functional signatures in the human gut microbiome associated to Crohn’s disease and obesity, such as increased abundance of modules for lipopolysaccharide biosynthesis, iron transport and acetate production. With
studies establishing correlations between features and responsiveness, stratification of subjects based on these microbiome features in an intervention could be a promising approach selecting for increased response efficacy.

**Metabolite-based stratification strategies**

Although 16S rRNA gene and metagenome data provide insight into which microbes are present and into their functional capacity, these approaches do not reflect actual microbial activity. Excreted metabolites on the other hand are the end result of metabolic activity and those produced by the microbiota can directly impact numerous processes in the body. Also, metabolic diversity influences nutrient requirements and responses to diet between individuals (32). Hence, stratification based on metabolic profiles provides another way of stratifying populations based on microbiome features (Table 1). Metabonomics, i.e. profiling of all produced metabolites (metabonome) has demonstrated to be powerful in discriminating subjects with differences in their health status as well as human populations from different geographic locations with their traditional long-term dietary habits (33).

Untargeted metabonomics has demonstrated to be successful for identification of disease biomarkers in human blood, urine and faecal samples (20-22), which subsequently aids in disease diagnosis and proposed guidelines for potential therapeutics. In addition, Africans and African Americans demonstrated completely different metabonome profiles in urine and faeces which are in line with their microbiota compositional differences (8). Such distinct features between subjects can be used to microbiome-based stratification.

In addition to metabonomics, monitoring of specific metabolite levels in a targeted approach is another way to distinguish subjects with potential responsiveness to a given intervention. For example, faecal propionate and butyrate levels, which are considered beneficial and suggested as biomarkers for IBS diagnosis (23), could be used as targets for stratification. The drawback of this approach is that it provides limited to no insight in which phylogenetic groups are involved in the microbial activities displayed, and for some metabolites it is difficult to separate microbial from host activities.
Towards stratification-focused interventions to promote human health

Gut microbiome homeostasis is extremely important for maintaining overall human health, and its dysfunction or changes to its composition/activity have been associated with not only intestinal diseases (e.g., IBS, IBD, and colorectal cancer), but also extra-intestinal disorders (e.g., obesity, type 2 diabetes mellitus, and metabolic syndrome) (34, 35). Hence, understanding underlying mechanisms that govern these associations as well as predicting the efficacy of diets or other treatments in order to cure the respective disease or improve its symptoms remains a major challenge. An additional challenge lies in the fact that individuals often respond differently to similar or identical diets (18) or other treatments, and thus individuals can be differentiated into responders and non-responders. In this section, we summarise the associations between specific gut microbial features and a selected number of well-studied disorders including IBD, IBS, and metabolic syndrome, how these associations are linked to response variation and how these findings could lead to microbiome-based stratification of subjects with improved prediction of the efficacy of interventions.

Inflammatory bowel disease (IBD)

IBD is a group of chronic inflammatory conditions of the gut of which ulcerative colitis (UC) and Crohn’s disease (CD) are the most common. Both conditions share a lot of similarities with regards to disease symptoms, but location of inflammation is one of the key differences between the two. In CD patients, inflammation could be found in any part of the GI tract, while in UC patients it is exclusively restricted to the inner lining of the colonic and rectal mucosa (36). Although the onset and maintenance of IBD is largely unknown, mounting evidence supports the notion that the microbiome plays a crucial role. There are a variety of studies that have shown marked differences in microbiota composition between IBD and healthy controls which generally shown reduced diversity and butyrate production capacity in IBD (36-38). Similarly, the faecal metabonome of IBD patients has been found to be very distinct from that of healthy subjects and even enables discrimination between CD and UC (22), showing a clear association between disease and microbiota activity. FMT interventions in subjects suffering from IBD offer a
promising approach to treat IBD, but studies performed so far have shown varying success. Although from a medical point of view this is often disappointing, such studies are needed to identify retrospectively, whether there are specific microbial features that are associated to relapse and remission. Taking UC as example, one study demonstrated that patients that have relatively high relative abundance of *Clostridium* clusters IV and XIVa, and butyrate production capacity in their microbiota, are more likely to enter sustained remission after FMT (39). Moreover, Zhu *et al.* (40) recently found that in mice, colitis could be ameliorated by tungstate treatment, which prevented the expansion of *Enterobacteriaceae* via inhibiting molybdenum-cofactor-dependent microbial respiratory pathways, whilst influencing the microbiota composition minimally. These examples of intervention studies revealed different microbial characteristics that could be used to select subjects based on specific microbial features prior to a next intervention study.

**Irritable bowel syndrome (IBS)**

IBS is characterised by increased intestinal sensation to triggers reflected by chronic or recurrent intestinal symptoms in the intestine in the absence of other pathological disorders (41). Although there is no cure for IBS, in general the low fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAP) diet had been proven to ameliorate gut symptoms in adult IBS patients (42). However, it has to be noted that despite the evidence supporting the high efficacy of a low FODMAP diet, nearly a quarter of adult IBS patients does not show a positive response to the diet (43). Among all the taxonomic changes in those non-responders, a lower relative or absolute abundance of *Bifidobacterium* was found to be consistent in most studies (44, 45). Similarly, the baseline gut microbiota composition in children with IBS was found to be related to the efficacy of low FODMAP diet intervention as well. For example, in an IBS study in children, Chumpitazi *et al.* (46) demonstrated that the faecal microbiota of responders was enriched at baseline in i) taxa with known greater saccharolytic metabolic capacity (e.g., *Bacteroides, Ruminococcaceae, Faecalibacterium prausnitzii*) and ii) two Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologues that are related to carbohydrate metabolism. However, other studies have found that baseline microbiota composition data currently cannot accurately predict response to a low FODMAP diet (47). In contrast, Rossi and co-authors (48) were able to predict
responses of patients with IBS to a low FODMAP diet with a mean accuracy of 97% based on 15 features in faecal volatile organic compound profiles before the intervention, suggesting that the metabolic function of bacteria may be a suitable biomarker in determining response. This is a promising observation, as the efficacy of the low-FODMAP dietary intervention can be improved by metabolite-based stratification of subjects. Therefore, the efficacy of low FODMAP diet in IBS patients could be improved if we take the microbial features into account for stratification of subjects.

**Metabolic syndrome**

Metabolic syndrome is a collection of conditions associated with metabolic disorder and increased risk of developing cardiovascular diseases. It has not only been shown to associate with an aberrant gut microbiota, but is also highly influenced by long term dietary patterns (49). In a recent barley beta glucans intervention study, it was observed that three participants with higher relative abundance of *Bifidobacterium* spp. and *Akkermansia muciniphila* before the intervention experienced total cholesterol level reduction, while the others who had lower amounts or even none of these microbial groups did not (50). Similarly, higher relative abundance of *A. muciniphila* at baseline was associated with a healthier metabolic status in overweight/obese humans, characterised by the improvement in glucose homoeostasis, blood lipids and body composition (15). Recently the first intervention study with *A. muciniphila* as a probiotic in obese and overweight subjects has been published and demonstrated improvement of some metabolic parameters, supporting a causal role of this microorganism (51).

The relative contribution of diet and gut microbiome to fat accumulation has been characterised by a recent study, which indicated that certain nutrients alone were hardly able to affect fat accumulation, whereas specific gut bacteria contributed to host adiposity irrespective of dietary intake (52). This indicated that modulation of gut microbiota composition might be a target for losing visceral fat mass. Moreover, a 6-month intervention study described that subjects with high *Prevotella/Bacteroides* ratio appeared to more easily lose body fat on a high fibre and whole grain diet than subjects with a low *Prevotella/Bacteroides* ratio (53). Other dietary components involved in weight loss have also been reported with microbiota associations. Capsaicin, a compound obtained from chili peppers, has a potential in controlling
obesity, and one study has shown that the \textit{Firmicutes}/\textit{Bacteroidetes} ratio as well as the relative abundance of the genus \textit{Faecalibacterium} was increased by capsaicin intervention, accompanied with higher plasma level of glucagon-like peptide 1 (11). Meanwhile, these beneficial effects were mainly found among subjects that were clustered into the \textit{Bacteroides} enterotype rather than the \textit{Prevotella} enterotype. Similarly, in the case of diabetes, a recent acarbose intervention study showed that patients with higher baseline relative abundance of \textit{Bacteroides} in their microbiota responded better, characterised by plasma secondary bile acids, reduced BMI and improved insulin resistance, compared with those with a higher relative abundance of \textit{Prevotella} (54). This highlights the potential of microbiota-based pre-treatment selection for better predicting antidiabetic metabolic benefits. Collectively, all the above-mentioned examples provided microbial hints that could be used for stratifying subjects based on microbial features in order to optimally select the right target group for a given intervention.

\textbf{Potential strategies to increase the success rate of intervention}

An ideal scenario for personalised nutrition would be that for each individual a personal microbiome-based analysis would be done based on which health-care practitioners (e.g., dieticians, clinicians) could provide the individual with personalised dietary advices and/or medication. A hallmark study by Zeevi et al. (55) described a first concept towards such a personalised nutritional strategy. A high variability in the response to identical dietary components was observed between subjects, which could be accurately predicted by making use of a device that integrated blood parameters, dietary habits, physical activity, body measurements and the gut microbiota. This study showed that personalised dietary recommendations may modify elevated postprandial blood glucose levels, possibly leading to diminished disease symptoms in type 2 diabetes mellitus. Although the same study demonstrated a significant increase in bacterial species that are generally considered beneficial when following a healthy diet (with low postprandial glycaemic responses level) compared to a control group, the intervention only lasted for one week and it would be very insightful to continue this study for months or even years.
Although very promising, such a personalised dietary recommendation approach is not immediately applicable yet for disorders with unknown biomarkers or biomarkers that cannot be monitored continuously. Hence, a good intermediate step between this complete personal approach and universal recommendations, are stratification patterns as mentioned earlier. To reach this, we propose a five-step approach.

**Figure 2. Schematic representation of a five-step approach to apply the microbiome-based stratification in personalised nutrition.**

**Step 1:**
- Identification of:
  - Possible microbiome-based stratification options
  - Certain diet-related metabolic and inflammatory diseases
- Literature & “omics” data

**Step 2:**
- Predicted response: +
- Screen subjects and predict response to the planned intervention.

**Step 3:**
- Outcome:
  - +, Full response
  - -, Multiple rounds
- Perform dietary intervention, check the outcome and alter the prediction algorithm accordingly.

**Step 4:**
- New Intervention
- Full response
- 100% efficacy
- Step 2 & 3
- Step 4

**Step 5:**
- Apply the microbiome-based stratification in real-life to allow health-care professional to give more personalised advice.
approach for a microbiome-based stratification, as shown in Fig. 2, to ultimately bridge the gap between general recommendations and more personalised nutrition.

The first step is to determine potential associations between microbial characteristics within the microbiome and certain diseases or conditions that could be used as targets for a dietary intervention and allow easy stratification of subjects. As mentioned in section 3, there are a variety of examples that could be used as start for microbiome-based stratification options. Evidently, this step requires a focus on a specific target group of subjects with a potentially matching intervention, such as the low-FODMAP diet for IBS patients as mentioned previously. Ideally targets for screening and subsequent stratification should be present in faeces, blood or urine as these are relatively easy and cheap to obtain, and their sampling is already integrated in the current health-care system. However, for this to be feasible, it might be that specific attention should be paid to storage methods as metabolites are often not very stable. In addition, targets should preferably be amenable to screening using relatively easy-to-apply assays, such as PCR-based approaches to identify and/or quantify a specific microbial taxon or functional gene or metabolites that can easily been detected by high-performance liquid chromatography (HPLC) or gas chromatography (GC).

The second step is to screen subjects based on the selected target for the dietary intervention and predict who will respond positively to the intervention. During this step, in principle the same rules apply compared to stratification of subjects based on other in- or exclusion criteria, including optimal intervention set-up and power calculations to determine the number of subjects. The third step will be the implementation of dietary intervention based on the prediction in the second step, collect the data, and evaluate the accuracy of the prediction. Based on the outcome of the prediction, fine-tuning or altering the prediction algorithm might be needed which will subsequently lead to the fourth step, which is to perform a new intervention study with the responders that were predicted to respond based on microbiome-based stratification pattern. This will not only lead to determine the improvement of the prediction, but also indicate whether the response in the same individual is reproducible or coincidental. Ideally, a 100% success rate of the new intervention will be achieved. However, it is likely that multiple rounds of validation of the accuracy of specific microbiome-based stratification pattern will be needed.
The fifth and final step will be incorporating the microbiome-based stratification set-up in real-life settings. This step will certainly need the involvement of health professionals to implement it in such a way that it allows providing a more personalised advice to individual subjects as well as to make its implementation accessible for larger populations. For the latter part educating people will be an important aspect. We also foresee this real-life implementation will drive innovation in the private sector. This could include the development of devices to measure the target molecules which are used for the stratification in a home setting. Typical examples of such devises include e-noses to detect specific odorous metabolites or chips that measure blood glucose levels continuously in diabetic individuals. Another private sector that may benefit from this implementation concerns those involved in development of specific food supplements or probiotics. Not only novel products may be produced, but microbiome-based stratification could also lead to better definition of target populations that will benefit from these products.

It is evident that this proposed five-step approach will result in non-responders for which the respective intervention will not offer a solution. However, the benefit of predicting accurately which subjects will not respond to the respective intervention is that these non-responders can focus on finding alternative interventions using the same five-step approach.

**Future research**

Although there are increasing numbers of studies investigating the gut microbiome, mechanistic insights with respect to health and how the microbiome can be affected by a dietary intervention remains largely unknown. As we indicated in last section, identifying a robust and specific response between a given intervention and disease is the next key step to apply existing microbiome knowledge into real life nutritional recommendation. To achieve this, mining of massive omics and metabolite data sets complemented with mechanistic studies, as well as standardising methodologies between laboratories will lay a solid foundation for the further clinical studies. This should be followed by setting in- and exclusion criteria of target diseases/disorders and stratification strategies based on the predicted efficacy of the intervention on the microbiome. In addition, more information on the effectiveness of prediction algorithms will be needed. Since inconsistencies are often encountered
in gut microbiome studies, large-scale clinical studies should also be employed to confirm the reproducibility of predictions and findings.

**Conclusion**

The gut microbiome is vastly influenced by diet and ultimately affects human health. However, it is evident that gut microbiomes vary greatly among individuals, and as a result traditional stratifiers for grouping subjects, such as age groups, gender, disease and respective subtypes, might not be sufficient to obtain high efficacy of dietary interventions that target the gut microbiome. Hence, this inter-individual variability of gut microbiomes should be taken into account. Therefore, individual microbiome-based stratification, as an intermediate step towards personalised recommendations, may be a promising strategy to improve the success rate of certain dietary treatments. This is still a challenging approach as studies often show inconsistent, even contrasting associations between health parameters and specific characteristics of the microbiome. Nevertheless, the first steps moving forward into this field have been taken and are promising (14). We are convinced that implementing characteristics of the microbiome, such as differences in composition, functional capacity and/or its activity, as stratifiers for targeted dietary interventions will not only lead to improved understanding of the microbiome in health and disease, but also lead to innovations that will ultimately lead to personalised dietary recommendations in a real-life setting.

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Chapter 2

References


Microbiome-Based Stratification to Guide Dietary Interventions to Improve Human Health


Chapter 2

Chapter 3

Placebo-Induced Clinical Improvement in Irritable Bowel Syndrome Patients Can Be Robustly Predicted by Baseline Host- and Microbiota-Derived Data from an Observation Cohort Despite Cohort Heterogeneity

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Abstract

**Background:** Increasing evidence has suggested that the gut microbiome plays an important role in the pathophysiology of irritable bowel syndrome (IBS). However, IBS is a heterogeneous clinical condition with varying symptoms, and treatment efficacy varies due to patients’ heterogeneity. To date, a robust microbiota signature that could help predict potential improvement after intervention remains scarce. Therefore, this study aims to identify microbial composition signatures of IBS patients who achieved a clinical improvement based on the IBS Severity Scoring System (IBS-SSS).

**Methods:** We analysed data from an intervention cohort \((n = 63)\), who received one of four dietary treatments (chondroitin sulphate, pea inner fibre, resistance starch, and protein hydrolysate cow milk) or placebo (maltodextrin) for four weeks, and from an independent observation cohort \((n = 55)\), who did not receive any intervention over the same period. In the intervention cohort, subjects reported their daily gastrointestinal complaints and stool patterns. In both cohorts, faecal samples were collected for microbiota composition profiling by 16S ribosomal RNA gene sequencing, and multiple questionnaires (e.g., IBS-SSS) were completed at baseline (T0) and at week 4 (T1). A reduction in IBS-SSS score by more than 50 points over time was classified as ‘clinical improvement’. Using host- and microbiota-derived data as input, we also performed cross-cohort prediction of clinical improvement using a machine learning (ML) approach.

**Results:** In the intervention cohort, subjects with clinical improvement \((n = 28)\) showed a distinct and favourable development in gastrointestinal complaints compared to subjects without clinical improvement \((n = 35)\). Despite cohort heterogeneity in host and microbiota characteristics at baseline, subjects who achieved a clinical improvement were characterised by a higher IBS-SSS score and lower relative abundances of several genera belonging to the family *Lachnospiraceae*. The ML model, trained on the observation cohort, predicted clinical improvement outcome in the placebo group \((n = 13)\) in the intervention cohort with an accuracy of 92% (12/13), while the accuracy was 50% (25/50) in the treatment groups \((n = 50)\).

**Conclusion:** We identified a conserved host and microbiota signature of clinical improvement across cohorts, and highlighted the potential of ML in identifying subjects who may or may not achieve a clinical improvement from placebo. These findings can contribute to the design of more personalised IBS clinical trials.
Keywords: irritable bowel syndrome; microbiome signatures; personalised nutrition; placebo; machine learning; Lachnospiraceae
Introduction

Irritable bowel syndrome (IBS) is one of the functional gastrointestinal (GI) disorders with recurrent abdominal pain and a change in bowel habits diagnosed by the Rome IV criteria (1). The prevalence of IBS is 10 to 20% in Western countries, and it negatively affects the quality of life (QoL) and working productivity of patients (2). Thus, effective treatments can have both societal and economic importance. Diet has been shown to be a source of triggers associated with IBS symptoms in a majority of patients with IBS (3, 4). Therefore, dietary modifications have frequently been applied as a treatment to reduce IBS symptoms (5). For instance, increasing soluble dietary fibre has demonstrated treatment efficacy in IBS (6), similar to a modified diet which is low in fermentable oligosaccharides, disaccharides, and monosaccharides and polyols (FODMAPs) (7). However, the precise pathophysiologic mechanisms of IBS remain unclear and differ in magnitude and extent from subject to subject. Consequently, to date, a universal treatment that relieves symptoms for all patients is still not available, in part due to the vast patient heterogeneity (4). For example, in a study comparing the response of IBS patients to different treatments, soluble fibre (e.g., psyllium seed husks) was the most-often-used treatment which was given to 118 patients. In these patients, 17% responded positively, while 75% and 8% showed minor or even negative responses, respectively (8). Moreover, clinical trials with IBS patients often observe high placebo response rates (35-40%) (9, 10).

Although the aetiology of IBS remains incompletely understood (11), an aberrant gut microbiome has been found associated with the onset and the subsequent development of IBS (12, 13). Emerging evidence has further shown that the individual-specific response to dietary interventions is associated with specific microbiome signatures (14, 15). Hence, the gut microbiome could serve as a pool of biomarkers to help with the diagnosis, patient stratification or even personalised treatment in IBS (16). Distinct gut microbiome signatures associated with IBS subtypes have been described recently based on a total of 942 IBS subjects and 942 non-IBS controls (17). Moreover, a distinct microbiome subtype (pathogenic-like) was identified with an enhanced clinical response to a low FODMAPs diet (18). It has also been shown that the gut microbiome can potentially be used to predict the response to a low FODMAPs diet (19). However, observations are not consistent
between studies. Furthermore, the validity of predictive models is also challenged by the inconsistencies observed in clinical characteristics among subjects, such as the severity of symptoms, which also exhibit correlations with the gut microbiome (20). Hence, identifying reproducible and robust insights into microbiome signatures of clinical improvement in IBS patients, which could be used to predict an individual’s response to a specific dietary intervention, has been a formidable challenge due to discrepancies in experimental procedure and cohort heterogeneity between studies.

With the advent of artificial intelligence (AI), there is a growing expectation for a significant increase in AI-based IBS management (21). For example, AI showed high potential in developing personalised diets for alleviating symptoms of IBS patients. In a study with IBS patients who received a 6-week personalised diet designed by an AI algorithm based on individual gut microbiome signatures, 78% (11 out of 14) patients had switched their symptom severity from severe to moderate based on the Rome IV criteria, with significantly reduced IBS severity scores (22). Machine learning (ML), as a subfield of AI, opens a window into discovering potential hidden patterns in gut microbial composition and other features of a certain group of subjects, providing leads, i.e. biomarkers, to diagnose diseases or predict treatment efficacy (21). For instance, Su et al. have developed a faecal microbiome-based ML model for diagnosis of multi-class diseases including IBS (23). Nevertheless, a successful diagnosis does not automatically result in effective treatment. Furthermore, the effectiveness of ML-based prediction is often restricted to intra-cohort datasets, primarily due to the presence of cohort heterogeneity resulting from variations in selection criteria, experimental procedures, and observational factors (24).

Recently, one intervention study and one observation study in two Dutch IBS cohorts from the Wageningen area, with nearly identical experimental set-up and procedures were conducted. We hypothesised that combining the clinical metadata and microbial composition data from these two cohorts might offer great potential for cross-cohort comparisons and outcome prediction using ML. Hence, this study aimed to validate the clinical relevance of clinical improvement, identify faecal microbiota signatures of IBS subjects who would achieve a clinical improvement and determine whether placebo- or treatment-induced clinical improvement in the intervention cohort can be predicted at baseline, based on data from the observation cohort, using an ML approach.
Materials and Methods

Data from an intervention cohort ($n = 63$) and an observation cohort ($n = 55$) of individuals with IBS were analysed to identify subjects who achieved a clinical improvement, based on improvements in their IBS-SSS scores over time. Microbiota composition of subjects who showed a clinical improvement was compared to that of subjects who did not, to identify faecal microbiota signatures associated with clinical improvement. Subsequently, baseline clinical metadata and microbiota data from the observation cohort were used as input to develop ML algorithms to predict which individuals would show clinical improvement in the intervention cohort.

Study design

The detailed clinical trial design and data collection methods for the intervention cohort study (May-July 2021) can be found in the Supplemental materials. The observation cohort study (September-December 2018) has been described previously (25). Briefly, the intervention cohort comprised 63 IBS subjects who were allocated to one of four different dietary treatment arms including Chondroitin sulfate (Bioiberica S.A.U., Spain) ($n = 13$), Pea Inner Fibre (I50M, Roquette, France) ($n = 13$), Resistant starch (Ingredion, USA) ($n = 10$), and Protein hydrolysate cow milk (Ingredia, France) ($n = 14$), or a placebo arm (maltodextrin, GLUCIDEX® IT 19 P, Roquette, France) ($n = 13$) for a 4-week period. The observation cohort consisted of 55 IBS subjects who received no intervention over the same period. In both cohorts, faecal samples were collected, and multiple questionnaires were completed at baseline (T0) and at week 4 (T1) (see Fig. 1A, B).
Microbiota-Based Prediction of IBS Placebo Response

A

IBS patients 
(n = 63)

T0

Intervention

T1

Wk 1  Wk 2  Wk 3  Wk 4

• IBS-SSS
• IBS-QoL
• HADS
• Stool frequency
• Stool consistency
• FFQ (T0 only)

Microbiota composition

• Daily GI complaints
• Daily stool frequency
• Daily stool consistency

B

IBS patients 
(n = 55)

T0

Observation

T1

Wk 1  Wk 2  Wk 3  Wk 4

• IBS-SSS
• IBS-QoL
• HADS
• Stool frequency
• Stool consistency
• FFQ (T0 only)

Microbiota composition

C

Intervention cohort 
(n = 63)

Clinical improvement : ≥ 50 points reduction in IBS-SSS

Observation cohort 
(n = 55)

Intervention-induced

Arbitrary

Clinical improvement (+)  
(n = 28)
Clinical improvement (-)  
(n = 35)
Clinical improvement (+)  
(n = 14)
Clinical improvement (-)  
(n = 41)

Comparison

C1
C2
C3
C4
Chapter 3

**Questionnaire data collection**

For both cohorts, clinical assessments were performed at baseline (To) and at week 4 (T1) using the validated IBS-SSS questionnaire, IBS quality of life (IBS-QoL), and hospital anxiety and depression score (HADS) (**Fig. 1A**). A food frequency questionnaire (FFQ) was completed only at To to assess dietary habits of subjects in both cohorts. In the intervention cohort, throughout the 4-week intervention daily questionnaires were completed by subjects concerning GI complaints (i.e., abdominal pain, bloating, flatulence), stool frequency and stool consistency. In the observation cohort, stool frequency and stool consistency were only reported at To and T1.

The IBS-SSS is a comprehensive scoring system that incorporates five main symptom domains: abdominal pain severity, duration of abdominal pain, bloating/distension, satisfaction with bowel habits, and IBS-related quality of life. Each symptom domain is rated on a scale from 0 to 100, and the sum score ranges from 0 to 500 (26). IBS-SSS sum scores are categorised into remission (scores lower...
than 75), mild (scores between 75 to 175), moderate (scores between 175 to 300), and severe (scores higher than 300). In this study, clinical improvement was defined as a reduction by more than 50 points over time in IBS-SSS sum score, which was previously defined as clinically relevant (26). Clinical improvement was further differentiated into intervention-induced type, including treatment-induced and placebo-induced, in the intervention cohort or arbitrary type in the observation cohort (Fig. 1C). The IBS-QoL is a validated 34-item questionnaire that provides information on the quality of life, where a higher score represents a higher quality of life (27). HADS is a self-assessment scale that reflects the states of anxiety and depression (28). The FFQ is a 83-item semiquantitative questionnaire that assesses habitual dietary intake of the previous month (29, 30). Habitual dietary fibre intake was calculated using the Dutch Food Composition table. GI complaint scores were reported on a scale of 0-10, where a higher score represents more complaints. Stool frequency was reported as the number of defecations per day. Stool consistency was assessed using the Bristol stool form scale, which is a 7-point scale ranging from 1 (hard stools) to 7 (diarrhoea) (31).

**Faecal microbiota composition profiling**

Faecal samples from the intervention cohort (n = 126 faecal samples) and observation cohort (n = 110 faecal samples) were used for microbiota composition profiling via sequencing the V4 region of the 16S ribosomal RNA (rRNA) gene using Illumina HiSeq2500 technology, following the same procedure as described previously (32). Briefly, DNA was isolated from 0.25 gram wet faeces using a previously published protocol (33). Isolated and purified DNA was further used for amplification of the V4 region of the 16S rRNA gene with barcoded primers 515F (5’-GTGY CAGC MGCC GCGG TAA-3’) (34) and 806R (5’-GGAC TACN VGGG TWTC TAAT-3’) (35) in duplicate PCRs. PCR was performed in 50 µL reactions including 10 µL 5× HF buffer (Thermo Fisher Scientific, Vilnius, Lithuania), 1 µL dNTPs (10 mM, Thermo Fisher Scientific), 0.5 µL Phusion Hot start II DNA polymerase (2 U/µL, Thermo Fisher Scientific), 36.5 µL nuclease free water (Promega, Madison, WI, USA), 1 µL DNA template (20 ng/µL) and 1 µL sample-specific barcode-tagged primers (10 µM) (36). The PCR program was set as follows: 98 °C for 30 s, followed by 25 cycles of 98 °C for 10 s, 50 °C for 10 s, 72 °C for 10 s, with a final extension of 7 min at 72 °C. The PCR product was then purified with the CleanPCR kit (CleanNA, The
Netherlands), and quantified using the Qubit dsDNA BR Assay kit (Invitrogen by Thermo Fisher Scientific, Eugene, OR, USA). An equimolar mix of purified PCR products was securely packed on dry ice and promptly dispatched to Novogene (Cambridge, United Kingdom) for sequencing. Raw sequence data were pre-processed using NG-Tax 2.0 with default settings (36, 37). Amplicon sequencing variant (ASV) picking and taxonomic assignments were performed using the SILVA 138.1 database.

Sequence data analyses were performed in R (version 4.2.2). The pre-processed data were used for alpha diversity (within-sample diversity) and beta diversity (between-sample diversity) calculation at ASV level, as implemented in the phyloseq (38) and picante (39) packages. Alpha diversity metrics including inverse Simpson and phylogenetic diversity were calculated. Beta diversity was visualised via principal coordinates analysis (PCoA), based on unweighted UniFrac distance (considering presence/absence and phylogenetic relatedness of ASVs) (40) and weighted UniFrac distance (considering relative abundance and phylogenetic relatedness of ASVs) (41). In addition, 16S rRNA gene read count data were transformed to microbial relative abundance, as implemented in the microbiome package (42). Taxa were further aggregated at genus level and predominant taxa were selected based on average relative abundance above 0.5% across the whole datasets from two cohorts.

**Machine learning**

In this study, we built a random forests (RF) ML classifier (43). RF is a powerful approach that is also suitable for datasets with small sample size, and it has been also applied to 16S rRNA gene sequence data (23, 44, 45). The datasets used to train and test the RF classifier included clinical metadata and microbial composition data. The metadata consisted of gender, age, BMI, IBS-QoL, HADS, and dietary fibre intake. Stool frequency and consistency data were excluded due to null values in cases of no defecation. The microbial composition data included centred log-ratio (clr) transformed (46) abundance data of microbial taxa that were aggregated at genus or order level.
Figure 2 Machine learning workflow. (A) Framework for machine learning (ML) model training and testing. Datasets from each cohort were randomly divided into a training set (80% of samples) and a test set (20% of samples). The samples in the training set were used for training the ML classifier using 5-fold cross validation. Next, the ML classifier was tested on the remaining 20% of samples that were not used for training (test set). This procedure was repeated five times. Model performance was evaluated and important features were identified. (B) Framework for ML model validation. Cross-cohort model validation was performed by applying the model trained on the data from the training cohort (i.e., observation cohort) to the data of the validation cohort (i.e., intervention cohort) to predict clinical improvement outcome.
To train and test the ML model (Fig. 2A), the datasets from each cohort were randomly divided into a training set (80% of samples) and a test set (the remaining 20% of the samples). The training set was used to train an RF classifier using 5-fold cross-validation (CV), which involved dividing the training set into five non-overlapping CV folds. One of the five CV folds was used as a validation set, and the remaining CV folds were used to train the RF model. The 5-fold CV procedure was repeated five times. To correct for potential bias derived from an unbalanced dataset, a synthetic minority over-sampling technique (SMOTE) was applied (47). Subsequently, the RF classifier was evaluated using the test set to determine its performance on samples not used for training, and important features were identified. Model performance was characterised using the area under the receiver operating characteristic (ROC) curve (AUC), with a value of 0.5 for a random classifier. Feature importance was calculated to identify important features, with a higher score indicating a higher contribution in distinguishing subjects who showed a clinical improvement from those that did not.

To validate and assess the predictive power of the ML models, both training and validation cohorts are needed (Fig. 2B). In our study, the observation cohort served as the training cohort with the aim to assess whether the model can predict the clinical improvement of subjects in the intervention cohort, which served as the validation cohort. The ML model was trained using the baseline clinical metadata and microbial composition data from the training cohort, as described in Fig. 2A. To further improve the predictive power, an unsupervised feature selection was conducted. Redundancy analysis (RDA) was performed to identify significant metadata factors that relate to the variability in microbial composition. The top two predominant microbial taxa at order level were selected. Subsequently, combined datasets of selected metadata and microbiota features from the training cohort were used for model training and testing. Then, combined datasets of the same selected features from the validation cohort were used as input for the trained model, which then outputted a classification for each subject in the validation cohort based on the ML model’s predictions. We measured the predictive power of the models in terms of sensitivity (the percentage of subjects correctly predicted to have a clinical improvement), specificity (the percentage of subjects correctly predicted not to have a clinical improvement), and accuracy (the percentage of correctly predicted subjects
among all subjects). These metrics were calculated based on the comparison of the predicted outcome with the true outcome of each subject within the validation cohort.

**Statistical analysis**

All statistical analyses were conducted in R (version 4.2.2). Continuous data were shown as mean ± standard deviation if normally distributed or median [interquartile range] when skewed. Differences of continuous data between T0 and T1 were compared by paired sample t-test when normally distributed or paired Wilcoxon test when not normally distributed. Differences of continuous data between two cohorts or between clinical improvement groups were compared with independent sample t-test when normally distributed or Mann-Whitney U test when not normally distributed. Linear regression was carried out by fitting a linear model to the data using the *lm* function in the *stats* package. In the intervention cohort, dynamics of GI complaints (e.g., abdominal pain, bloating, flatulence), stool frequency and stool consistency over time within each clinical improvement group (fixed effect: time, random effect: subject) or between clinical improvement groups (fixed effect: time*group, random effect: subject) were assessed using generalised linear mixed-effects models (GLMMs) for repeated measures using the *glmer* function in the *lme4* package (48). Categorical data are shown as counts and percentage. Percentages of categorical data were compared with Fisher’s exact test. Permutational multivariate analysis of variance (PERMANOVA) was used to test significant differences between groups (beta diversity), as implemented in the *vegan* package (49). RDA analysis was performed with the *vegan* package. Permutation tests (n = 999) were used to determine the significance of variance explained by each metadata factor. All figures were generated using the *ggplot2* package (50). In all cases, p values of 0.05 or lower were considered statistically significant.

**Ethics**

The intervention cohort study was approved by the Medical Ethics Review Committee Utrecht, the Netherlands (NL75824.041.20), and was registered at ClinicalTrials.gov on 10 March 2021 (NCT04790422). The observation cohort study was approved by the Medical Ethics Review Committee Wageningen, the Netherlands (NL64950.081.18), and was registered at ClinicalTrials.gov on 25 October 2018 (NCT03720314).
Data availability

Clinical metadata are available upon reasonable request (contact: Lonneke Janssen Duijghuijsen, Wageningen Food & Biobased Research, Wageningen University & Research, Wageningen, The Netherlands, janssenduijghuijsen@wur.nl). Raw 16S-rRNA gene sequence data have been deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under accession number PRJEB56219 for the intervention cohort study, and PRJEB44533 for the observation cohort study. The R code for the statistical analysis used in this study is available at a public repository on GitLab (https://git.wur.nl/afsg-microbiology/publication-supplementary-materials/2023-liu-ibs_clinical_comparison_study).

Results

In this study, we aimed to identify microbial composition signatures of IBS patients who achieved a clinical improvement. To optimally use and compare the data of both cohorts, we first validated the clinical relevance of the definition of 'clinical improvement' by analysing daily GI complaints data of subjects from an intervention cohort with IBS subjects. Furthermore, we investigated the dynamics over time and the signatures of clinical improvement in relation to questionnaires as well as faecal microbiota composition within an intervention and an observation cohort. Finally, we compared the cohort-specific clinical improvements and performed cross-cohort predictions of clinical improvement using baseline datasets.

Baseline demographic characteristics of subjects in two cohorts

Baseline demographic characteristics of subjects in the two cohorts are summarised in Table 1. Subjects in the intervention cohort (n = 63) and the observation cohort (n = 55) were not significantly different with respect to age, BMI or gender distribution. However, subjects in the intervention cohort had significantly higher IBS-SSS scores (226 [154–256], median [interquartile range]) than those in the observation cohort (140 [100–250]) (p = 0.021). In addition, the distribution of IBS severity groups was different between the cohorts (p = 0.001), with a higher fraction of moderate (58.7%) IBS subjects in the intervention cohort and a higher fraction of mild (43.6%) IBS subjects in the observation cohort. In contrast to the severity differences, no significant difference between the two cohorts was observed.
with respect to quality of life, anxiety and depression, stool frequency, stool consistency, and dietary fibre intake \( (p > 0.05) \).

**Table 1 Baseline demographic characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Intervention cohort ( n = 63 )</th>
<th>Observation cohort ( n = 55 )</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td>37.7 ± 13.0</td>
<td>40.3 ± 14.3</td>
<td>0.289</td>
</tr>
<tr>
<td><strong>BMI, kg/m^2</strong></td>
<td>23.6 ± 2.9</td>
<td>22.8 ± 2.8</td>
<td>0.123</td>
</tr>
<tr>
<td><strong>Female, n (%)</strong></td>
<td>53 (84.1)</td>
<td>46 (83.6)</td>
<td>0.895</td>
</tr>
<tr>
<td><strong>IBS-SSS, sum scores</strong></td>
<td>226 [154–256]</td>
<td>140 [100–250]</td>
<td><strong>0.021</strong></td>
</tr>
<tr>
<td><strong>Severity groups (^a), n (%)</strong></td>
<td></td>
<td></td>
<td><strong>0.001</strong>**</td>
</tr>
<tr>
<td>Remission ((&lt; 75))</td>
<td>0 (0)</td>
<td>5 (9.1)</td>
<td></td>
</tr>
<tr>
<td>Mild ((75-175))</td>
<td>21 (33.3)</td>
<td>24 (43.6)</td>
<td></td>
</tr>
<tr>
<td>Moderate ((175-300))</td>
<td>37 (58.7)</td>
<td>16 (29.1)</td>
<td></td>
</tr>
<tr>
<td>Severe ((≥ 300))</td>
<td>5 (7.9)</td>
<td>10 (18.2)</td>
<td></td>
</tr>
<tr>
<td><strong>IBS-QoL, sum scores</strong></td>
<td>75.7 [61.4–80.1]</td>
<td>75.7 [57.4–85.3]</td>
<td>0.662</td>
</tr>
<tr>
<td><strong>HADS, sum scores</strong></td>
<td>11 [7.5–16.5]</td>
<td>9 [6–16]</td>
<td>0.208</td>
</tr>
<tr>
<td>Stool frequency, ( n ) of defeation per day</td>
<td>1 [1–2]</td>
<td>1 [1–2]</td>
<td>0.801</td>
</tr>
<tr>
<td>Stool consistency, predominant mode score</td>
<td>4 [3–6]</td>
<td>4 [3–6]</td>
<td>0.491</td>
</tr>
<tr>
<td>Clinical improvement (+) (^b), n (%)</td>
<td>28 (44.4)</td>
<td>14 (25.5)</td>
<td><strong>0.036</strong></td>
</tr>
<tr>
<td><strong>Dietary fibre intake, grams per day</strong></td>
<td>23.9 ± 7.67</td>
<td>26.3 ± 8.52</td>
<td>0.107</td>
</tr>
</tbody>
</table>

\(^a\): severity groups are based on IBS-SSS scores at baseline (To)

\(^b\): clinical improvement is based on change in IBS-SSS scores over time

Continuous data were shown as mean ± standard deviation or median [interquartile range] when skewed. Differences of age, BMI, and dietary fibre intake were tested with the independent sample \( t \)-test. Differences of IBS-SSS, IBS-QoL, HADS, stool frequency, and stool consistency were tested with Mann-Whitney \( U \) test. Percentage of gender, severity groups, and clinical improvement were tested.
with Fisher’s exact test. Abbreviations: BMI: body mass index, HADS: hospital anxiety and depression score, IBS-SSS: IBS severity scoring system, IBS-QoL: IBS quality of life.

Validation of clinical relevance of ‘clinical improvement’

Based on previous research, ‘clinical improvement’ was defined as a reduction in IBS-SSS score by more than 50 points (26). Overall, 44.4% of the subjects (28/63) in the intervention cohort achieved a clinical improvement, independent of specific treatment, in comparison to 25.5% of the subjects (14/55) in the observation cohort (Table 1, p = 0.036). Daily GI complaints (i.e., abdominal pain, bloating and flatulence) were only reported within the intervention cohort. Subjects reported on average low scores (abdominal pain: 2.4 ± 2.1, bloating: 2.8 ± 2.3, flatulence: 2.4 ± 2.2), indicating no or mild GI complaints during the intervention period. However, by fitting a GLMM to the data of daily GI complaints and stool pattern (i.e., stool frequency and stool consistency), we found that subjects who achieved a clinical improvement did not have exacerbations of abdominal pain (Fig. 3A, p = 0.441) and bloating (Fig. 3B, p = 0.089), but had a small relieve of flatulence (Fig. 3C, p = 0.003). In contrast, subjects defined as having no clinical improvement reported a small but significant increase in abdominal pain (p < 0.001) and bloating (p < 0.001), but stable flatulence (p = 0.104). Additionally, a separation of developing pattern over time was observed between both groups with respect to abdominal pain (p = 0.046), bloating (p = 0.006), and flatulence (p = 0.025), with more favourable scores for those who achieved a clinical improvement, while stool frequency and stool consistency did not show a significant change in either of the two groups during the intervention (Fig. 3D, E, p > 0.05).
Microbiota-Based Prediction of IBS Placebo Response

A

Abdominal pain

Clinical improvement (+) \( p = 0.4405 \)
Clinical improvement (-) \( p = 3.2846 \times 10^{-5} \)
Clinical improvement (+) vs (-) \( p = 0.0474 \)

Days

B

Bloating

Clinical improvement (+) \( p = 0.08637 \)
Clinical improvement (-) \( p = 0.00021 \times 10^{-5} \)
Clinical improvement (+) vs (-) \( p = 0.00457 \)

Days

C

Flatulence

Clinical improvement (+) \( p = 0.002001 \)
Clinical improvement (-) \( p = 0.1306 \)
Clinical improvement (+) vs (-) \( p = 0.025 \)

Days

D

Stool frequency

Clinical improvement (+) \( p = 0.1855 \)
Clinical improvement (-) \( p = 0.8828 \)
Clinical improvement (+) vs (-) \( p = 0.3351 \)

Days

E

Stool consistency

Clinical improvement (+) \( p = 0.2395 \)
Clinical improvement (-) \( p = 0.8376 \)
Clinical improvement (+) vs (-) \( p = 0.6954 \)

Days

Group

- Clinical improvement (+)
- Clinical improvement (-)
Figure 3 Dynamics of GI complaints and stool patterns of subjects in the intervention cohort. Dynamics of (A) abdominal pain, (B) bloating, (C) flatulence, (D) stool frequency, and (E) stool consistency within each clinical improvement group. The dots, which represent the mean value of each parameter within each improvement group, were connected by lines. A smooth line with 95% confidence level interval (shaded area) for predictions from a generalised linear model was added. Dynamics within each clinical improvement group were tested by fitting a generalised linear mixed-effects model (GLMM) to the data with time as fixed effect and subject as random effect. For comparison between groups, GLMMs were fitted with (time*group) as fixed effects and subjects as random effect. Green: clinical improvement (+), red: clinical improvement (−).

To determine whether clinical improvement was associated to baseline symptom severity level, linear regression was performed (Fig. 4). We found that IBS-SSS score reduction (i.e., improvement) was positively related with baseline IBS-SSS score in both the intervention cohort ($r = 0.388$, $R^2 = 0.249$, $p < 0.001$) and observation cohort ($r = 0.841$, $R^2 = 0.423$, $p < 0.001$), indicating that higher baseline severity scores were related with a higher reduction in IBS-SSS score over time. This was also reflected in the proportion of subjects who achieved a clinical improvement, which increased with the level of severity in both cohorts (Fig. S1, $p = 0.013$ and $p < 0.001$, respectively). In addition, we also investigated whether there was a relation between IBS-SSS score reduction and baseline IBS-QoL score (Fig. S2). Surprisingly, there was no relation in the intervention cohort ($r = 0.003$, $R^2 = 0$, $p = 0.889$), whereas a negative relation was observed in the observation cohort ($r = -0.084$, $R^2 = 0.137$, $p = 0.005$), indicating that lower QoL at baseline was related with a higher reduction in IBS-SSS score in the observation cohort.
To determine whether subjects identified as clinically improved and non-clinically improved showed distinct dynamics over time with respect to questionnaire and microbiota observations, we compared and contrasted both groups within each cohort (Fig. 1C, C1). As expected, in both cohorts, subjects who achieved a clinical improvement reported significantly reduced IBS-SSS scores (Fig. 5A, Table S1, $p < 0.001$ and $p = 0.001$, respectively), and those who did not achieve a clinical improvement did not ($p > 0.05$) or showed the opposite ($p = 0.004$) in the
Chapter 3

intervention- and observation cohort, respectively. In addition, IBS-QoL scores were significantly increased in subjects who achieved a clinical improvement in the intervention cohort, indicating an increased quality of life (Fig. 5B, \( p < 0.001 \)), while no differences or worsening of QoL were observed in subjects without a clinical improvement in both cohorts. Moreover, no significant differences in HADS, stool frequency, or stool consistency were observed between groups, in both cohorts (Fig. 5C, D, E, \( p > 0.05 \)).
Microbiota-Based Prediction of IBS Placebo Response

A

clinical improvement (+) clinical improvement (-)

IBS-SSS

T0 T1 T0 T1

p = 3.99e-06**

observation cohort

p = 0.00106**

B

clinical improvement (+) clinical improvement (-)

IBS-QoL

T0 T1 T0 T1

p = 1.29e-05****

observation cohort

C

clinical improvement (+) clinical improvement (-)

HADS

T0 T1 T0 T1

p = 0.00384**

observation cohort

D

clinical improvement (+) clinical improvement (-)

Stool frequency

T0 T1 T0 T1

E

clinical improvement (+) clinical improvement (-)

Stool consistency

T0 T1 T0 T1

67
Microbial alpha diversity and beta diversity were subsequently investigated. Alpha diversity metrics, including inverse Simpson and phylogenetic diversity, did not show significant differences over time for both groups in both cohorts (Table S2). With respect to beta diversity, PERMANOVA (Table 2) showed no significant changes of faecal microbiota composition over time within each cohort based on pairwise unweighted (Fig. 6A) and weighted (Fig. 6B) UniFrac distances. In addition, dynamics of predominant microbial taxa at genus level did not show significant differences over time in either of the two cohorts for subjects that achieved a clinical improvement (Table S3). However, in subjects without a clinical improvement, decreased relative abundance of *Coprococcus* (*p* = 0.014) and increased relative abundance of *Paraprevotella* (*p* = 0.018) were observed in the intervention cohort, whereas only *Parabacteroides* (*p* = 0.037) showed an increased relative abundance in the observation cohort (Fig. S3, Table S3).
Table 2 Permutational multivariate analysis of variance (PERMANOVA) of faecal microbiota composition

<table>
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<tr>
<th>Cohort</th>
<th>IC</th>
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<td>T0</td>
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<td>Comparison</td>
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<td>T0</td>
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Beta diversity (between-sample diversity) of faecal microbiota composition was tested by PERMANOVA. $R^2$ represents the proportion of variance explained by grouping factor, $p$ value represents the significance between grouping factors. Abbreviations: IC: intervention cohort, OC: observation cohort, CI: clinical improvement, PERMANOVA: permutational multivariate analysis of variance.
Host and microbiota signatures of clinical improvement at baseline

Baseline host and microbiota characteristics of subjects with clinical improvement (n = 28 and 14 in the intervention and observation cohort, respectively) and those without clinical improvement (n = 35 and 41) within each cohort were subsequently investigated (Fig. 1C, C2). In comparison to subjects without a clinical improvement, subjects who achieved a clinical improvement had significantly higher baseline IBS-SSS scores in both cohorts (Table S1, p = 0.003 and p < 0.001, respectively), concomitant with higher baseline BMI (p = 0.020) in the intervention cohort, but lower baseline IBS-QoL scores (p = 0.007) in the observation cohort.

Baseline microbiota alpha diversity metrics scores were not significantly different between subjects with- and without clinical improvement in the two cohorts (Table S2). PERMANOVA revealed significant differences in composition between subjects with- and without clinical improvement in the intervention cohort at baseline based on unweighted UniFrac distances (Fig. 6A, Table 2, R² = 3.105%, p = 0.026), but not for weighted UniFrac distances (Fig. 6B, Table 2, R² = 1.927%, p = 0.294). This suggests that baseline microbiota signatures of subjects with clinical improvement in the intervention cohort were mainly attributed to the presence/absence of taxa. In contrast, no significant differences were observed between subjects with- and without clinical improvement in the observation cohort, neither based on unweighted UniFrac (Fig. 6A, Table 2, R² = 1.304%, p = 0.845) nor weighted UniFrac (Fig. 6B, Table 2, R² = 2.174%, p = 0.296) distances.

When comparing the microbiota composition at baseline between subjects with- and without clinical improvement, subjects in the intervention cohort who achieved a clinical improvement were characterised by higher relative abundance of Methanobrevibacter (Fig. 7, Table S3, p = 0.040), but lower relative abundance of Lachnospiraceae ND3007 group (p = 0.004) and Lachnospira (p = 0.014), both
belonging to the *Lachnospiraceae* family. Since the genus *Methanobrevibacter* is only detected in high relative abundance in a fraction of IBS subjects (51), we compared the prevalence of *Methanobrevibacter* in subjects with- and without clinical improvement. This revealed that this genus was detected in a significantly higher fraction (43%) of the subjects with clinical improvement as compared to 17% of the subjects without clinical improvement in the intervention cohort ([Fig. S4A](#), Fisher's exact test: $p = 0.048$). This is in line with the differences in beta diversity based on unweighted UniFrac distances ([Table 2](#)) as *Methanobrevibacter* is phylogenetically distant from all bacteria (52) and therefore its presence in some of the subjects has a large impact on the phylogenetic distance-based algorithm used in the unweighted UniFrac calculations.
**Figure 7** Baseline relative abundance of distinct genera between subjects with- and without clinical improvement in two cohorts. Data of relative abundance at genus level. Each dot represents the data from an individual subject. In each group, the boxplot was added with vertical line inside the box representing the median, and the left and right ends correspond to the first and third quartiles (the 25th and 75th percentiles). Genera belonging to the *Lachnospiraceae* family were labelled with red text. Significance was tested via Mann-Whitney *U* test. Green: clinical improvement (+), red: clinical improvement (−).
The observation cohort showed distinct differences with respect to microbiota composition between subjects with- and without clinical improvement compared to the intervention cohort (Fig. 1C, C2 right). The relative abundance of *Parabacteroides* was higher (Fig. 7, Table S3, \( p = 0.024 \)), while that of *Subdoligranulum* \( (p < 0.001) \), and three genera belonging to the *Lachnospiraceae*, namely *Fusicatenibacter* \( (p = 0.024) \), *Dorea* \( (p = 0.003) \), and [*Ruminococcus* gauvreauii] group \( (p = 0.036) \), was significantly lower in subjects with clinical improvement, compared to subjects without clinical improvement. Prevalence of *Methanobrevibacter* was similar \( (41−43\%) \) in both groups (Fig. S4B, Fisher’s exact test: \( p = 1 \)), which was in contrast to the results in the intervention cohort. Collectively, these results suggest that baseline microbiota compositional differences between the two clinical improvement groups were cohort-dependent, but the majority of the distinct genera belonged to *Lachnospiraceae*, with lower relative abundances in subjects who achieved a clinical improvement.

**Baseline comparison of subjects with clinical improvement between intervention cohort and observation cohort**

Since cohort-specific differences in microbiota composition were observed between subjects with- and without clinical improvement, we explored whether there were baseline differences between subjects who achieved a clinical improvement in the intervention cohort \( (n = 28) \) and those who achieved a clinical improvement in the observation cohort \( (n = 14) \) (Fig. 1C, C3). Subjects who achieved a clinical improvement in the intervention cohort were characterised by relatively lower IBS-SSS scores (Table S1, \( p = 0.030 \)), but higher BMI \( (p = 0.018) \) compared to subjects who achieved a clinical improvement in the observation cohort. No significant baseline difference in microbial alpha diversity or composition (beta-diversity) was observed (Table S2, Fig. 6, Table 2). When considering specific genera (Fig. 8, Table S3), subjects who achieved a clinical improvement in the intervention cohort showed higher baseline relative abundance of *Bifidobacterium* \( (p = 0.016) \), and *Subdoligranulum* \( (p = 0.005) \) compared to subjects with clinical improvement in the observation cohort. In contrast, subjects with a clinical improvement in the observation cohort showed higher baseline relative abundance of *Lachnospiraceae* ND3007 group \( (p = 0.026) \), *Oscillospiraceae* NK4A214 group \( (p = 0.011) \),
**Clostridium sensu stricto 1** ($p = 0.021$), and **Butyrivibrio** ($p = 0.006$) compared to subjects with clinical improvement in the intervention cohort. Since the differences at baseline between clinically improved subjects from both cohorts can be due to cohort-specificity or due to baseline microbiota signature...
differences, we also compared whether differences exist between cohorts at baseline in subjects who did not achieve a clinical improvement (Fig. 1C, C4). Subjects from the intervention cohort who did not achieve a clinical improvement were characterised by higher IBS-SSS scores (Table S1, $p = 0.003$) but lower phylogenetic diversity (phylogenetically weighted richness) (Table S2, $p = 0.013$) compared to subjects who did not achieve a clinical improvement in the observation cohort. Additionally, PERMANOVA, based on unweighted UniFrac distances, revealed significant differences microbiota composition in subjects without a clinical improvement between the intervention cohort and the observation cohort at baseline (Fig. 6A, Table 2, $R^2 = 3.890\%$, $p = 0.003$), but not for weighted UniFrac distances ($R^2 = 2.269\%$, $p = 0.117$). Furthermore, 11 genera were significantly different in relative abundance between cohorts for subjects without clinical improvement (Fig. S5), of which only the genera Oscillospiraceae NK4A214 group and Butyricicoccus overlapped with the differences observed for subjects with clinical improvement. It is noteworthy that the relative abundance of predominant genera such as unclassified Lachnospiraceae ($p = 0.021$) and Bacteroides ($p = 0.004$) was significantly higher in subjects without clinical improvement in the intervention cohort at baseline, while the relative abundance of Prevotella 9 ($p = 0.042$) was higher in the subjects without clinical improvement in the observation cohort at baseline. Overall, these results suggest that intrinsic heterogeneity between the cohorts may hamper the identification of signature microbes to distinguish subjects with or without clinical improvement between intervention cohort and observation cohort.

**Predicting clinical improvement outcome in the intervention cohort using machine learning models trained on the observation cohort**

The aforementioned results showed that both subject demographic characteristics and microbiota composition differed between subjects with- and without clinical improvement, although in a cohort-dependent manner. Therefore, we employed a machine learning (ML) approach, using random forests, to assess whether clinical improvement can be accurately predicted in one cohort based on baseline clinical metadata and microbiota data characteristics of the other cohort. Initially, the ML models were trained using either metadata, microbiota data at genus level, or both. Metadata alone yielded an area under the curve (AUC) of 0.529 and 0.487 in the intervention cohort and observation cohort, respectively (Fig. 9). On the
other hand, microbiota data alone resulted in an AUC of 0.693 and 0.652 in the intervention cohort and observation cohort, respectively. ML models trained with both metadata and microbiota data, on average, outperformed the models trained with either metadata or microbiota data alone, with an AUC of 0.684 and 0.697 in the intervention cohort and observation cohort, respectively (Fig. 9). Hence, the metadata in combination with the microbiota data were selected to train the models.

When looking into the important features of each model, however, there were no common features shared by the different models (Fig. 10). This is in line with the intrinsic heterogeneity between the cohorts as indicated in the previous section. Nevertheless, it is worth noting that nine out of 20 most important feature taxa belonged to the Lachnospiraceae. More specifically, these included Lachnospiraceae ND3007 group, Lachnospira, [Eubacterium] xylanophilum group, an unclassified...
genus within *Lachnospiraceae*, and *Lachnospiraceae* UCG-004 in the intervention cohort and *Dorea*, *Lachnospiraceae* FCS020 group, *[Eubacterium] ventriosum* group and *[Ruminococcus] gauvreauii* group in the observation cohort (**Fig. 10**).
Nonetheless, we aimed to predict the outcome of subjects from the intervention cohort using the ML model trained on baseline datasets from the observation cohort. To improve the predictive power, an unsupervised selection of features was performed on the observation cohort datasets only. For metadata factors, RDA analysis followed by a permutation test showed that dietary fibre intake \((p = 0.031)\) and gender \((p = 0.025)\) significantly explained most of the variation in microbiota composition (Fig. 11).

**Figure 11** Redundancy analysis (RDA) between metadata factors and microbiota composition at genus level of subjects in the observation cohort. RDA plot illustrating the relationship between metadata factors \((n = 7, \text{red arrows})\) and relative abundance of microbial taxa at genus level in subjects \((n = 55, \text{grey dots})\) from the observation cohort. The microbiota composition data were centred log-ratio transformed. Arrows indicate the direction and magnitude of the effects of factors. Permutation tests \((n = 999)\) were used to determine the significance of variance explained by each metadata factor. Only dietary fibre intake and gender were significant and showed a \(p\)-value below 0.05. Percentages given for each axis indicate the amount of variation in microbiota data explained. Abbreviations: RDA: redundancy analysis.
For microbiota data, the *Lachnospirales* and *Oscillospirales* orders were selected as they collectively accounted for ~60% of all taxa and were present in all subjects (Fig. S6). Hence, these four features were selected for ML model training and testing. This model attained an AUC of 0.664, sensitivity of 0.507, and specificity of 0.680, which could be regarded as a moderately effective classifier (53) (Fig. 12).

By applying this model to predict the clinical improvement of subjects from the intervention cohort (Table 3), only 15 out of 28 subjects with clinical improvement were correctly classified (sensitivity = 0.536), and 22 out of 35 subjects without clinical improvement were correctly classified (specificity = 0.629), resulting in an accuracy of 0.587. However, when looking specifically at subjects who consumed

![Figure 12](image_url)

**Figure 12 Performance of ML model trained on datasets of selected features from the observation cohort.** The receiver operating characteristic (ROC) curve of the model trained by the datasets from the observation cohort was visualised. Area under the ROC curve (AUC) was calculated. Each model was trained by gender and baseline dietary fibre intake, centred log-ratio transformed abundance data of order *Lachnospirales* and *Oscillospirales* of each subject within the observation cohort. Model performance is reflected by AUC, sensitivity and specificity. Abbreviations: AUC: area under curve, ML: machine learning, ROC: receiver operating characteristic.
placebo \((n = 13)\) of which four subjects achieved clinical improvement and nine subjects did not) and those who consumed the dietary treatment components \((n = 50)\) of which 24 subjects achieved clinical improvement and 26 subjects did not) in the intervention cohort, the outcome for 12 out of 13 subjects in the placebo group was correctly predicted (sensitivity = 0.750 \((3/4)\), specificity = 1 \((9/9)\), accuracy = 0.923 \((12/13)\)) while the outcome was only correctly predicted for 25 out of 50 subjects who consumed the active treatment components (sensitivity = 0.500 \((12/24)\), specificity = 0.500 \((13/26)\), accuracy = 0.500 \((25/50)\)).

**Table 3 Clinical improvement outcome prediction of subjects in placebo or treatment group from the intervention cohort**

<table>
<thead>
<tr>
<th>Group</th>
<th>Clinical improvement (+) ((n))</th>
<th>Clinical improvement (−) ((n))</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reference Prediction Sensitivity</td>
<td>Reference Prediction Specificity</td>
<td></td>
</tr>
<tr>
<td>All subjects</td>
<td>28 15 0.536</td>
<td>35 22 0.629</td>
<td>0.587</td>
</tr>
<tr>
<td>((n = 63))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>4 3 0.750</td>
<td>9 9 1</td>
<td>0.923</td>
</tr>
<tr>
<td>((n = 13))</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Treatments</td>
<td>24 12 0.500</td>
<td>26 13 0.500</td>
<td>0.500</td>
</tr>
<tr>
<td>((n = 50))</td>
<td></td>
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</table>

Using the model trained by datasets from the observation cohort \((n = 55)\), clinical improvement of subjects in the intervention cohort \((n = 63)\), subsequently divided into placebo group \((n = 13)\) or treatment group \((n = 50)\), was predicted. Predictive performance was shown as the sensitivity (percentage of correctly predicted subjects with clinical improvement), specificity (percentage of correctly predicted subjects without clinical improvement) and accuracy (percentage of all correctly predicted subjects).

This indicates that host and microbiota features of placebo-induced clinical improvement largely overlapped with those of arbitrary clinical improvement in the two cohorts included in this study, and that placebo responsiveness can potentially be predicted at baseline with a ML model trained on an observation cohort.
Discussion

By analysing clinical metadata and faecal microbiota data from both an intervention cohort and an observation cohort of IBS subjects, we identified subjects who achieved a clinical improvement based on their improvements in IBS-SSS scores over time. This clinical improvement based on IBS-SSS scores was characterised by improvements in daily GI complaints among subjects in the intervention cohort. Although there were no significant shifts in questionnaire- or faecal microbiota-related observations over time, we found that subjects who achieved a clinical improvement had higher baseline IBS-SSS scores and lower relative abundances of a panel of genera belonging to the *Lachnospiraceae* family, which varied among the cohorts. Despite this cohort heterogeneity, an ML model trained on baseline host- and microbiota-derived data from the observation cohort allowed for a robust prediction of clinical improvement outcomes for subjects in the placebo group from the intervention cohort.

The paradigm of a reduction in IBS-SSS score by more than 50 points is of significant clinical relevance

IBS-SSS is a simple and easy-to-use scoring system, which has been widely used in research trials to quantify symptom severity with a reproducible reliability within 24 h (26, 54), although a consensus on the severity of IBS is still lacking. For instance, in a clinical trial in which 52 IBS patients received either probiotics or placebo for eight weeks, a gradual reduction of IBS-SSS score was observed in both groups every two weeks correlating with improved QoL, satisfaction with bowel habit and fewer days with pain (55). However, another study showed that assessment of clinical improvement or response in IBS is still challenging due to the variation of severity levels between patients as well as the fluctuation over time within one patient (56). A reduction by more than 50 points in IBS-SSS scores was previously shown to correlate with an improvement of IBS severity based on measurements conducted in hospitals (26), and so this definition of clinical improvement has been applied in numerous trials to assess the response of patients. For example, in a faecal microbiota transplantation (FMT) trial where 165 IBS patients received either placebo, 30 g FMT or 60 g FMT, the effectiveness of FMT in the treatment of IBS has been demonstrated (57). In addition, responders (IBS-SSS score reduction by more than 50 points after
three months) accounted for 23.6%, 76.9% and 89.1% of participants in each group, respectively, which was accompanied by significant improvements in QoL and fatigue in FMT groups. Similarly, researchers also identified responders to 4-week low FODMAP dietary intervention, by looking at the reduction of IBS-SSS scores (19). Therefore, a reduction by more than 50 points in IBS-SSS scores could be regarded as a solid definition of clinical improvement, although the definition is still being argued (58).

To determine whether this definition of clinical improvement was related with improvement of IBS symptoms in our study, we compared and contrasted daily GI complaints over time between IBS patients that were classified as clinically improved or not improved based on IBS-SSS score reduction by more than 50 points over four weeks. Indeed, the results showed a distinct but favourable developing pattern of GI complaints and QoL in patients that reached clinical improvement in contrast to those who did not. This was in line with previous studies, including a prospective survey study involving 858 patients with IBS, where a positive correlation was found between the severity of IBS symptoms and the patients’ QoL (59). These findings collectively indicated the identification of clinically improved subjects with a reduction by more than 50 points in their IBS-SSS score is indeed associated with an overall improvement for these IBS patients over time and therefore a good marker for the classification of IBS patients who positively responded to a treatment.

**Baseline host and microbiota signatures of clinical improvement are cohort-dependent**

Although several studies have investigated the baseline factors that determine the progression of IBS (60, 61), most of them have neglected discrepancies in clinical metadata. Patients with severe IBS at baseline (as defined with IBS-SSS), were found to be more likely to achieve the greatest reduction in symptoms (62). Likewise, in our study, we observed that baseline IBS-SSS scores were positively associated with a reduction in IBS-SSS over time, irrespective of cohort, consistent with a higher frequency of clinical improvement in patients with more severe symptoms. Surprisingly, we also observed this trend with patients in the observation cohort who were just followed over time without treatment. This may explain why high placebo effects are frequently observed in intervention studies with IBS, which confines the possibility of finding an effective dietary therapy. Nevertheless, our findings uphold
the notion that symptom improvement has greater potential in patients with higher IBS-SSS scores. This conclusion suggests that severity-based stratification holds considerable promise for clinical application. In fact, pre-screening patients with higher severity was already implemented in other clinical trials. For example, only moderate or severe patients (IBS-SSS score > 175) were included in a low FODMAPs dietary intervention trial (19). Furthermore, researchers observed a decrease in the variability of treatment response when they exclusively enrolled symptomatic IBS patients with IBS-SSS scores > 150 for a 12-week probiotic or placebo intervention (63).

On the other hand, apart from the IBS-SSS score, we also observed that subjects who achieved a clinical improvement had cohort-dependent host signatures at baseline. Specifically, subjects in the intervention cohort had higher BMI values, while those in the observation cohort exhibited lower QoL. Although the link between obesity and IBS remains incompletely understood, overweight and obese IBS patients had reported to have greater symptom severity compared IBS patients with normal weight (64). However, the prevalence of IBS among obese adults is notably cohort-dependent (65), suggesting the solid link between high BMI and high chance of improvement is susceptible to cohort heterogeneity. Nevertheless, differences in BMI observed in our study were small, and importantly, all patients were classified as normal weight according to the World Health Organization’s criteria (BMI lower than 24.9). Regarding IBS-QoL, a Dutch IBS cohort underwent a 5-year follow-up study in which researchers implemented a multivariable regression model to identify baseline predictors related to QoL at follow-up (66). This study revealed that patients with higher QoL at baseline were more likely to experience higher QoL at follow-up, suggesting that baseline QoL is an important predictor of future QoL. However, no consistent improvements in QoL were observed in relation to symptom severity. Consequently, the limited value of baseline QoL in implying clinical improvement remains apparent.

As IBS clinical treatment decisions are often based on prognostic predictions of disease progression, we endeavoured to identify baseline predictors that could foresee a favourable improvement outcome. A comprehensive literature review has shown that the baseline faecal microbiome has predictive capacity for clinical response to dietary interventions in IBS patients (67). For example, responders to a low FODMAP diet were characterised by higher baseline relative abundances of taxa
with known higher saccharolytic metabolic capacity (e.g., *Faecalibacterium prausnitzii*) (60). However, there is limited consensus on the specific microbiome signatures that could serve as a distinguishing tool between patients who are likely to achieve clinical improvement and those who are not, prior to treatment. In our study, cohort-dependent baseline microbiota signatures were observed. Specifically, both prevalence and relative abundance of *Methanobrevibacter* were higher in subjects with clinical improvement in the intervention cohort, whereas that was *Parabacteroides* in the observation cohort. These findings underscore the significant differences in microbiota composition between cohorts, even among individuals from the same geographical area and recruited based on similar criteria. It is noteworthy that these two cohort studies were conducted in different seasons. As seasonal differences in microbiota composition have been previously identified (68), future comparative studies need to take trial execution season into account.

Supplementation of *Bifidobacterium* spp. has previously shown its efficacy in improving IBS symptoms (69). In the observation cohort, it has been reported that the relative abundance of *Bifidobacterium* in individuals with IBS was significantly lower compared to that in healthy controls (25). As such, an intervention study was subsequently conducted with the ambition to increase the relative abundance of *Bifidobacterium* (*van den Belt* et al., in prep; for study design, see Supplementary materials). However, a challenge arose during the interpretation of the intervention’s efficacy due to baseline differences between the intervention and observation cohorts. Specifically, the intervention cohort already exhibited a higher relative abundance of *Bifidobacterium* compared to the observation cohort, likely due to inherent heterogeneity between the cohorts.

Despite of cohort heterogeneity and accompanied fact that at genus level we did not find a consistent genus associated with clinical improvement, it was striking that in each cohort five distinct genera from the family *Lachnospiraceae* were found with lower relative abundance at baseline in subjects who achieved a clinical improvement as compared to those who did not. *Lachnospiraceae* is a known core taxon of the adult gut microbiota (70), accounting for approximately 10% of the total gut microbiota (71). Moreover, members of *Lachnospiraceae* have shown the capacity to produce butyrate from lactate and acetate via the butyrate kinase or butyryl-CoA:acetate CoA-transferase (BCoAT) pathway (72). Butyrate could help improve colon motility. In a rat model of IBS, exogenous luminal infusion of 5 mM butyrate
accelerated the colonic transit time (73). Additionally, butyrate has been shown to have anti-inflammatory properties and to support the integrity of the gut barrier, which could help alleviate IBS symptoms (74). It was found that the relative abundance of *Lachnospiraceae* was significantly lower in patients with IBS compared to healthy controls (75), and that this reduction was associated with more severe IBS symptoms. In another rat model study, the majority of taxa belonging to *Lachnospiraceae* were reduced in rats under stress, together with the downregulation of expression of occludin, which is essential for maintaining the integrity of the gut barrier (76). The authors further confirmed a positive correlation between four *Lachnospiraceae*-related ASVs and occludin expression. By supplementing those rats with another species belonging to *Lachnospiraceae*, namely *Roseburia hominis*, decreased mRNA expression of occludin was prevented and butyrate production was restored. Taken together, lower levels of *Lachnospiraceae* contributed to elevated IBS symptoms, which was possibly due to reduced butyrate production. Among the five distinct genera including *Lachnospiraceae* ND3007 group, *Lachnospira*, *Fusicatenibacter*, *Dorea*, and [*Ruminococcus* gauvreauii] group, between subjects with- or without clinical improvement at baseline observed in our study, metagenomic analyses have identified *Lachnospira* and *Dorea* as the main genera within the *Lachnospiraceae* family (77). *Lachnospira* is strongly associated with high fibre diets and is involved in the production of short-chain fatty acids (SCFAs) (78), which have beneficial effects on intestinal barrier integrity and gut-brain interaction via the microbiota-gut-brain axis (79). For instance, a 4-week starch- and sucrose-reduced diet (increased fibre and other carbohydrates) intervention in IBS patients increased the relative abundance of *Lachnospira* and another genus from the *Lachnospiraceae* family, namely *Lachnospiraceae* UCG-001, which was correlated with reduced GI symptoms (80). These results suggested that *Lachnospira* had a positive impact on alleviating IBS symptoms. However, it should be noted that not all genera within the *Lachnospiraceae* family exhibit the same characteristics. For example, *Dorea* has been shown to promote intestinal gas production, which can contribute to abdominal discomfort (81). Furthermore, studies have found a higher abundance of *Dorea* in the faecal microbiota of paediatric IBS patients (82), and in the descending colonic microbiota in diarrhoea-predominant IBS patients, compared to respective healthy controls (81). The latter study also observed a positive relationship between the relative abundance of *Dorea* and IBS-SSS scores.
Collectively, the *Lachnospiraceae* family may play a significant role in IBS, although the beneficial and harmful effects vary across genera, underscoring the importance of examining individual genera in studies investigating the role of the microbiome in IBS.

**Baseline observation cohort datasets trained ML model robustly predicted outcomes of subjects in placebo group despite cohort heterogeneity**

The ML model trained with a combined dataset of metadata and microbiota data revealed that the cohort heterogeneity hampered the identification of a robust baseline microbiota signature of subjects with clinical improvement. Despite this, half of the microbial features belonged to the *Lachnospiraceae*. This suggests that a robust baseline microbiota signature of subjects with clinical improvement is characterised by lower relative abundances of a panel of taxa within the *Lachnospiraceae*. The predictive power of the ML model trained on the observation cohort was also confined by the cohort heterogeneity. Similarly, ML-based brain imaging prediction was also limited by cohort heterogeneity (83). Another reason might be the balance of datasets. The performance of an ML model relies to a large extent on the balance of data used for model training. A balanced dataset (outcome ratio $\approx 1$) holds higher potential to perform well, whereas an imbalanced dataset negatively affect the accuracy of class predictions (84). In our study, the ratio between subjects with- and without-clinical improvement was only 0.341 in the observation cohort. Collectively, discrepancies in host and microbiota characteristics and an imbalanced outcome within the cohort, interfered with the prediction of clinical improvement.

Nevertheless, it is remarkable to note that the outcome in 12 out of 13 (92%) patients in the placebo group of the intervention cohort was correctly predicted at baseline from the ML model trained by the observation cohort, while this was only 50% for the group receiving any of the dietary treatments ($n = 50$). IBS is a heterogeneous condition with high response rates in the placebo group. In fact, a meta-analysis of 73 randomised controlled trials in adult IBS patients demonstrated that approximately 35 to 40% of subjects in the placebo group attained a clinical response (10), which is in line with the placebo response rate in our intervention cohort (31%, 4 out of 13 patients responded). The placebo response is still poorly
understood and consequently, high placebo response rates continue to confine the possibility of finding an effective dietary therapy. Therefore, minimising or even predicting placebo response can be highly relevant for clinical practice. The clinical improvement in the observation cohort could be regarded as an arbitrary type, and it has a robust predictive power to the clinical improvement in the placebo group, which suggests that the placebo responders in the intervention cohort share similar features as those who reached arbitrary clinical improvement in the observation cohort. Therefore, our study indicates that placebo response may be accurately predicted based on longitudinal observations and this may assist in leveraging the placebo response to optimise therapeutic response rates in the management of IBS via ML.

**Strengths and limitations**

The strength of our study is that it involved two IBS cohorts from the same area in the Netherlands, and that all procedures, including timepoint of sampling, laboratory analyses and questionnaire procedures were identical. This gave us the unique opportunity to compare and contrast the two cohorts without suffering from demographic and technical confounders. This also allowed for accurate ML modelling to perform cross-cohort prediction of subjects who would achieve a clinical improvement.

Our study also had a few limitations, which include the fact that the number of (placebo) subjects was relatively small, although sufficiently powered for the primary objective (i.e., determining the bifidogenic effects of a 4-week dietary supplement intervention). Moreover, despite the nearly identical demographic features and identical procedures, we observed clear cohort heterogeneity, which is a general feature of studies with cohorts that is difficult to interpret and seems impossible to overcome. It should also be noted that these two trials were executed in different seasons (around June vs. around November), which might explain part of the differences and should be considered in future studies. Nevertheless, although the prediction accuracy was hampered due to this cohort heterogeneity, the ML approach managed to predict the outcome of 12 out of 13 (92%) subjects in the placebo group in the intervention cohort using the model trained by the observation cohort and thereby showing that placebo responders during an intervention share similar features with those that show arbitrary variation over time.
Conclusions

In conclusion, our study validated the robustness and significant importance of the paradigm of classifying clinical improvement by more than 50 points reduction in IBS-SSS scores. Despite cohort heterogeneity in host- and microbiota-based characteristics, a consensus of a higher IBS-SSS score and lower relative abundance of a panel of genera within the Lachnospiraceae family was identified in IBS patients who would achieve a clinical improvement after four weeks. By training the ML model with the baseline host- and microbiota-derived data from the observation cohort, clinical improvement outcome of patients in the placebo group could be robustly predicted at baseline. This offers great potential for clinical practise as potential non-placebo responders may already be selected prior to the intervention and thereby improving the determination of treatment efficacy.

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Author contributions

The author contributions were as follows: Z.L. and E.G.Z. conceived the original idea; M.v.d.B. executed the intervention cohort study and collected questionnaire data; Z.L. and T.W. acquired the microbiome data from the intervention and observation cohort study, respectively; B.J.M.W. supervised patient recruitment, data and sample collection; Z.L. performed all the data analysis and made all the figures and tables; K.K. and D.M.H. assisted in the machine learning algorithms development; E.G.Z. contributed substantially to the data interpretation; B.J.M.W., N.d.W., H.S., and E.G.Z. jointly supervised the work; Z.L. wrote the original draft. All authors critically revised the manuscript.

Competing interests

The authors declare that they have no competing interests.
Supplementary materials

Set-up of the intervention cohort study

Study participants: This study was conducted at Wageningen University & Research, in the Netherlands. Subjects were recruited using the in-house subject database, advertisements on social media like Facebook and Instagram, and via the website and social media of the Dutch IBS patient association. All subjects willing to participate in the study signed the informed consent form. Inclusion criteria were: IBS patients meeting the Rome IV criteria, age between 18–65 years, and BMI between 18.5–30 kg/m². The main exclusion criteria were: having GI or other relevant diseases, history of intestinal surgery, antibiotic treatment in the 3 months before the study, use of medication or irregular dietary supplements that could interfere with the study outcomes, or following a FODMAP-restricted diet. Eligibility was assessed using an online screening questionnaire.

Study design and procedures: A randomised, double-blind, placebo-controlled parallel intervention study was conducted, with a total duration of four weeks. Subjects completed daily questionnaires via an EMA app (LifeData, LLC), to report information about their stool frequency, stool consistency by using the Bristol Stool Scale, and IBS-related complaints such as bloating, flatulence, and abdominal cramping. Furthermore, supplement compliance and medication intake was reported. At baseline and after the four weeks of intervention subjects completed a food frequency questionnaire (FFQ), a validated questionnaire to assess symptom severity (IBS-SSS), the validated 34-item Irritable Bowel Syndrome Quality of Life questionnaire (IBS-QoL), and the validated screening Hospital Anxiety and Depression score (HADS). Faecal samples were collected at baseline and after 4 weeks of intervention. After collection, the samples were immediately frozen at home and transported to the research facility on dry ice within 14 days. Subsequently, the faecal samples were stored at −80 °C until further analysis.

Dietary intervention products: The four tested dietary compounds were Chondroitin sulfate (Bioiberica S.A.U., Spain), Pea Inner Fibre I50M (Roquette, France), Resistant starch (Ingredion, USA), and Protein hydrolysate cow milk (Ingredia, France). The placebo compound consisted of Maltodextrin (GLUCIDEX® IT 19 P, Roquette, France). Each of these compounds was consumed twice a day, one sachet in the morning and one sachet in the evening together with two spoons of
banana compote. The Pea Inner Fibre contained 10 g fibre per day and resistant starch contained 20 g fibre per day. Because a sudden increase in fibre might lead to GI complaints, subjects in all arms had a five day run-in period where they consumed only one sachet, followed by both sachets for the remainder of the four weeks. The Chondroitin sulfate compound arm consisted of a daily dose of 1.2 g and the Protein hydrolysate cow milk arm consisted of a daily dose of 300 mg. Both compounds were mixed with Maltodextrin to fill up the dosages to equal 10 g. The second dose of 10 g, which was consumed in the evening, consisted of the placebo compound for both arms.

**Flow diagram:**

Figure S1 Fraction of subjects with- and without clinical improvement within each severity group at baseline. Number and percentage of subjects with- and without clinical improvement in the (A) intervention cohort and (B) observation cohort. Significance between severity groups was tested by Fisher’s exact test. Green: clinical improvement (+), red: clinical improvement (−).
Figure S2 Linear regression between IBS-SSS reduction and baseline IBS-QoL. Data of IBS-SSS reduction and baseline IBS-QoL in each subject. A smooth line with 95% confidence level interval (shaded area) for predictions from a linear model was added. Linear regression was performed via fitting a linear model to the data from each cohort. $r$ quantifies the strength of the linear relationship, $R^2$ represents the proportion of variation in IBS-SSS reduction explained by baseline IBS-QoL, and $p$ represents the significance of linear regression. Blue: intervention cohort, yellow: observation cohort.
Figure S3 Relative abundances of genera that differ in relative abundance between T0 and T1. Data of relative abundance at T0 and T1 were visualised with boxplots showing median and interquartile range. Violin plots added rotated kernel density plot on each side, which showed the full distribution of the data. Data at T0 and T1 within each subject were connected by grey lines. Significance was tested using paired samples Wilcoxon test. Green: clinical improvement (+), red: clinical improvement (−).
Figure S4 Fraction of detectable methanogen carriers at baseline. The fractions of detectable methanogen carriers at baseline in the (A) intervention cohort and (B) observation cohort. Subjects who had detectable levels of methanogens (i.e., *Methanobrevibacter*) in the faeces at baseline (T0) were classified as a carrier. The difference on fraction between clinical improvement (+) and clinical improvement (−) were compared with Fisher’s exact test.
Figure S5 Baseline relative abundances of distinct genera in subjects without clinical improvement in two cohorts. Data of relative abundance at the genus level were visualised. Each dot represents the data from each subject. In each group, the boxplot was added with horizontal line inside the box representing the median, and the lower and upper ends correspond to the first and third quartiles (the 25th and 75th percentiles). Significance was tested via Mann-Whitney U test. Blue: intervention cohort, yellow: observation cohort.
Figure S6 Relative abundances of microbial taxa at order level of subjects in the observation cohort. Relative abundances of predominant taxa at order level were visualised. Subjects were ranked in ascending order of relative abundance of Lachnospirales from left to right. Lachnospirales had an average relative abundance of 34.5%, followed by Oscillospirales (25.4%).
Table S1 Demographic characteristics of subjects in two cohorts

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</table>
Data were shown as mean ± standard deviation when normally distributed or median [interquartile range] when skewed. Differences between T0 and T1 were tested using paired sample t-test or Wilcoxon test when skewed. Differences between clinical improvement groups or cohorts were tested with independent sample t-test or Mann-Whitney U test when skewed. Abbreviations: BMI: body mass index, HADS: hospital anxiety and depression score, IBS-SSS: IBS severity scoring system, IBS-QoL: IBS quality of life.
Table S2 Faecal microbiota alpha diversity metrics including inverse Simpson and phylogenetic diversity

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<th>Cohort</th>
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<th>p value</th>
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<td>To</td>
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Data are shown as median [interquartile range]. Differences between T0 and T1 were tested using paired samples Wilcoxon test, while differences between clinical improvement groups or cohorts were tested with Mann-Whitney U test.
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* indicates significant difference.
### Chapter 3

#### Cohort

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#### Time

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$p$ values and statistics for the comparison of intervention and observation cohorts.
### Microbiota-Based Prediction of IBS Placebo Response

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| g__Eubacterium_coprostanoligenes_group | 0.93 [0.66-1.49] | 0.98 [0.57-1.54] | 0.66 [0.39-1.18] | 0.75 [0.25-1.18] | 0.85 [0.36-1.23] | 1.16 [0.52-1.04] | 1.21 [0.79-1.49] | 1.38 [0.95-1.95] | 0.91 4 [0.26 5 0.29 0.34 0.08 0.16 0.61 0.0 0.06*]
| g__Lachnospiraceae ND3007_group | 0.56 [0.25-0.86] | 0.5 [0.23-0.73] | 0.95 [0.71-1.38] | 0.96 [0.33-1.48] | 1.14 [0.67-1.99] | 1.49 [1.06-1.86] | 1.01 [0.62-1.62] | 1.05 [0.3-0.5-0.5] | 0.91 4 [0.26 9 0.52 0.62 0.83 0.04* 0.0 0.71 6*]
| g__Erysipelotrichaceae UCG-003 | 0.99 [0.37-1.48] | 0.57 [0.17-1.16] | 1.05 [0.56-1.42] | 0.75 [0.46-1.73] | 0.8 [0.04-1.34] | 0.57 [0.26-1.28] | 0.46 [0.29-0.93] | 0.65 [0.18-1.41] | 0.11 4 [0.3 0.63 1 0.27 0.27 0.66 0.43 0.03 26*]
| g__Holdemanella | 0.1 [0-1.72] | 0 [0-1.63] | 0 [0-0.74] | 0 [0-0.26] | 0.06 [0-0.89] | 0.13 [0-0.86] | 0 [0-1.33] | 0 [0-1.49] | 0.66 4 [0.19 0.44 0.63 0.3 0.61 0.98 0.32 0.62 0.06*]
| g__Ruminococcus_torques_group | 0.56 [0.34-1.09] | 0.55 [0.35-0.95] | 0.84 [0.42-1.15] | 0.7 [0.39-0.97] | 0.7 [0.3-1.08] | 0.87 [0.37-0.87] | 0 [0.3-1.05] | 0.49 [0.3-1.05] | 0.15 7 [0.33 0.06 0.3 0.62 0.05 0.95 0.35 0.85 0.75 0.19 0.43*]
| g__Alistipes | 0.59 [0.26-1.08] | 0.45 [0.19-0.76] | 0.59 [0.25-0.79] | 0.47 [0.28-0.68] | 1.11 [0.31-1.69] | 1.77 [0.39-2] | 0.5 [0.27-0.96] | 0.72 [0.38-1.38] | 0.72 7 [0.37 0.20 0.20 0.08 0.78 0.11 0.11 0.99 0.99 0.75 0.00]
### Microbiota-Based Prediction of IBS Placebo Response

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Intervention cohort (IC, $n = 63$)</th>
<th>Observation cohort (OC, $n = 55$)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
<td>Clinical improvement (+) (IC (+), $n = 28$)</td>
<td>Clinical improvement (+) (OC (+), $n = 14$)</td>
<td>Clinical improvement (+) (IC (+), $n = 14$)</td>
</tr>
<tr>
<td><strong>Time</strong></td>
<td>To</td>
<td>T1</td>
<td>To</td>
</tr>
<tr>
<td><strong>Comparison</strong></td>
<td>CI (+)</td>
<td>CI (−)</td>
<td>CI (+)</td>
</tr>
<tr>
<td><strong>g__Phascolarctobacterium</strong></td>
<td>0.37</td>
<td>0.26</td>
<td>0</td>
</tr>
<tr>
<td><strong>erium</strong></td>
<td>[0-1.11]</td>
<td>[0-1.15]</td>
<td>[0-1.31]</td>
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<tr>
<td><strong>g__Streptococcus</strong></td>
<td>0.29</td>
<td>0.33</td>
<td>0.32</td>
</tr>
<tr>
<td><strong>NK4A214_group</strong></td>
<td>[0.09-0.85]</td>
<td>[0-0.84]</td>
<td>[0-1.09]</td>
</tr>
<tr>
<td><strong>g__Oscillospiraceae</strong></td>
<td>0.26</td>
<td>0.29</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>NK4A136_group</strong></td>
<td>[0.12-0.45]</td>
<td>[0-0.61]</td>
<td>[0-1.05-0.4]</td>
</tr>
<tr>
<td><strong>g__Methanobrevibacter</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>g__Lachnospiraceae</strong></td>
<td>[0-0.45]</td>
<td>[0-0.64]</td>
<td>[0-0.6]</td>
</tr>
<tr>
<td><strong>g__Romboutsia</strong></td>
<td>0.52</td>
<td>0.56</td>
<td>0.84</td>
</tr>
<tr>
<td><strong>NK4A136_group</strong></td>
<td>[0.24-0.93]</td>
<td>[0-1.15]</td>
<td>[0.44-1.24]</td>
</tr>
<tr>
<td><strong>g__Clostridium_sens</strong></td>
<td>0.29</td>
<td>0.16</td>
<td>0.23</td>
</tr>
<tr>
<td><strong>u_stricto1</strong></td>
<td>[0-0.77]</td>
<td>[0-0.55]</td>
<td>[0-0.63]</td>
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<td><strong>f__Muribaculaceae</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>g__Unclassified</strong></td>
<td>[0-0.85]</td>
<td>[0-0.93]</td>
<td>[0-0.38]</td>
</tr>
<tr>
<td><strong>g__Romboutsia</strong></td>
<td>0.38</td>
<td>0.49</td>
<td>0.39</td>
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</tbody>
</table>

**p** values are given for significant changes in microbial abundance.
<table>
<thead>
<tr>
<th>Cohort</th>
<th>Intervention cohort (IC, n = 63)</th>
<th>Observation cohort (OC, n = 55)</th>
<th>( p ) value</th>
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<tr>
<td>Group</td>
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<td>Clinical improvement (-) (CI (-), ( n = 35 ))</td>
<td>Clinical improvement (+) (CI (+), ( n = 14 ))</td>
</tr>
<tr>
<td></td>
<td>IC</td>
<td>IC</td>
<td>OC</td>
</tr>
<tr>
<td>Time</td>
<td>T0</td>
<td>T1</td>
<td>T0</td>
</tr>
<tr>
<td></td>
<td>CI</td>
<td>CI</td>
<td>CI</td>
</tr>
<tr>
<td></td>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td></td>
<td>0.14 [0.07-0.72]</td>
<td>0.16 [0.08-0.87]</td>
<td>0.11 [0.07-0.75]</td>
</tr>
<tr>
<td>g__Clostridia_UCG-014_f__ unclassified__</td>
<td>0.31 [0.12-0.76]</td>
<td>0.3 [0.13-0.84]</td>
<td>0.46 [0.05-0.72]</td>
</tr>
<tr>
<td>g__Oscillospiraceae__</td>
<td>0.38 [0.16-0.99]</td>
<td>0.4 [0.15-0.72]</td>
<td>0.7 [0.21-0.75]</td>
</tr>
<tr>
<td>g__Parabacteroides__</td>
<td>0.36 [0.0-0.58]</td>
<td>0.28 [0.21-0.41]</td>
<td>0.28 [0.22-0.66]</td>
</tr>
<tr>
<td>g__Butyricicoccus__</td>
<td>0.25 [0.0-0.37]</td>
<td>0.31 [0.0-0.69]</td>
<td>0.57 [0.24-1.12]</td>
</tr>
<tr>
<td>g__Lachnospira__</td>
<td>0.33 [0.1-0.69]</td>
<td>0.38 [0.0-0.64]</td>
<td>0.48 [0.29-0.7]</td>
</tr>
<tr>
<td>g__[Ruminococcus]<em>gauvreauii_group</em>_</td>
<td>0.33 [0.1-0.69]</td>
<td>0.38 [0.0-0.64]</td>
<td>0.48 [0.29-0.7]</td>
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**Microbiota-Based Prediction of IBS Placebo Response**

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Intervention cohort (IC, n = 63)</th>
<th>Observation cohort (OC, n = 55)</th>
<th>p value</th>
</tr>
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<td></td>
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<td>Clinical improvement (+) (n = 14)</td>
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<td></td>
<td>Clinical improvement (−) (n = 35)</td>
<td>Clinical improvement (−) (n = 41)</td>
<td></td>
</tr>
<tr>
<td>Group</td>
<td>To</td>
<td>T0</td>
<td>IC</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>T1</td>
<td>IC</td>
</tr>
<tr>
<td></td>
<td>T0</td>
<td>T0</td>
<td>OC</td>
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<tr>
<td></td>
<td>T1</td>
<td>T1</td>
<td>OC</td>
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<tr>
<td>Time</td>
<td>To</td>
<td>To</td>
<td>IC</td>
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<tr>
<td></td>
<td>T1</td>
<td>T1</td>
<td>IC</td>
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<tr>
<td></td>
<td>T0</td>
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<td>OC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OC</td>
</tr>
</tbody>
</table>

| g__Barnesiella | 0.38 [0.09-0.69] | 0.24 [0-0.6] | 0.28 [0-0.66] | 0.28 [0-0.66] | 0.43 [0.19-0.48] | 0.35 [0.07-0.78] | 0.36 [0.11-0.94] | 0.49 [0.11-1.12] | 0.25 0.19 0.67 0.67 0.29 0.88 0.93 0.21 |
| g__Paraprevotella | 0 [0-0.48] | 0 [0-0.52] | 0 [0-0.48] | 0 [0-0.95] | 0 [0-0.62] | 0 [0-0.41] | 0 [0-0.41] | 0 [0-0.46] | 0.01 0.94 0.62 0.89 0.48 0.61 0.87 |

Taxa selection was based on average relative abundance above 0.5% across the whole datasets. Data were shown as median [interquartile range]. Differences between To and T1 were tested using paired samples Wilcoxon test, while differences between clinical improvement groups or cohorts were tested with Mann-Whitney U test.
References


Chapter 3


43. Tin Kam H. Random decision forests, p 278-282. In (ed),


Chapter 3


Chapter 4

Impact of Donor Individuality, Faeces Collection Date, and Culture Medium Type on Microbiota Composition and Metabolic Activity in Human Faecal Batch Culture

Zhuang Liu*, Jacoline Gerritsen, Hauke Smidt, Erwin G. Zoetendal

* Corresponding author

A slightly adapted version is under review in Applied and Environmental Microbiology.
Abstract

**Background:** Faecal batch culture (FBC) studies often rely on a single faecal sample collection and the use of one type of medium for cultivation, bringing challenges to the interpretation of results and the comparison between studies. **Methods:** This study investigated the impact of donor individuality, faeces collection date, and culture medium type on microbiota composition and metabolic activity in an FBC setting with the fibre polydextrose (PDX) as carbon and energy source. FBCs were inoculated with faecal microbiota from three healthy donors sampled at three different days (day 1, 2 and 30), using either basal or rich culture medium with PDX as carbon source. Microbiota composition and metabolic activity were determined after 0, 6, 12, and 24 h of incubation. **Results:** Microbiota composition variation explained by donor individuality dropped from 51% to 16% during incubation, while that explained by medium and PDX supplementation increased from 0% to 17% and 20%, respectively. Independent of the medium, the genera *Erysipelotrichaceae UCG-003, Blautia* and *Fusicatenibacter* were stimulated by PDX supplementation. In basal medium *Bacteroides* and *Anaerostipes* grew better, whereas *Bifidobacterium, Faecalibacterium* and *Megasphaera* grew better in rich medium. Metabolite variation was explained up to 50% by PDX supplementation during incubations, with butyrate being produced at the highest concentrations among all metabolites. Faeces collection date explained less than 3% of the variation in both microbiota and metabolite composition. **Conclusion:** Donor individuality had the most profound impact on microbiota succession while medium and PDX supplementation had larger impacts on metabolic activity in FBCs in this study. We recommend that variations in medium and donor samples should be considered when planning and interpreting in vitro incubation studies.

**Keywords:** human gut microbiota; in vitro; batch culture; donor individuality; faeces collection date; culture medium type; polydextrose; fibre
Introduction

The human gastrointestinal (GI) tract is residence to trillions of microbes (mainly bacteria) that are commonly referred to as the “gut microbiota”, which is crucial to overall host health and disease (1, 2). The composition of the gut microbiota varies widely among individuals, and many factors such as host genetic background (3), antibiotic exposure (4), dietary habits (5), geography (6), and age (7) contribute to its diversity. Over the past decades, much progress has been achieved thanks to the development of molecular, mostly DNA-based approaches, with a phylogenetic framework provided by 16S ribosomal RNA (rRNA) gene sequencing (8), and functional insights unravelling by metagenomics (9, 10) as well as cultivation of pure and defined mixed (faecal) cultures (11). However, most in vivo studies are, in fact, a snapshot of which microbial groups are there, and how abundant they are. The lack of mechanistic insights often limits our understanding of the functional role of the different microbes. To this end, in vitro models can to some extent fill the gap between in vivo studies and single and defined mixed culture studies (11). Furthermore, in vitro models also allow the investigation of mechanistic effects of different dietary components on microbiota composition and metabolic activity independent of the host, making them cost-effective alternative approaches for gut microbiota research.

A range of in vitro models has been developed to mimic colonic fermentation, ranging from simple batch culture models to sophisticated multi-stage continuous culture models, and to artificial digestive systems (12). Among those models, the faecal batch culture (FBC) model is particularly employed to study production of short-chain fatty acids (SCFAs) and other metabolites from dietary compound fermentation by gut microbiota due to its low cost and simple settings (13). A recent study from our group reported good reflection of in vivo observations by FBC by comparing clinical intervention data and FBC data with respect to carbohydrate degradation (14). However, there are many choices to be made in the experimental set-up of FBCs that can influence compositional dynamics and activity of the microbiota that will be observed. For example, for running an FBC model study usually a single time-point faeces sample is used, bringing challenges to the interpretation of results and the comparison between studies as variation within an individual over time could be sometimes substantial (15).
established inter-individual variability in microbiota composition is often not taken into account as it is common practice to pool faeces from different donors as inoculum (16, 17). In addition, the consequence of the choice of cultivation medium must not be underestimated as differences in e.g., nutrient availability can have a drastic effect on the selectivity of a given medium for microbial growth. This selectivity is not only determined by variations in nutrient composition, but also in nutrient load (18, 19). Last but not least, the methods used for sampling and cryopreservation of faeces will determine which microbes will survive these procedures, notably when subjects are requested to collect samples at home. The gut microbiota contains a wide variety of microbes with different survival rates when exposed to oxygen varying from strict anaerobes to facultative anaerobes. Hence, several protocols have been developed to optimise these procedures in order to maximise recovery of viable microbes (20). Although complete preservation of all viable microbes in the original sample will be challenging, a recent study showed that fast introduction of anoxic conditions to faecal samples after defecation resulted in limited loss of viability (14).

In this study, our aim was to elucidate the impact of donor individuality, faeces collection date and culture medium type on microbiota composition and metabolic activity in an FBC setting. We used the fibre polydextrose (PDX), as model carbon and energy source as previous studies have shown that it stimulates the growth of a consortium of different microbes (21, 22), the composition of which differed between subjects (23). Using faecal microbiota from three healthy adult donors, this study aimed to answer the following three research questions: 1) to what extent are microbial activity (SCFAs, gaseous metabolites, pH) and microbiota composition in FBCs with PDX as carbon source affected by donor individuality, faeces collection date and culture medium type; 2) which groups of bacteria are stimulated in a medium- and PDX-dependent manner; 3) how does microbial viability vary in inocula prepared from faeces collected at different dates.

**Materials and Methods**

**Faecal material collection and storage**

Faecal material was obtained from three healthy donors at three different days (Fig. 1). Subjects with an intake history of antibiotics six months and pre- or
probiotics one month prior to the first sampling were excluded. Three healthy young female adults were recruited. No dietary restrictions or recommendations were given prior to sampling. The donors provided their faecal material at two successive days (day 1 and 2) and one month later (day 30) for this study (Fig. 1A). An informed written consent was obtained from each donor. Each donor defecated into a user-friendly and hygienic stool collection device, the Fecotainer (Excretas Medical BV, Enschede, the Netherlands). To create anoxic conditions during transportation, Anaercult® A mini (Merck KGaA, Darmstadt, Germany) was activated with 10 mL nuclease-free water and placed inside the device. Together with the device, two opened AnaeroGen (AnaeroGen™ 3.5 L Sachet, Thermo Scientific, Waltham, Massachusetts, US) bags were put into the transportation box (AnaeroPack™ 7.0 L Rectangular Jar, Thermo Scientific). Afterwards, the faecal material was transported to the laboratory within 3 h (14). The device was opened inside an anaerobic chamber filled with 96% nitrogen and 4% hydrogen. A portion of around 5 g faeces was

Figure 1 Schematic overview of the study set up. (A) faeces collection, (B) glycerol cryopreservation and storage of faecal inoculum, concurrent with microbial viability test at pre- and post-storage, (C) in vitro incubation and (D) sample collection and measurement. Abbreviations: CON: control, PDX: polydextrose, rRNA: ribosomal RNA, SCFAs: short-chain fatty acids, SIEM: standard ileal efflux medium.
instantly frozen at −20 °C for microbiota composition analysis. Another 10 g faeces was mixed with an anoxic solution consisting of 4 mL dialysate (Tritium Microbiologie, Eindhoven, the Netherlands), 19 mL nuclease-free water and 7 g glycerol, resulting in a 25% (w/w) faecal inoculum as previously described (24). The inoculum was then transferred into a serum bottle, sealed with a butyl rubber stopper and aluminium cap inside the anaerobic chamber, and stored at −80 °C (Fig. 1B).

**Microbial viability test**

Before and after the storage at −80 °C, 1 mL of each faecal inoculum was mixed with 2.5 µL of 20 mM propidium monoazide (PMA) dye (Biotium, Fremont, CA, USA). The samples were then incubated in the dark for 5 min, exposed to light in a PMA-Lite™ LED photolysis device (Biotium, Fremont, CA, USA) for light-induced crosslinking of PMA to dsDNA for 15 min at room temperature, and centrifuged at 4 °C at 5,000 × g for 10 min. After discarding the supernatant, the pellets were stored at −20 °C for DNA isolation and microbiota composition analysis.

**Culture medium preparation**

Bicarbonate buffered CP medium (25, 26) served as the basal medium in this study. Per litre of medium, it consists of 0.41 g KH₂PO₄, 0.53 g Na₂HPO₄·2H₂O, 0.3 g NH₄Cl, 0.3 g NaCl, 0.1 g MgCl₂·6H₂O, 0.11 g CaCl₂·2H₂O, 4.0 g NaHCO₃, 0.24 g Na₂S·9 H₂O, 0.5 g L-cysteine, 0.5 g resazurin, 1 mL acid stock solution (50 mM HCl, 1 mM H₃BO₃, 0.5 mM MnCl₂, 7.5 mM FeCl₂, 0.5 mM CoCl₂, 0.1 mM NiCl₂, 0.5 mM ZnCl₂, 0.1 mM CuCl₂), and 1 mL alkaline stock solution (10 mM NaOH, 0.1 mM Na₂SeO₃, 0.1 mM Na₂WO₄, 0.1 mM Na₂MoO₄). After boiling and cooling down, the headspace was exchanged to 80% nitrogen and 20% carbon dioxide. After autoclaving, 1 mL vitamin stock solution (20 mg/L biotin, 200 mg/L nicotinamide, 100 mg/L p-aminobenzoic acid, 200 mg/L thiamine (vitamin B1), 100 mg/L panthotenic acid, 500 mg/L pyridoxamine, 100 mg/L cyanocobalamine (vitamin B12), 100 mg/L riboflavin) was added through a 0.2 µm filter.

2-(N-morpholino) ethane sulfonic acid (MES) buffered standard ileal efflux medium (SIEM, Tritium Microbiologie B.V., Eindhoven, The Netherlands)) (27) served as the rich medium in this study. Per litre of medium, it consists of 50 ml BCO (60 g/L bacto peptone, 60 g/L casein, and 1 g/L ox bile), 16 mL salts solution (156.3 g/L K₂HPO₄, 281.3 g/L NaCl, 28.13 g/L CaCl₂·2H₂O, 0.31 g/L FeSO₄·7H₂O, 0.63 g/L
hemin, 99% porcine), 10 mL 50 g/L MgSO₄, 0.5 g L-cysteine, 1 mL vitamins (1 mg/L menadion, 2 mg/L D(+)-biotine, 0.5 mg/L Vitamin B12, 10 mg/L D(+) pantothenate, 5 mg/L aminobenzoic acid, 4 mg/L thiamine HCl and 5 mg/L nicotinamide adenine dinucleotide free acid), 0.5 g resazurin and 100 mL MES buffer (1M, pH = 6.0). Medium was prepared in the anaerobic chamber according to the manufacturer’s instructions.

**Carbon and energy source for incubations**

PDX, kindly provided by Winclove Probiotics B.V. (Amsterdam, the Netherlands) was used as a carbon and energy source for incubations. It contains a minimal 90% of polymer and an average degree of polymerisation of 12. PDX has a high solubility, allowing the preparation of 50% w/w solution at 20 °C. Sterilised PDX was obtained by filtering the 50% w/w solution through a 0.2 µm filter.

**Inoculation and incubation**

Inoculation and incubation were conducted in CP medium and SIEM, with PDX or nuclease-free water as control (CON) (Fig. 1C). Inoculation was carried out inside of the anaerobic chamber filled with 96% nitrogen and 4% hydrogen. 10 mL incubation vials were used in this study, whereby 5 mL medium was added to each vial. Cryo-conserved faecal inoculum was carefully thawed at 4 °C and added to the incubation vial at 0.1% (w/v) in duplicate. PDX was added at 1% (w/v). After completion, the vial was sealed with a butyl rubber stopper and aluminium cap inside the anaerobic chamber. For CP medium, the headspace gas was subsequently exchanged to 80% nitrogen and 20% carbon dioxide with a final pressure of 1.7 atmospheres. For SIEM, the headspace gas remained unchanged, i.e., 96% nitrogen and 4% hydrogen. Cultures were incubated in the dark at 37 °C statically for 24 h.

**Sample collection**

Samples were collected at t = 0, 6, 12, and 24 h after inoculation (Fig. 1C). Specifically, at each time point, two incubation vials (biological replicate) in each group were used for sample collection (Fig. 1D). Firstly, the cumulative gas production along the incubation was determined by measuring the headspace gas pressure using GMH 3100 Series (GREISINGER, GHM Messtechnik GmbH, Regenstauf, Germany). Secondly, 0.2 mL headspace gas samples were taken for
composition measurement. Thirdly, three aliquots of 1mL culture were then added into 1.5 mL sterilised Eppendorf tubes. After centrifuging at 4 °C at 21,130 × g for 10 min, the supernatant was separated from the pellet. One aliquot of the supernatant was used for pH measurement, the remaining two aliquots of supernatant and pellet were stored at −20 °C for SCFAs measurement and microbiota composition analysis, respectively.

**Metabolic activity parameters**

Gas production was calculated with gas pressure (unit: psi) using the Ideal Gas Equation at 37 °C (28). Headspace gas composition including methane, carbon dioxide and hydrogen was measured using a CompactGC4.0 gas chromatograph (Global Analyser Solutions, Breda, The Netherlands), equipped with a thermal conductivity detector (TCD) and a Carboxen 1010 column. The settings for measuring were: pressure: 325 kPa, valve (injection) oven: 60 °C, column oven temperature: 140 °C, TCD temperature: 110 °C, filament temperature 175 °C, backflush time 30 s. Data were processed by the software Chromeleon (version 7.2.9, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The concentrations of methane, carbon dioxide and hydrogen in the headspace were calculated based on the standard air pressure and the molar volume to convert the measured percentage to mmol/litre culture medium.

pH of the supernatant was measured by using ProLine Qis B210 pH meter (ProSense, Oosterhout, The Netherlands). Concentrations of SCFAs in the supernatant were measured via High-Performance Liquid Chromatography (HPLC, Shimadzu Prominence-I LC2030C-Plus, Shimadzu, Duisburg, Germany using a Shoedex SH1821 column (Showa Denko, New York, USA) and RID-20A refractive index detector (Shimadzu). Before injection, pre-treatment with Carrez A and B was carried out to de-proteinate the samples. Carrez A solution consists of 0.1 M K₄Fe(CN)₆·3H₂O and Carrez B solution consists of 0.2 M ZnSO₄·7H₂O. Both solutions were stored at 4 °C. Briefly, 500 µL of supernatant was mixed with 250 µL cold Carrez A, followed by mixing with 250 µL cold Carrez B. Next, the sample was centrifuged at 4 °C at 21,130 × g for 5 min, and the clear supernatant was collected for measurement. The conditions and settings of the HPLC instrument were as follows: eluent: 0.01 N H₂SO₄, eluent flow rate: 1 mL/min, column oven temperature: 54 °C,
flow rate: 0.8 mL/min, internal standard: 10 mM DMSO. All data were processed by software Chromeleon (version 7.2.9, Thermo Fisher Scientific).

**DNA isolation**

Total DNA was isolated from faecal samples, PMA-treated inocula and collected FBC pellets following previously published protocol (29). Briefly, after resuspending samples with Stool Transfer and Recovery (STAR) buffer (Roche, Basel, Switzerland), the samples were repeatedly bead beaten with beads in MP FastPrep-24 5G (MP Biomedicals, Irvine, CA, USA) at 5.5 ms for 3 × 1 min. DNA was purified from the samples using the Maxwell 16 Tissue LEV Total RNA purification Kit custom-adapted for DNA extraction (cat no. AS 1220, Promega, Madison, Wisconsin, USA) in a Maxwell 16 Mdx instrument (Promega). After purification, the DNA concentration was measured using NanoDrop 2000 (Thermo Scientific, Waltham, Massachusetts, USA).

**Quantitative PCR (qPCR) analysis**

Quantification of the total bacterial 16S rRNA gene copy number was performed on the FBC pellet samples using the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA). The primers used for amplification were 5’-TCCT ACGG GAGG CAGC AGT-3’ and 5’-GGAC TACC AGGG TATC TAAT CCTG TT-3’ (30). Each reaction mixture of 10 µL consisted of 5 µL BioRad iQ SYBR Green Supermix (BioRad), 0.2 µL forward primer (10 µM), 0.2 µL reverse primer (10 µM), 2.6 µL nuclease-free water and 2 µL template DNA (1 ng/µL). qPCR was performed using the following protocol: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and elongation at 72 °C for 15 s. A melting curve from 60 °C to 95 °C in steps of 0.5 °C was added at the end. All qPCRs were performed in triplicates. Data were analysed using Bio-Rad CFX Maestro software version 2.0.

**Microbiota composition analysis**

Microbiota composition was determined by sequencing the V4 region of the 16S rRNA gene using Illumina Hiseq2500 technology, following the same procedure as described previously (14). Briefly, isolated and purified DNA was used for amplification of the V4 region of 16S rRNA gene with barcoded primers 515F (5’-
GTGY CAGC MGCC GCGG TAA-3') (31) and 806R (5'-GGAC TACN VGGG TWTC TAAT-3') (32) in duplicate PCRs. PCR was performed in 50 µL system including 10 µL 5× HF buffer (Thermo Fisher Scientific, Vilnius, Lithuania), 1 µL dNTPs (10 mM, Thermo Fisher Scientific), 0.5 µL Phusion Hot start II DNA polymerase (2 U/µL, Thermo Fisher Scientific), 36.5 µL nuclease-free water (Promega, Madison, WI, USA), 1 µL DNA template (20 ng/µL) and 1 µL sample-specific barcoded primer (10 µM) (33). The PCR product was purified with the CleanPCR kit (CleanNA, The Netherlands), and quantified using the Qubit dsDNA BR Assay kit (Invitrogen by Thermo Fisher Scientific, Eugene, OR, USA). An equimolar mix of purified PCR products was prepared and sent to Novogene (Cambridge, United Kingdom) for sequencing. Raw sequence data were processed using NG-Tax 2.0 with default settings (33, 34). Amplicon Sequencing Variant (ASV) picking and taxonomic assignments were performed using the SILVA 132 database.

**Statistical analysis**

All statistical analyses were conducted in R (version 4.1.1). All figures were generated using ggplot2 R package (35). 16S rRNA gene sequencing read counts were transformed to microbial relative abundance, as implemented in the microbiome R package (36). The absolute abundance of each taxon was calculated by multiplying the relative abundance with the total bacterial 16S rRNA gene copy number. Weighted UniFrac (37) and unweighted UniFrac (38) distance-based principal coordinates analysis (PCoA) was used to visualise microbial community variation at the ASV level. Permutational multivariate analysis of variance (PERMANOVA) was used to test for significant differences between groups, as implemented in the vegan R package (39). Furthermore, at each incubation time point, contribution of each explanatory variable (i.e., donor individuality, faeces collection date, culture medium type and PDX supplementation) to the weighted and unweighted UniFrac based partitioning distance was assessed using the adonis2 function in the vegan package. Redundancy analysis (RDA) was performed on SCFA concentrations using the rda function in the vegan package using stepwise forward and backward model selection. In addition, at each incubation time point, a permutation test was performed to estimate the contribution of each explanatory variable (i.e., donor individuality, faeces collection date, culture medium type and PDX supplementation) to the overall
variation of SCFA concentrations, using the \textit{permu.hp} function in the \textit{rdacca.hp} R package (40).

Differences with respect to metabolic activity parameter and absolute abundance of microbial taxa between groups were analysed by fitting linear mixed-effects models (LMMs) to each dataset with donor individuality, faeces collection date, culture medium type, PDX supplementation, and incubation time as the fixed effects and each duplicate as the random effect using the \textit{lmer} function from \textit{lmerTest} package (41). False discovery rate (FDR) correction based on Benjamini–Hochberg procedure was used to correct for multiple testing. In all cases, (adjusted) $p$ values $\leq 0.05$ were considered statistically significant.

\textbf{Data availability}

Raw sequence data are available at the European Nucleotide Archive under the accession number PRJEB56217.

\section*{Results}

Incubations of faecal microbiota from three different healthy adult donors collected at three different dates were carried out in two different media to elucidate the impact of donor individuality, faeces collection date and culture medium type on microbiota composition and metabolic activity in FBCs with PDX as carbon and energy source. In addition, microbial viability in the inocula was determined to investigate its variation at different collection dates.

\textbf{Growth in two different media with or without PDX as carbon and energy source}

CP medium served as the basal medium, while standard ileal efflux medium (SIEM) served as the rich medium in this study. As expected, in almost all CP medium incubations without PDX microbial growth was not observed as this medium does not contain any available energy source (Fig. S1). Only some growth was observed in the sample of donor 2 and this appeared to be limited to an unclassified member belonging to the family \textit{Enterobacteriaceae}. Since CP medium without PDX does not provide an energy source for growth, we speculate this taxon grew on some residual energy source present in the respective faecal samples. Because of this very
limited growth, samples in the control group (CON) with CP medium were excluded from all downstream analyses. Growth was observed in all other incubations, including SIEM without PDX. Growth in this medium was expected as SIEM contains bacto peptone and casein as nitrogen sources, which can also serve as energy sources for proteolytic microbes.

Impact of donor individuality, faeces collection date, culture medium type, and PDX supplementation on microbiota succession

PERMANOVA revealed a significant contribution of all examined variables to the microbiota succession in the FBCs. For unweighted UniFrac distances (presence and absence as well as phylogenetic relatedness of microbial taxa), the respective contributions were 34.4% for donor individuality, 21.1% for incubation time, 3.6% for PDX supplementation, 2.1% for culture medium type, and 2.0% for faeces collection date, whereas these were 9.9%, 35.1%, 8.9%, 5.4% and 2.5%, respectively, based on weighted UniFrac distances (relative abundance and phylogenetic relatedness of microbial taxa). A clear separation between donor 1 and donors 2 & 3 was observed based on unweighted UniFrac distance calculations, while this was not the case for weighted UniFrac (Fig. 2A, B). The presence of methanogens (i.e., Methanobrevibacter) in donor 1 (Fig. S2A) explained the high contribution of donor individuality to the unweighted UniFrac distances as Methanobrevibacter is phylogenetically distant from all bacteria, and therefore its presence in some of the samples has a strong impact on the phylogenetic distance-based algorithm used in the unweighted UniFrac calculations.
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Specifically, at each incubation time point, donor individuality had a significant contribution ($p = 0.001$) to microbiota variation both for weighted UniFrac (50.49-15.90%) and unweighted UniFrac distances (81.10-30.27%), although the contribution decreased gradually during the incubation (Fig. 2D, E). With a similar trend but lower contribution, faeces collection date could explain 16.02% and 6.91% of the variation based on weighted and unweighted UniFrac distances at 0 h, which decreased to 5.69% and 2.74%, respectively, at 24 h. In contrast, culture medium type had no contribution at 0 h as expected, but its contribution increased to 16.47% (weighted UniFrac) and 15.19% (unweighted UniFrac) at the end of the incubation (24 h). PDX supplementation also displayed increased contribution to 20.06% and 8.59% at 24 h based on weighted and unweighted UniFrac distances, respectively.

**Different microbial taxon groups observed during incubations**

Due to donor individuality, and variations in media and carbon source, total microbial numbers as well as microbiota composition varied drastically between the incubations. Overall, the variation observed was reflected by 10 predominant genera across all incubations (Fig. 3). When looking into the details of their growth individually, different patterns could be identified for specific groups of taxa by fitting linear mixed-effects models (LMMs) to their absolute abundance dynamics (Fig. 4, Table S1).
Figure 3 Microbial succession of the predominant microbial taxa during the incubation. Top 10 taxa at genus level were selected based on the ranking of the average absolute abundance across the entire dataset, other genera are summarised as “others”. Results from duplicates were averaged for visualisation. Abbreviations: CON: control, PDX: polydextrose, SIEM: standard ileal efflux medium.
Independent of whether growth occurred in CP medium or SIEM, in all subjects the genera *Erysipelotrichaceae UCG-003*, *Blautia*, and *Fusicatenibacter* were stimulated by PDX ([Fig. 4, Table S1], [LMMs-PDX: coefficient > 0, \( p < 0.001 \)], [LMMs-SIEM: coefficient > 0, \( p > 0.05 \)]), suggesting a medium-independent PDX stimulation manner. Although an increase in abundance of the genera *Anaerostipes* and *Bacteroides* was observed in all incubations, these taxa grew best in CP medium supplemented with PDX ([LMMs-PDX: coefficient > 0, \( p = 0.008 \) and 0.613, respectively], [LMMs-SIEM: coefficient < 0, \( p = 0.006 \) and \( p < 0.001 \), respectively]) whereas *Bifidobacterium*, *Faecalibacterium* and *Megasphaera* grew best in SIEM supplemented with PDX ([LMMs-PDX: coefficient > 0, \( p < 0.001 \), \( p < 0.001 \) and \( p = 0.028 \), respectively], [LMMs-SIEM: coefficient > 0, \( p < 0.001 \), \( p < 0.001 \) and \( p = 0.001 \), respectively]), suggesting medium-dependent stimulation towards specific taxa. Remarkably, the genus *Megasphaera* was only observed in samples from donor 3, reinforcing the strong impact of individuality in microbial taxa at genus level on the incubations ([Fig. 4]).
Figure 4 Dynamics of predominant microbial taxa during the incubation. Absolute abundances (log10 transformed) of each predominant taxon at genus level were plotted with the size of each circle representing the level, and the colour of each circle representing each taxon group. Blue: PDX stimulated taxa (medium-independent); green: PDX stimulated taxa (CP-preferred); red: PDX stimulated taxa (SIEM-preferred); navy-blue: proteolytic taxa. Abbreviations: CON: control, PDX: polydextrose, SIEM: standard ileal efflux medium.
We did not observe any taxon that specifically grew in SIEM without PDX, but some taxa demonstrated better performance in SIEM compared to CP medium independent of the presence of PDX. One of the important differences between both media is the presence of ox-bile as well as of an organic nitrogen source, i.e., bactopeptone and casein, suggesting that these taxa might engage in proteolytic activity. Taxa stimulated in SIEM included the genera *Coprococcus* and an unclassified genus within the family *Enterobacteriaceae* (Fig. 4, Table S1, [LMMs-PDX: coefficient < 0, \( p = 0.024 \) and 0.075, respectively], [LMMs-SIEM: coefficient > 0, \( p < 0.001 \)]).

**Impact of donor individuality, faeces collection date, culture medium type, and PDX supplementation on metabolic activity variation**

Incubation of faecal microbiota from three different donors collected at three different dates in two different media demonstrated different response to PDX with respect to the metabolic activity parameters (Fig. S3). RDA revealed significant contribution of culture medium type (\( p = 0.012 \)) and PDX supplementation (\( p = 0.002 \)) to the overall variation in SCFA concentrations (Fig. 2C). RDA performed separately for different timepoints showed that the relative contribution of culture medium type gradually decreased after 6 h (from 51.59% at 6 h to 8.31% at 24 h), whereas contribution of PDX supplementation increased over time (from 9.33% at 6 h to 49.94% at 24 h) (Fig. 1F). Although the impact of these variables over time was similar to that observed for microbiota composition using the weighted UniFrac algorithm, PDX supplementation explained 50% of the variability on metabolic output at the end of the incubation, which contrasts to its relatively lower contribution to microbiota compositional observations (<20%) (Fig. 2).

By fitting LMMs to the metabolic activity parameter dynamics, the impact of those variables were tested (Table S2). Indeed, the supplementation of PDX promoted the production of all metabolites measured in this study, including acetate (+4.55 mmol/L, \( p < 0.001 \)), propionate (+0.71 mmol/L, \( p < 0.001 \)), butyrate (+2.05 mmol/L, \( p < 0.001 \)), gas production (+64.30 ml/L, \( p = 0.004 \)), carbon dioxide (+0.72 mmol/L, \( p = 0.008 \)), hydrogen (+0.84 mmol/L, \( p < 0.001 \)), and pH (−0.22, \( p < 0.001 \)) (Fig. S3, Table S2).

Culture medium type had a profound impact on all metabolic parameters measured in this study, except for propionate. Specifically, compared to CP medium, SIEM incubations showed significantly higher concentrations of acetate (+2.67
mmol/L, \( p < 0.001 \)), butyrate (+1.67 mmol/L, \( p < 0.001 \)), hydrogen (+1.55 mmol/L, \( p < 0.001 \)), but significantly less gas (−146.90 ml/L, \( p < 0.001 \)) (Fig. S3, Table S2).

Strikingly, hydrogen exclusively accumulated at 24 h in SIEM plus PDX incubations (Fig. S3F). Although carbon dioxide was also observed, a comparison between SIEM and CP could not be made as the latter one already contains carbon dioxide in the headspace as part of the carbonate buffer used with this medium. This difference in the buffer may also explain the significant lowering of pH (−3.02, \( p < 0.001 \)) during SIEM incubations. No methane production was observed in any of the incubations despite the presence of the genus *Methanobrevibacter* in the samples of donor 1 (Fig. S2A).

A limited impact of donor individuality on metabolic parameters was observed over time, notably towards the end of the incubations (Fig. 2F). Compared to donor 1, it was found that donor 3 incubations had relatively higher acetate (Fig. S3, Table S2, +2.10 mmol/L, \( p < 0.001 \)) and donor 2 had relatively lower propionate (−0.31 mmol/L, \( p = 0.034 \)) concentrations. In line with the compositional observations, the impact of faeces collection date was limited (<8.14%). Significantly lower concentrations of propionate (−0.48 mmol/L, \( p = 0.001 \)) and butyrate (−1.23 mmol/L, \( p = 0.002 \)) were found in samples from incubations with faeces obtained on day 30 compared with day 1, mainly due to the lower microbial growth (Fig. S1) in incubations of faecal microbiota from donor 2 on day 30.

**Differences in viable microbiota composition of faecal inocula before and after cryopreservation**

To determine the fraction of viable microbes in the inocula before and after −80 °C storage, microbial viability was determined with propidium monoazide (PMA), which selectively penetrates only into “dead” cells where it binds to double stranded DNA, inhibiting downstream PCR amplification. Because PMA does not enter viable cells, their DNA is amplified (42).

Before incubations were performed, faecal samples were collected, and subsequently, inocula were prepared and stored at −80 °C as glycerol stocks. To determine whether the storage at −80 °C impacted the viability of microbes and thereby may explain differences in observations between incubations, inoculum samples were treated with PMA before and after cryopreservation to determine the viable fraction. Although there was no significant impact on the presence and absence
of viable microbes based on unweighted UniFrac distances ($p = 0.288$, Fig. 5A), weighted UniFrac distances revealed a significant difference with respect to relative abundance of viable microbiota after the cryopreservation ($p = 0.039$, Fig. 5B). At phylum level, the viable microbiota was predominated by *Firmicutes*, *Bacteroidetes* and *Actinobacteria* (Fig. 5C). The relative abundances of viable *Actinobacteria* ($p = 0.039$) and *Bacteroidetes* ($p = 0.012$) were increased whereas that of *Firmicutes* decreased ($p = 0.008$) (Fig. 5D). In addition, the fraction of viable *Actinobacteria* in the sample collected from donor 2 and 3 at day 30 was drastically lower compared to the other days, especially with respect to viable *Bifidobacterium* (Fig. S2B). Since *Methanobrevibacter* was observed in samples from donor 1, but methane production was not observed, we also checked its viability in the inocula (Fig. S2A). *Methanobrevibacter* was detected in all samples from donor 1 before and after PMA treatment, suggesting that loss of viability cannot explain the absence of methane. Although the absence of hydrogen in the CP medium incubations may explain the lack of methane production, we speculate for the SIEM incubations that there was no niche for methanogenic activity despite the production of hydrogen (Fig. S3F).
Role of Donor Sample and Media on Faecal Batch Culture

A. Unweighted UniFrac

B. Weighted UniFrac

C. Relative abundance of different phyla across different storage conditions and donor samples.

D. Relative abundance of Actinobacteria, Bacteroidetes, and Firmicutes across pre-storage and post-storage conditions.

Donor:
- Donor 1
- Donor 2
- Donor 3

Date:
- Day 1
- Day 2
- Day 30

Storage:
- Pre-storage
- Post-storage

Phylum:
- Actinobacteria
- Bacteroidetes
- Firmicutes
- Others

Statistical significance:
- p = 0.0391*
- p = 0.0117*
- p = 0.00781**
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Using faecal microbiota from three donors, this study aimed to answer the following three research questions: 1) to what extent does donor individuality, faeces collection date and culture medium type explain the variation with respect to metabolite production and microbiota composition in FBCs with PDX as carbon source, 2) which groups of bacteria are stimulated in a medium- and PDX-dependent manner, 3) how does microbial viability vary in faecal inocula collected at different dates.

Donor individuality rather than faeces collection date determined microbiota composition in FBCs

With the restriction imposed by donor individuality, it has always been challenging to draw robust conclusions with respect to the fermentation of a variety of carbohydrates, even the succession of bacteria (43-45). Additionally, although gut microbiota has been considered as a stable ecosystem with strong resilience, the fluctuation caused by, e.g., diet changes, still exists, something that has been ignored in most in vitro studies (46). In our study, by partitioning the different factors that may contribute to microbiota succession, donor individuality was found to be a large determining factor of the variation of microbiota composition in FBCs. In agreement with our findings, An et al. (14) also found that subject individuality explained distinct portions of the overall microbiota composition variation, using the same FBC setting as used in this study, and investigating degradation of various carbohydrates with faecal microbiota from adults and elderly. For FBCs, the microbial composition of the faecal inoculum is the starting point for the microbial succession during
incubation, and it also determines the overall microbiota composition over time. In our study, the presence of *Methanobrevibacter* was only observed in donor 1, and *Megasphaera* only in donor 3. This might partially contribute to the drastically distinct microbiota composition and dynamics. In another FBC study, it was also shown that the response of faecal microbiota to wheat bran and inulin was largely dependent on donor (47). These findings collectively indicated that donor individuality associated microbiota variation is an important contributing factor in FBC studies, which is lost when pooled faecal samples are used as inocula.

In contrast, faeces collection date exerted limited impact, with less than 3% of variation attributed to it. Although the fluctuation of microbiota composition has been followed for example at the resolution of days (46) or weeks (48), relatively few studies took that into account while performing *in vitro* incubations. As found in our study, the fluctuation of microbiota over a short (i.e., one day) or longer period (i.e., one month) was minimal, and it had limited contribution to the variation of microbiota composition and SCFA production.

**PDX supplementation shaped microbial community and activity in a medium-dependent manner**

SIEM has been widely used in studies with batch and continuous fermentation models of faecal microbiota as it represents well the proximal colon environment (e.g., lower pH, higher nutrition load compared to distal colon), where the fibre degradation starts. In comparison, CP medium is a basal medium developed mostly for the isolation and physiological characterisation of pure cultures given its defined ingredient composition. In addition to that, higher pH and the lack of protein in CP medium also to some extent represent the conditions in the distal colon where most easily used substrates are scarce. As for the carbon and energy source, PDX was selected as it showed stimulation of different microbes in an individual-specific manner (21-23). In an *in vivo* situation, it was found that around 10% PDX (0.8 g out of 8 g consumption) ends up in the faeces of healthy individuals (23). Additionally, a colon simulator study demonstrated a sustained degradation of PDX throughout the colon model (49). These findings indicate fermentation of PDX across the whole colon. Therefore, the combination of SIEM and CP medium provides complementary information, and thus can contribute to improved understanding of PDX.
fermentation by microbes, as carbohydrates degrading bacteria have species-specific preferences towards conditions such as pH and available energy source (50, 51).

Starting from 12 h, culture medium type and PDX supplementation also played important roles in the variation of microbiota composition. Despite the differences between media used in this study, most taxa stimulated by PDX were overlapping. Of note, a group of taxa (i.e., Erysipelotrichaceae UCG-003, Blautia and Fusicatenibacter) were dependent on PDX to grow, with limited growth in CON incubations with both media. However, culture medium type also exerted taxa-specific growth support with SIEM-preferred PDX stimulated taxa (e.g., Bifidobacterium) and CP medium-preferred PDX stimulated taxa (e.g., Bacteroides). The pH differences between CP medium and SIEM might contribute to the preference as growth of Bacteroides was favoured in relatively higher (neutral) pH, while Bifidobacterium showed preference towards lower pH (51, 52). Additionally, the growth of Bifidobacterium spp. relies greatly on amino acids or peptides (53), which were available in SIEM rather than CP medium. Most Bacteroides spp. are capable of producing SCFAs via degrading complex carbohydrates (54). These findings collectively indicated that not only energy source determines which taxa successfully grow but also the medium/conditions in which they are offered.

Without PDX, nearly no bacterial growth or metabolic activity were observed in CP medium, whilst a group of genera (e.g., Coprococcus) could still grow in SIEM, regardless of the supplementation of PDX. This indicated that these genera are independent on PDX to grow. In agreement with our finding, Coprococcus was also previously found to be enriched in SIEM without any additional carbohydrates (14). In healthy individuals, the increase in Coprococcus was found to be positively associated with levels of branched-chain fatty acids (BCFAs, e.g., iso-butyrate and iso-valerate), which are the main end product of branched-chain amino acid (BCAA) degradation (55, 56). These findings collectively suggested Coprococcus is involved in protein degradation. Considering the nutrient differences between SIEM and CP medium such as peptone and casein in SIEM, this suggests that Coprococcus is involved in the proteolytic degradation of these substrates.

Although the microbiota composition as well as the compositional dynamics were found to be individual-specific in our study, metabolic activity, e.g., SCFA production showed no significant difference among incubations in our study, indicating a donor-independent SCFA production manner. Similarly, by looking at
the SCFA production from several substrates with faecal microbiota from three
different donors at three different days, another FBC study showed that the amount
and ratio of SCFAs were dependent on the type of substrate rather than the individual
(57). In our study, SCFA production was explained best by culture medium type and
PDX supplementation, with the highest contribution from the latter at 24 h. There is
no doubt that the nutrients provided by the culture medium selectively dictate the
metabolic activity in FBCs (18). Higher concentrations of SCFAs were found in the
rich medium (i.e., SIEM) compared to the basal medium (i.e., CP medium) in our
study. As the main end product of fibre degradation, SCFA concentrations were
elevated with the supplementation of PDX as expected. Not only in in vitro models
(58), colonic fermentation of PDX also induced higher SCFA production in a human
intervention trial, notably acetate and butyrate (59).

In conclusion, our study demonstrated that donor individuality and medium
choice impact the microbiota succession and metabolic activity during FBC when the
same substrate is used as carbon and energy sources. We recommend for future FBC
studies to use individual faecal samples rather than pooling because within the same
medium we saw clear differences in microbiota composition when inoculated with the
same substrate. In addition, we also recommend the use of basal and rich media to
determine which microbes are stimulated by a certain substrate as these may reflect
the variation in conditions along the colon as the proximal colon is richer in nutrients
compared to the distal colon.

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Author contributions

The author contributions were as follows: conceptualisation: Z.L., J.G., H.S.,
writing original draft: Z.L.
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Competing interests

The authors declare that they have no competing interests.
Supplementary materials

Figure S1 Total bacterial 16S rRNA gene copy numbers during the incubation. Results from the duplicates were averaged for visualisation. Abbreviations: CON: control, PDX: polydextrose, rRNA: ribosomal RNA.
Figure S2 Relative abundance of *Methanobrevibacter* and *Bifidobacterium* in fresh faeces and in PMA-treated faecal inocula pre- and post-storage. The relative abundance of (A) *Methanobrevibacter* and (B) *Bifidobacterium* in fresh faeces, as well as in PMA-treated faecal inocula pre- and post-storage. Abbreviations: PMA: propidium monoazide.
Figure S3 Concentrations of SCFAs and pH in the cultures, gas production, concentrations of carbon dioxide and hydrogen in the headspace during the incubation. Concentrations of (A) acetate, (B) propionate, and (C) butyrate in the cultures. (D) Gas production, concentrations of (E) carbon dioxide and (F) hydrogen in the headspace, and (G) pH during the incubation. Red lines represent (SIEM + CON), green lines represent (CP + PDX), and blue lines represent (SIEM + PDX). Methane was not observed in any of the incubations. CON: control, PDX: polydextrose, SIEM: standard ileal efflux medium.
### Table S1 Significance test results by fitting linear mixed-effects models (LMMs) to the data of absolute abundance of predominant genera

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By fitting LMMs to the data of absolute abundance of predominant genera, the significance of the impact of PDX supplementation and culture medium type were tested. The coefficient represents the change in the mean response associated with the given level of variables. Abbreviations: LMM: linear mixed-effects model, PDX: polydextrose, SIEM: standard ileal efflux medium.
Table S2 Significance test results by fitting linear mixed-effects models (LMMs) to metabolic activity parameter data

<table>
<thead>
<tr>
<th>Variables</th>
<th>Level</th>
<th>Coefficient</th>
<th>p value</th>
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<tr>
<td>Acetate</td>
<td></td>
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<tr>
<td>Faeces collection date</td>
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<td>−0.32</td>
<td>0.537</td>
</tr>
<tr>
<td></td>
<td>Day 30</td>
<td>−0.73</td>
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<td>Donor individuality</td>
<td>Donor 2</td>
<td>+0.40</td>
<td>0.440</td>
</tr>
<tr>
<td></td>
<td>Donor 3</td>
<td>+2.10</td>
<td>&lt;0.001</td>
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<tr>
<td>PDX supplementation</td>
<td>PDX</td>
<td>+4.55</td>
<td>&lt;0.001</td>
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<tr>
<td>Culture medium type</td>
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<td>+2.67</td>
<td>&lt;0.001</td>
</tr>
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<td>Propionate</td>
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<td>−0.31</td>
<td>0.034</td>
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<td>Donor 3</td>
<td>−0.14</td>
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<td>−0.30</td>
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<td>PDX supplementation</td>
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<td>+0.72</td>
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By fitting LMMs to the data of metabolic activity parameters, the significance of the impact of faeces collection date, donor individuality, PDX supplementation and culture medium type were tested. The coefficient represents the change in the mean response associated with the given level of variables. Abbreviations: LMM: linear mixed-effects model, PDX: polydextrose, SIEM: standard ileal efflux medium.

<table>
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<tr>
<th></th>
<th>Culture medium type</th>
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<th>PDX supplementation</th>
<th>Culture medium type</th>
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<td><strong>Hydrogen</strong></td>
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References


Chapter 5

In Vitro Screening of Butyrate-Boosting Dietary Fibres to Stimulate Bacterial Butyrate Production in the Intestine of Ulcerative Colitis Patients

Zhuang Liu*, Eleni Tsompanidou, Diana Kusuma, Ben J. M. Witteman, Hauke Smidt, Jacoline Gerritsen†, Erwin G. Zoetendal†

* Corresponding author † Shared last authorship

Manuscript in preparation.
Abstract

**Background:** Short-chain fatty acids (SCFAs) such as butyrate are considered beneficial for human health and are produced by intestinal bacteria during the fermentation of dietary fibres (DF). Intestinal butyrate production capacity is determined by microbiota composition and DF intake and is found to be compromised in active ulcerative colitis (UC) patients. Recently, sustained remission in UC patients, induced by faecal microbiota transplantation, was found to be associated with increased butyrate-producing capacity. Importantly, this sustained remission was only observed in patients with low relative abundance of *Bacteroidetes* in their faeces during active disease. We hypothesised that in UC patients butyrate production could be boosted by specific DF intake, and that intervention efficacy would depend on faecal *Bacteroidetes* levels. **Methods:** *In vitro* batch cultures were inoculated with faecal microbiota from UC patients that were classified as either high *Bacteroidetes* (HB, *n* = 5) or low *Bacteroidetes* (LB, *n* = 6), with a cut-off value of 10% relative abundance. Cultures were supplemented with one of four butyrate-boosting DF prototypes (A: [50% golden kiwi powder + 50% resistant starch], B: [30% golden kiwi powder + 70% resistant starch], C: [40% acacia gum + 40% partially hydrolysed guar gum + 20% resistant starch], D: [45% acacia gum + 15% golden kiwi powder + 40% partially hydrolysed guar gum]) or no additional carbohydrate as blank control. Headspace gas production and composition (i.e., methane, carbon dioxide, hydrogen), SCFA concentrations and microbiota composition (16S ribosomal RNA gene sequencing) were determined after 0, 24, and 48 h of incubation. **Results:** DF prototypes C and D stimulated the highest SCFA production, including butyrate, with the lowest gas production in LB incubations, whereas no differences in SCFA production among the four prototypes were observed in the HB incubations, supporting a *Bacteroidetes* level-dependent butyrate-boosting capacity in UC patients. Moreover, LB incubations led to selective enrichment of a wider range of potential butyrate-producing bacteria (i.e., *Subdoligranulum*, *Megasphaera*, and *Holdemanella*) in addition to *Bifidobacterium* and *Lactobacillus*, while potential inflammation-inducing bacteria (e.g., members of the family *Enterobacteriaceae* and genus *Prevotella*) were selectively enriched in HB incubations. **Conclusion:** (i) Butyrate production during *in vitro* batch incubations with faeces from UC patients
Faecal \textit{Bacteroidetes} Level Determined Response to Fibre in UC

could be stimulated by DF. (ii) Successful increase in butyrate production is dependent on DF mixture composition and faecal levels of \textit{Bacteroidetes}.

\textbf{Keywords:} ulcerative colitis; inflammatory bowel disease; microbiota; batch incubation; dietary fibre; prebiotics; butyrate; \textit{Bacteroidetes}
Ulcerative colitis (UC) is one of the chronic inflammatory bowel diseases (IBD) and is characterised by mucosal inflammation affecting the gastrointestinal tract extending from colon to rectum (1). It is intricately linked to symptoms encompassing abdominal pain, bloody diarrhoea, and urgency to defecate (2). Longstanding UC was shown to increase the risk of colorectal cancer (CRC) by around two-fold in comparison to the general population (3, 4). UC patients have been shown to have an aberrant gut microbiota composition compared to healthy controls, which was often characterised by lower microbial diversity, a lower relative abundance of the phylum Firmicutes, but a higher level of Bacteroidetes, however, it still remains unclear whether the UC-associated aberrant gut microbiota is the cause or consequence of UC (5-7). The clinical course of UC is marked by flare-ups during which symptoms temporarily exacerbate, and remission when symptoms disappear, in which around 50% of patients have a relapse (i.e., entering flare-ups) every year (8). However, the underlying mechanisms are still poorly understood. As a consequence, there is unfortunately no satisfactory therapeutic strategy available although the prevalence and incidence of UC continue to rise globally (2). Therefore, efforts have been focused on inducing remission and preventing relapse of UC (9).

The gut microbiome is shaped by diet, among other factors, and diet has been shown to play an important role in the development and maintenance of UC (10, 11). However, the roles of specific dietary components in UC remain unclear. Among different component groups, it has been shown that long-term DF intake is associated with a lower risk of IBD (12). Short-chain fatty acids (SCFAs) such as butyrate are produced by gut bacteria when they break down food components, especially DF, and SCFAs are considered beneficial for our health (13). Specifically butyrate is associated with beneficial health effects, and extensive evidence has shown its positive effects on UC as it has anti-inflammatory effects and serves as an energy source for colonic mucosal cells (14). Therefore, increased colonic butyrate levels might help ameliorate mucosal inflammation. Most butyrate-producing bacteria belong to the phylum Firmicutes, which is frequently the most dominant phylum in the gut (15). In addition, by quantifying the gene encoding butyryl-CoA: acetate-CoA transferase (BCoAT) that is a key enzyme in butyrate synthesis and reflects butyrate-producing
capacity, compromised butyrate-producing capacity was found in patients with active UC compared to those in remission (16). Therefore, targeting butyrate-producing capacity via DF intervention is a treatment strategy of great clinical application potential. Indeed, the usage of butyrate enema in the treatment of UC exerted therapeutic action (17). Furthermore, targeting butyrate production in the gut, administration of *Plantago ovata* seeds as a rich DF source significantly increased faecal butyrate levels and showed effectiveness equal to 5-ASA (5-aminosalicylic acid; a primary therapeutic regimen for modulating inflammation in IBD patients) in maintaining remission in UC (18). The efficacy of extra direct butyrate supplementation or indirect stimulation of its production implies that restoring butyrate levels would have therapeutic effects on UC.

Nevertheless, there is no one-size-fits-all DF intervention approach as often only a selection of patients responds positively (19-21). The reason for these differences between studies or between individual responses remains speculative. The response to dietary intervention is susceptible to inter-individual variability in gut microbiota composition (22). Remarkably, after receiving faecal microbiota transplantation (FMT), subjects that had sustained remission (so-called ‘responders’) were characterised by increased BCoAT gene levels and relative abundances of butyrate-producing bacteria, and had a baseline microbiota characterised by a relatively low level of *Bacteroidetes* (median [interquartile range]: 3.2 [2.5–7.3] %), which was more similar to that of healthy individuals (4.6 [2.5–13.3] %) (5). In contrast, subjects who did not go into remission, and thus those who did not respond to the FMT (so-called ‘non-responders’), had a relatively high baseline *Bacteroidetes* level (13.3 [4.3–30.1] %). The association between members of the *Bacteroidetes* and UC activity has been previously identified (23). Thus, we speculated that UC microbiota with a lower level of *Bacteroidetes* (less than around 10%) provides more ecological niches for butyrate-producing bacteria (mostly *Firmicutes*) to grow. In addition, IBD patients showed varying responses to around ten different DF subtypes (24), suggesting the intervention efficacy also depends on the type of DFs. Therefore, we speculated that both DF composition and faecal microbiota composition might contribute to the distinct responses. Accordingly, UC-targeted DF candidate selection and identification of concrete microbial biomarkers that enable clinicians to identify patients more likely to respond to specific DF beforehand and push them back to remission is needed.
Among DF subtypes, only soluble and/or fermentable DF can be utilised in the colon by gut microbiota (25). In turn, increased DF consumption also brings excess gas production, which should be avoided as it may lead to undesirable abdominal pain and bloating (26). Of note, a mixture of DFs was shown to outperform an individual DF to promote butyrate production (13, 27). Therefore, a DF mixture with a higher butyrogenic and lower gas-producing (i.e., higher tolerance) capacity would be ideal. Moreover, good tolerance has been reported in humans when consumed at 20-40 g per day (28-30). Intrinsīc fibre extracts that are rich in various type of DFs may be more promising as candidates than pure components as recently reviewed (31). Based on a combination of literature review and unpublished in vitro data (Wincoloe B.V.) on the prebiotic effects, four distinct types of DF were selected based on their butyrogenic and/or acetogenic effects. These DF types were acacia gum, golden kiwi powder, partially hydrolysed guar gum, and resistant starch. Based on this knowledge, we developed four potential DF prototype mixtures consisting of two or three of the abovementioned DFs to favour high butyrate and low gas production.

In this study, we applied in vitro batch incubation of these four different DF prototypes with inocula derived from faecal samples of UC patients with either low or high Bacteroidetes levels. We hypothesised that DF could stimulate butyrate-producing capacity of inocula derived from faecal samples of UC patients, and that efficacy would be dependent on DF mixture composition and faecal Bacteroidetes levels.

**Materials and methods**

**Study setup**

Eleven UC patients were included in this study (Fig. 1A). Faecal samples were collected for calprotectin measurement, DNA isolation and inoculum preparation. Faecal microbiota composition was profiled by 16S ribosomal RNA (rRNA) gene amplicon sequencing using faecal DNA as template. Quantitative PCR (qPCR) was used to quantify the 16S rRNA gene copies of total bacteria and Bacteroidetes. According to the findings of a previous FMT study (5), we chose to set the cut-off value of Bacteroidetes relative abundance to 10%, with >10% defined as high Bacteroidetes (HB, n = 5) and ≤10% defined as low Bacteroidetes (LB, n = 6). Using these faecal samples as inocula, in vitro batch incubation was conducted with four
different DF prototypes or without any additional carbohydrate (blank control) at 37 °C for 48 h (Fig. 1B). Samples for gas production and composition, SCFA

**Figure 1 Schematic overview of the study set up.** (A) Eleven UC patients were recruited to provide faecal samples, which were used for calprotectin measurement, DNA isolation and inoculum preparation. Isolated DNA was subsequently used for faecal microbiota composition profiling (16S rRNA gene sequencing) and quantification of *Bacteroidetes* level (qPCR). (B) *In vitro* batch incubation of inocula derived from faecal samples was conducted with four different DF prototypes or NF water (blank control) at 37 °C for 48 h. (C) Gas production and composition, SCFA concentrations, and microbiota composition were measured for samples collected after 0, 24 and 48 h of incubation.

measurement, and microbiota composition profiling were collected in duplicate after 0, 24 and 48 h of incubation (Fig. 1C).

**Dietary fibre (DF) prototypes**

Four DF prototypes were provided by Wincove B.V. (Amsterdam, The Netherlands). Each prototype was formulated as combination of two or three individual fibre-containing preparations, namely acacia gum (AG) (> 90% DF; Nexira, Rouen, France), golden kiwi powder (GKP) (> 1.9% DF; Livaux™, Anagenix, New Zealand), partially hydrolysed guar gum (PHGG) (> 80% DF; Sunfiber® R, Taiyo GmbH, Gevelsberg, Germany), and resistant starch (RS) (> 60% DF; HI-MAIZE™ 260, Ingredion Germany GmbH, Hamburg, Germany). The composition of the different DF prototypes is summarised in Table 1.

**Table 1 Composition of each DF prototype**

<table>
<thead>
<tr>
<th>DF prototypes</th>
<th>Composition</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>AG</td>
</tr>
<tr>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>40.00%</td>
</tr>
<tr>
<td>D</td>
<td>45.00%</td>
</tr>
</tbody>
</table>

The composition percentages were defined in g/g. Abbreviations: AG: acacia gum, DF: dietary fibre, GKP: golden kiwi powder, PHGG: partially hydrolysed guar gum, RS: resistant starch.

**Faecal sample collection and inoculum preparation**

Due to the low burden and risk for participants, this study was exempted from medical ethical approval by the medical ethical committee of Wageningen University. A total of eleven adult participants (age: 18–60 years, BMI: 18–35 kg/m²) diagnosed with UC based on histological confirmation through endoscopy were recruited from Hospital Gelderse Vallei (ZGV) located in Ede, The Netherlands (Table 2). All subjects provided signed informed consent. A single faecal sample was collected with a faeces collection device (Fecotainer, Excretas Medical BV, Enschede, the
Faecal Bacteroidetes Level Determined Response to Fibre in UC

Netherlands) following the protocol as described previously (32). After collection, the faecal samples were transported to the laboratory within 3 h and homogenised in an anaerobic chamber filled with 96% nitrogen + 4% hydrogen. One aliquot of around 1 g was instantly frozen at −20 °C for calprotectin measurement and another aliquot of around 5 g was stored at −20 °C for DNA isolation. Inocula were prepared with another 10 g faeces, and then stored at −80 °C until inoculation. Briefly, for inoculum preparation, faeces were mixed with 4 mL dialysate (Tritium Microbiologie, Eindhoven, the Netherlands), 19 mL nuclease-free water and 7 g glycerol in the anaerobic chamber, resulting in a 25% (w/w) faecal inoculum as previously described (33).

Table 2 Characteristics of study subjects

<table>
<thead>
<tr>
<th>Subject characteristics</th>
<th>Total (n = 11)</th>
<th>High Bacteroidetes (HB, n = 5)</th>
<th>Low Bacteroidetes (LB, n = 6)</th>
<th>p value (HB vs LB)</th>
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<tbody>
<tr>
<td>Age (years) (mean ± SD)</td>
<td>49.1 ± 14.3</td>
<td>48.4 ± 13.2</td>
<td>49.7 ± 16.3</td>
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<td>Gender (% female)</td>
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<td>63.64%</td>
<td>60.00%</td>
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<tr>
<td>BMI (kg/m²) (mean ± SD)</td>
<td>27.1 ± 5.2</td>
<td>24.8 ± 4.7</td>
<td>29.0 ± 5.2</td>
<td>0.193</td>
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<tr>
<td>Faecal calprotectin level (µg/g)</td>
<td>84.3 ± 66.0</td>
<td>85.0 ± 64.7</td>
<td>83.7 ± 73.3</td>
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<tr>
<td>Inflammation (active/remission)</td>
<td>6/5</td>
<td>3/2</td>
<td>3/3</td>
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<tr>
<td>Faecal Bacteroidetes level (%)</td>
<td>8.7</td>
<td>14.4</td>
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<td>(median [interquartile range])</td>
<td>[4.7–14.1]</td>
<td>[13.8–31.5]</td>
<td>[4.2–5.6]</td>
<td></td>
</tr>
</tbody>
</table>

Continuous data are presented as mean ± standard deviation or median (interquartile ranges) when skewed. Independent sample t-test was performed to test the differences in age, BMI and faecal calprotectin level. Mann-Whitney U test was performed to test the differences in faecal Bacteroidetes level. Fisher’s exact test was performed to compare the proportion of gender and inflammation status. Abbreviations: BMI: body mass index, SD: standard deviation, HB: high Bacteroidetes, LB: low Bacteroidetes.
Faecal calprotectin measurement

The inflammation status of subjects was assessed by the faecal calprotectin level. Around 1 g of frozen faeces was used for the measurement using a sandwich enzyme-linked immunosorbent assay (EK-CAL Calprotectin ELISA, Bühlmann). The cut-off for positive inflammation was set at 50 µg/g according to the manufacturer’s recommendation.

In vitro batch incubation

Standard ileal efflux medium (SIEM, Tritium Microbiologie B.V., Eindhoven, The Netherlands) with 2-(N-morpholino) ethane sulfonic acid (MES) as the buffering system was used in this study (34). Medium was prepared according to the manufacturer’s instructions. One litre of medium consisted of 50 ml BCO (60 g/L bacto peptone, 60 g/L casein, and 1 g/L ox bile), 16 mL salts solution (156.3 g/L di-potassium hydrogen phosphate, 281.3 g/L sodium chloride, 28.13 g/L calcium chloride dihydrate, 0.31 g/L iron (II) sulfate heptahydrate, 0.63 g/L hemin 99% porcine), 10 mL MgSO4 (50 g/L magnesium sulphate), 0.5 g L-cysteine, 1 mL vitamins (1 mg/L menadion, 2 mg/L D(+)-biotine, 0.5 mg/L Vitamin B12, 10 mg/L D(+)pantothenate, 5 mg/L aminobenzoic acid, 4 mg/L thiamine HCL and 5 mg/L nicotinamide adenine dinucleotide free acid), 0.5 g resazurin and 100 mL MES buffer (1M, pH = 6.0).

In addition, each DF prototype was added at 1% (w/v) as the carbon source to the medium, while incubations without any additional carbohydrate served as blank control (Fig. 1B). Faecal inocula were added at 1% (w/v) before incubation. The incubations were carried out in 10 mL vials, each containing 5 mL of medium with an anoxic headspace composed of 96% nitrogen and 4% hydrogen. All cultures were incubated statically at 37 °C for a period of 48 h.

Sample collection and measurements

Samples were collected from all incubation vials at t = 0, 24 and 48 h after incubation (Fig. 1B). At each time point, two incubation vials (biological duplicates) from each DF prototype were used for sample collection (Fig. 1C).

Cumulative gas production was calculated via the Ideal Gas Equation at 37 °C using gas pressure, which was measured by GMH 3100 Series (GREISINGER, GHM
Messtechnik GmbH, Regenstauf, Germany) before sampling (35). Headspace gas composition was measured by taking 0.2 mL headspace gas sample using 1 mL sterilised syringes. Methane, carbon dioxide and hydrogen were measured using the CompactGC4.0 gas chromatograph (Global Analyser Solutions, Breda, The Netherlands), equipped with a thermal conductivity detector (TCD) and a Carboxen 1010 column. The settings were as follows: pressure: 325 kPa, valve (injection) oven: 60 °C, column oven temperature: 140 °C, TCD temperature: 110 °C, filament temperature 175 °C, backflush time 30 s. Data were processed by the software Chromeleon (version 7.2.9, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The concentrations of methane, carbon dioxide and hydrogen were calculated based on the standard air pressure and the molar volume to convert the measured percentage to mmol/litre culture medium.

Subsequently, the vials were opened, and two aliquots of 1 mL culture were collected into 1.5 mL sterile Eppendorf tubes. After centrifugation at 4 °C at 21,130 × g for 10 min, supernatant and pellet were separated and stored at −20 °C for SCFA measurement and microbiota composition analysis, respectively. SCFAs were measured via High-Performance Liquid Chromatography (HPLC, Shimadzu Prominence-I LC2030C-Plus, Shimadzu, Duisburg, Germany) using a Shoedex SH1821 column (Showa Denko, New York, USA) and RID-20A refractive index detector (Shimadzu). The samples were pre-treated with Carrez A and B for deproteination before injection (36, 37). Carrez A solution consists of 0.1 M K4Fe(CN)6·3H2O and Carrez B solution consists of 0.2 M ZnSO4·7H2O. Both solutions were stored at 4 °C. Briefly, 500 µL of supernatant was mixed with 250 µL cold Carrez A, followed by mixing with 250 µL cold Carrez B. Next, the sample was centrifuged at maximum speed (21,130 × g) at 4 °C for 5 min, and the clear supernatant was collected for measurement. The conditions and settings of the HPLC instrument were as follows: eluent: 0.01 N H2SO4, eluent flow rate: 1 mL/min, column oven temperature: 54 °C, flow rate: 0.8 mL/min, internal standard: 10 mM DMSO. All data were processed by Chromeleon.
Furthermore, a weighted SCFA-gas production ratio was calculated to estimate the contribution of DF to health-promoting effects (i.e., SCFA production) and tolerability (i.e., gas production). Briefly, this ratio was calculated by giving weights to each SCFA and gas production within 24 h of incubation using the following equation:

\[
\frac{1}{5} \left( 1 \times \frac{\text{acetate treatment } 0-24h}{\text{acetate blank } 0-24h} + 2 \times \frac{\text{propionate treatment } 0-24h}{\text{propionate blank } 0-24h} + 2 \times \frac{\text{butyrate treatment } 0-24h}{\text{butyrate blank } 0-24h} \right) - \left( \frac{\text{gas production treatment } 0-24h}{\text{gas production blank } 0-24h} \right)
\]

In this equation, SCFA production was considered as a positive value, with a higher weight factor for propionate and butyrate (2) versus acetate (1) since the health-effects of propionate and butyrate are considered more pronounced as compared to those of acetate. Gas production was considered as negative in the equation since strong gas production is problematic in terms of tolerating DF. Each value of the equation was normalised to the value in the blank incubation. Therefore, a higher ratio represents higher SCFA stimulation and/or lower gas production (i.e., higher tolerability).

**DNA isolation**

DNA was isolated from faeces and incubation pellets (obtained through centrifugation as described in the previous section) following a previously published protocol (38). Briefly, after resuspending faeces or pellets in Stool Transfer and Recovery (STAR) buffer (Roche, Basel, Switzerland), the samples were repeatably beaten with beads in an MP FastPrep-24 5G instrument (MP Biomedicals, Irvine, CA, USA) at 5.5 ms for 3 × 1 min. DNA was purified from samples using Maxwell 16 Tissue LEV Total RNA purification Kit customised for faecal DNA extraction (AS1220, Promega, Madison, Wisconsin, USA) in a Maxwell 16 MDx instrument (Promega). The DNA concentration was measured after purification, using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA).

**Quantitative PCR (qPCR)**

To determine the 16S rRNA gene copy numbers of total bacteria and *Bacteroidetes* in faeces, qPCR was performed using the CFX384 Touch™ Real-Time
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PCR Detection System (Bio-Rad, Hercules, California, USA). Each reaction mixture of 10 µL consisted of 5 µL BioRad iQ SYBR Green Supermix (BioRad), 0.2 µL forward primer (10 µM), 0.2 µL reverse primer (10 µM), 2.6 µL nuclease-free water and 2 µL template DNA (1 ng/µL). For total bacteria, the primers used were 331F: 5’-TCCT ACGG GAGG CAGC AGT-3’ and 797R: 5’-GGAC TACC AGGG TATC TAAT CCTG TT-3’, following the protocol: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and elongation at 72 °C for 15 s (39). For Bacteroidetes, the primers used were 934F: 5’-GGAR CATG TGGT TTAA TTCG ATGA T-3’ and 1060R: 5’-AGCT GACG ACAA CCAT GCAG-3’, following the protocol: initial denaturation at 95 °C for 3 min, followed by 39 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s (40). For each reaction, a melting curve from 60 °C to 95 °C in steps of 0.5 °C was added in the end. All qPCRs were performed in triplicates. Data were analysed using Bio-Rad CFX Maestro software version 2.0.

Microbiota composition profiling

Microbiota composition was determined by sequencing the V4 region of the 16S rRNA gene using Illumina Hiseq2500 technology, following the same procedure as described previously (32). Briefly, isolated and purified DNA was used for amplification of the V4 region of 16S rRNA gene with barcoded primers 515F (5’-GTGY CAGC MGCC GCGG TAA-3’) (41) and 806R (5’-GGAC TACN VGGG TWTC TAAT-3’) (42) in duplicate PCRs. PCR was performed in 50 µL system including 10 µL 5× HF buffer (Thermo Fisher Scientific, Vilnius, Lithuania), 1 µL dNTPs (10 mM, Thermo Fisher Scientific), 0.5 µL Phusion Hot start II DNA polymerase (2 U/µL, Thermo Fisher Scientific), 36.5 µL nuclease-free water (Promega, Madison, WI, USA), 1 µL DNA template (20 ng/µL) and 1 µL sample-specific barcoded primer (10 µM) (43).

The PCR product was purified with the CleanPCR kit (CleanNA, The Netherlands), and quantified using the Qubit dsDNA BR Assay kit (Invitrogen by Thermo Fisher Scientific, Eugene, OR, USA). An equimolar mix of purified PCR products was prepared and sent to Novogene (Cambridge, United Kingdom) for sequencing. Raw sequence data were processed using NG-Tax 2.0 with default settings (43, 44). Amplicon sequence variant (ASV) picking and taxonomic assignments were performed using SILVA 138 database. 16S rRNA gene sequence
read count data were transformed to microbial relative abundance, as implemented in the *microbiome* R package (45). Microbial diversity (inverse Simpson) and richness (phylogenetic diversity) were calculated at ASV level, as implemented in the *phyloseq* (46) and *picante* (47) R packages, respectively. Weighted UniFrac (48) and unweighted UniFrac (49) distance-based principal coordinates analysis (PCoA) were used to visualise microbial community variation at the ASV level.

**Statistical analysis**

Numerical variables were presented as means ± standard deviations or medians [interquartile ranges] when skewed. Independent sample *t*-test was performed to compare the difference between HB and LB subjects, or Mann-Whitney *U* tests when data was skewed. Categorical variables were presented as counts or percentages, and Fisher’s exact test was used to test for significant differences. For HB and LB incubations, differences between DF prototypes were tested with multiple independent sample *t*-tests, or Wilcoxon signed-rank tests when data was not normally distributed. False discovery rate (FDR) correction based on the Benjamini–Hochberg procedure was used to correct for multiple testing. For microbiota composition data, permutational multivariate analysis of variance (PERMANOVA) was used to test differences, as implemented in the *vegan* R package (50). All statistical analyses were conducted in R (version 4.2.2). All figures were generated using the *ggplot2* R package (51). In all cases, (adjusted) *p* values ≤ 0.05 were considered to indicate statistical significance.

**Data availability**

Raw sequence data are available at the European Nucleotide Archive under the accession number PRJEB56220.

**Results**

Faecal samples from eleven UC subjects were collected for calprotectin measurement, microbiota composition profiling, *Bacteroidetes* level quantification and inoculum preparation. Using inocula derived from faecal samples, we performed *in vitro* batch incubations with the addition of four different DF prototypes at 37 °C.
for 48 h. Gas production and composition, SCFA concentrations, and microbiota composition were determined after 0, 24 and 48 h of incubation.

Dynamics of gas production, gas composition and SCFA production during the incubation of inocula derived from faecal samples of UC subjects with different DF prototypes

Constant gas production was observed in all incubations (Fig. 2A), with carbon dioxide being the most abundant gaseous metabolite, followed by hydrogen and methane (Fig. 2B). There were no significant differences in cumulative gas production and carbon dioxide concentration between the prototypes at 24 and 48 h (Fig. S1A, B). However, significantly lower hydrogen concentrations were found with DF prototypes C and D, in comparison to DF prototype A at 24 ($p = 0.038$ and $0.007$, respectively) and 48 h ($p = 0.016$ and 0.02, respectively) (Fig. S1C), and lower hydrogen concentration in incubations with DF prototype D compared to those with DF prototype B at 24 h ($p = 0.012$). Notably, methane production was only observed in incubations of faecal inocula derived from faecal samples of four out of eleven subjects with slightly higher production in the DF incubations compared to blank controls (Fig. S2).
Figure 2 Gas production, composition and SCFA production during the incubation of UC subject-derived faecal inocula with different DF prototypes. Data of (A) cumulative gas production, (B) gas composition including CH$_4$, CO$_2$, and H$_2$ and (C) SCFAs including acetate, propionate and butyrate during the incubation. Each dot represents the mean value of biological duplicates. A-D stand for the four DF prototypes used in this study (see Table 1). Abbreviations: SCFAs: short-chain fatty acids.
To determine whether different potentially butyrogenic DF prototypes induced butyrate production by the microbiota of UC subjects, SCFA production over time was determined. Butyrate production was observed in all incubations and was consistently the highest produced SCFA (Fig. 2C). The fact that butyrate production was higher in the DF incubations compared to non-DF blank control incubations indicates that the faecal inocula, derived from faecal samples of UC subjects, harboured butyrate-producing bacteria that utilised the different DF prototypes. Butyrate production was significantly higher with DF prototype D as compared to DF prototype A at 24 and 48 h ($p = 0.04$ and $0.038$, respectively) (Fig. S1D), as well as with DF prototype C compared to DF prototype A at 48 h ($p = 0.022$). With respect to the other SCFAs, acetate concentrations did not significantly differ among any of the prototypes at 24 h, but significantly higher concentrations were observed with DF prototypes C and D as compared to DF prototype B at 48 h ($p = 0.013$ and $0.016$, respectively) (Fig. S1E). Moreover, DF prototypes C and D resulted in significantly higher propionate production compared to DF prototype B at 24 ($p = 0.004$ and $0.007$, respectively) and 48 h ($p = 0.004$ and $0.009$, respectively) (Fig. S1F). DF prototype C also resulted in higher propionate concentrations as compared to DF prototype A at 48 h ($p = 0.042$). In summary, the highest stimulation of total SCFAs (combing acetate, propionate and butyrate) was induced by DF prototypes C and D, both at 24 and 48 h (Fig. S1G). Remarkably, although at lower concentrations compared to the DF prototype incubations, butyrate was also the most abundantly produced SCFA in blank control incubations that were not supplemented with the DF prototypes (Fig. S1C). This suggested that faecal inocula, derived from faecal samples of the UC subjects, were able to produce butyrate without the addition of DF.

**Characteristics of HB and LB subjects**

To determine whether the efficacy of DF-induced butyrate production would be dependent on faecal *Bacteroidetes* levels, subjects were stratified into high *Bacteroidetes* (HB, $n = 5$) or low *Bacteroidetes* (LB, $n = 6$) (Table 2). As expected, HB subjects had significantly higher relative abundance of *Bacteroidetes* (%) compared to that in LB subjects (14.4 [13.8–31.5] vs 4.7 [4.2–5.6], $p = 0.004$). In turn, there were no significant differences with respect to age, gender distribution, BMI, faecal calprotectin level, and inflammation status between HB and LB subjects.
Faecal microbiota composition of HB and LB subjects

There was no significant difference between HB and LB subjects with respect to diversity and richness of faecal microbiota (Fig. S3A, B). PCoA based on unweighted UniFrac (based on presence/absence and phylogenetic relatedness of ASVs) showed no significant difference between the microbiota of HB and that of LB subjects ($p = 0.124$) (Fig. 3A), whilst significant differences were found based on weighted UniFrac distances which consider relative abundance and phylogenetic relatedness of ASVs ($p = 0.003$) (Fig. 3B). This indicated that differences in faecal microbiota composition were mainly attributed to the relative abundance rather than presence/absence of microbial taxa. As expected, this was reflected by the relative abundance of taxa at phylum level since we divided the subjects based on percentage of Bacteroidetes (Fig. 3C). Despite this, at genus level, there were only three genera significantly different between HB and LB subjects, most likely due to the small number of subjects (Fig. S3C).
Faecal *Bacteroidetes* Level Determined Response to Fibre in UC

**Figure 3** Faecal microbiota composition profile. PCoAs based on (A) unweighted UniFrac and (B) weighted UniFrac distances. Samples were visualised with blue dots representing HB subjects and yellow dots representing LB subjects. Significant differences between HB and LB subjects were tested with PERMANOVA. Relative abundance of taxa at phylum level were visualised with stacked bar plots within each subject (C). Subjects were ordered from left to right in descending order of *Bacteroidetes* level. Abbreviations: HB: high *Bacteroidetes*, LB: low *Bacteroidetes*, PCoA: principal coordinates analysis, PERMANOVA: permutational multivariate analysis of variance.
**Differences in microbiota composition between HB and LB incubations with DF prototypes**

Predominant taxa were investigated to characterise their response to different DF prototypes when incubated with inocula from HB and LB subjects. The overall compositional changes described above were mirrored in the changes of several taxa at genus level. In total, 18 bacterial genus-level taxa were found predominant (above 1% on average across all incubations) during the incubation (Fig. 4). Remarkably, some bacterial groups were selectively stimulated when faeces from LB subjects were used as inocula compared to that of HB subjects. Independent of the DF prototype used, the genera *Bifidobacterium*, *Subdoligranulum*, *Megasphaera*, *Holdemanella*, and *Lactobacillus* showed significantly higher relative abundances in LB incubations, while higher relative abundances of a genus-level taxon within the family Enterobacteriaceae and the genera *Ruminococcus 2*, *Prevotella 9*, and *Fusicatenibacter* were observed in HB incubations. Looking specifically at the different DF prototypes, the genera *Bifidobacterium* and *Subdoligranulum* (24 h only) reached significantly higher relative abundances in LB incubations compared to HB incubations when supplemented with DF prototypes A and B. In contrast, no bacterial taxon showed higher relative abundances in HB incubations compared to LB incubations when supplemented with DF prototypes C and D. Moreover, the genus *Blautia* showed higher relative abundances only with DF prototypes C and D in LB incubations, respectively, compared to HB incubations, while the genus *Faecalibacterium* showed slightly but significantly higher relative abundances with DF prototype A in HB incubations compared to LB incubations. Overall, these observations suggested that different microbial taxa in LB and HB incubations were selectively stimulated by specific DF prototypes.
Faecal *Bacteroidetes* Level Determined Response to Fibre in UC

![Graph showing relative abundance of various bacterial species at 24 and 48 hours for different groups.](image)

- **g. Bilidobacterium**
- **g. Faecalibacterium**
- **g. Blautia**
- **f. Enterobacteriaceae_g. unclassified**
- **g. Subdoligranulum**
- **g. Coprococcus_3**
- **f. Lachnospiraceae_g. unclassified**
- **g. Ruminococcus_2**
- **g. Bacteroides**
- **g. [Eubacterium].hallii_group**
- **g. Dorea**
- **g. Megasphaera**
- **g. Prevotella_9**
- **g. Holdemanella**
- **g. Eryisipelotrichaceae_UCG-003**
- **g. Lachnospiraceae_ND3007_group**
- **g. Fusicatenibacter**
- **g. Lactobacillus**

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Differences in metabolite production of HB and LB incubations with different DF prototypes

Differences in gas production and composition between DF prototypes were only found in HB incubations. Specifically, significantly lower gas production was observed with DF prototype C (vs DF prototype A at 24 h) (Fig. 5A) and DF prototype D (vs DF prototypes A and B at 48 h) concomitant with significantly lower hydrogen concentrations with DF prototype D (vs DF prototype A at 24 h) (Fig. 5C). No significant difference regarding carbon dioxide was found (Fig. 5B). With respect to SCFAs, only acetate showed differences among DF prototypes in HB incubations. In detail, DF prototypes C and D induced significantly higher acetate concentrations than DF prototypes A (48 h) and B (24 and 48 h), but higher total SCFA concentrations were only observed with DF prototype C as compared to DF prototype A at 48 h ($p < 0.05$, Fig. 5E, G).
Faecal *Bacteroidetes* Level Determined Response to Fibre in UC
In contrast to the HB incubations, gas production and composition were not significantly different between LB incubations with the different DF prototypes (Fig. 5A-C), whilst all SCFAs besides acetate were differently impacted by the DF prototypes in LB incubations. Specifically, DF prototypes C and D resulted in significantly higher butyrate (Fig. D), propionate (Fig. 5F), and total SCFA concentrations (Fig. 5G) compared to DF prototypes A and B. By combining gas production and SCFA concentrations, a weighted SCFA-gas ratio was calculated to reflect the overall beneficial effect of DF as it represents the trade-off between SCFAs and gas production. In both HB and LB incubations, DF prototypes C and D displayed higher SCFA-gas ratios in comparison to DF prototypes A (LB incubations only) or B (HB and LB incubations) (Fig. 5H). Overall, these observations indicated that the impact of DF supplementation on microbiota activity differed between inocula derived from faecal samples of HB- and LB subjects with generally higher variability in SCFA production between DF prototypes in the latter ones.

**Discussion**

Using a faecal batch incubation setup, this study aimed to investigate the potential of four different DF prototypes to stimulate butyrate production by inocula derived from faecal microbiota of UC patients. We furthermore assessed to what extent the efficacy of this stimulation was dependent on the faecal Bacteroidetes level. We found that butyrate production was extensively stimulated by all DF prototypes, among which DF prototypes C and D resulted in the highest concentration, concomitant with the highest total SCFA production and lowest hydrogen production, indicating their high fermentability and potentially positive effects to ameliorate mucosal inflammation in UC patients. Of note, due to the
heterogeneity of the subjects with respect to the faecal *Bacteroidetes* level, distinct patterns of fermentation metabolites and microbiota composition were observed between HB and LB incubations with generally higher variability in SCFA production between DF prototypes and number of butyrate-producing bacteria in the latter ones.

**DF stimulated butyrate-producing capacity of inocula derived from faecal samples of UC patients in a composition-dependent pattern**

In UC patients, the butyrate-producing capacity was found to be compromised (16), which was linked to the disease progress (14). Moreover, in an *in vitro* batch incubation study, around ten-fold less butyrate was produced from starch by the microbiota from UC patients compared to microbiota from healthy subjects, indicating the validity of reflecting compromised butyrate production in UC patients via batch cultivation (52). To improve butyrate production, DF intervention has been proven to be an effective approach. For instance, increased faecal butyrate concentrations were observed upon addition of 60 g of oat bran (corresponding to 12 g DF) to the daily diet of UC patients in remission (53). In our study, all four DF prototypes that contained various butyrogenic fibres stimulated butyrate production by inocula derived from faecal samples of UC patients. Butyrate plays a key role in colonocyte mucosal healing via inhibition of inflammation (54). Therefore, this finding reinforced the potential of DF to stimulate butyrate production and thus ameliorate colonic mucosal inflammation when administered to UC patients.

Of note, the extent that these DF prototypes promoted butyrate production relied on their composition, in which some fibres shared similar abilities. Specifically, DF prototypes A and B were composed of the same ingredients namely resistant starch (RS) and golden kiwi powder (GKP) but in different ratios, while DF prototypes C and D had around 40% of acacia gum (AG) and partially hydrolysed guar gum (PHGG), with additional 20% of RS for prototype C and 15% of GKP for prototype D. Nevertheless, the stimulation of butyrate production did not show any significant difference between DF prototypes A and B or between DF prototypes C and D. Given that the main difference between DF prototypes A and B or between DF prototypes C and D was the ratio of RS and GKP, this suggested that RS and GKP share a similar capacity for promoting butyrate production. Although there are currently no comparative studies available that would confirm this, our results suggest that the two DF preparations RS and GKP might be used as alternatives in
final products. Of note, when individually incubated with healthy microbiota, GKP was found to produce twice as much butyrate compared to RS (unpublished data, Winclove B.V.). This suggests that the butyrogenic capacity of GKP and RS might be dependent on the source of microbiota.

It is also worth mentioning that higher concentrations of SCFAs including butyrate were produced from DF prototypes C and D than from DF prototypes A and B accompanied by lower hydrogen concentrations. Besides SCFAs, hydrogen is also produced during bacterial fermentation. Hydrogen accumulation can be induced by carbohydrates with low fermentation efficacy as the electron flow within the system is regulated via the production of hydrogen (55). Therefore, lower hydrogen represents higher efficacy of carbohydrate fermentation. These results collectively implied that the mixture of AG and PHGG outperformed the mixture of GKP and RS with respect to boosting butyrogenic activity and fermentation efficacy in inocula derived from faecal samples of UC patients. Consistent with our findings, Titgemeyer et al also found that a mixture of AG and PHGG or AG alone induced higher SCFA production than oat fibre, which is a good source of RS (56). AG is slowly fermented and well-tolerated when consumed up to 30 g per day in healthy individuals (28), similar to PHGG (20-40 g per day) (29). Moreover, blending AG with fructo-oligosaccharides (FOS, a well-researched prebiotic) can extend the time of fermentation of FOS in the colon (57). Collectively, our study clearly demonstrated that RS and GKP shared similar fermentability, and AG and PHGG had higher butyrate-boosting capacity and fermentability. Thus, DF prototypes C and D might exert higher potential beneficial effects for UC patients than DF prototypes A and B.

Butyrate-producing bacteria were selectively stimulated in LB incubations

Discrete DF modification led to distinct butyrate production in healthy subjects (58). Likewise, an in vitro model study of healthy subjects revealed heterogeneous butyrate production by different RS sources and faecal inocula with distinct microbiota composition (59). Thus, successful stimulation of butyrate production was not only determined by carbon source but also by the microbiota composition of inocula from which butyrate-producing consortia were initially introduced. With fewer competing organisms from Bacteroidetes, it is tempting to speculate that butyrate-producing bacteria, mostly Firmicutes, occupied more
ecological niches in LB subjects. In this study, the eleven UC patients were grouped as high or low Bacteroidetes subjects based on a predefined cut-off value (i.e., 10%) (5). To this end, DF prototypes C and D induced higher SCFAs including butyrate production only in LB incubations. Notably, three genera (i.e., Subdoligranulum, Megasphaera, and Holdemanella) belonging to the Firmicutes showed higher relative abundances in LB incubations. Although the understanding of their physiological roles in the gut is still limited, faecal microbiota of active UC patients was featured with lower relative abundances of Subdoligranulum (60), Megasphaera (61), and Holdemanella (62). Accordingly, butyrate-producing capacity has been demonstrated in certain species within these genera such as Subdoligranulum variabile (63), Megasphaera elsdenii (64), and Holdemanella bioformis (65). Therefore, we could speculate that higher butyrate-producing capacity existed in the inocula derived from the faecal microbiota of LB subjects. Apart from the classical butyrate-producing pathway via BCoAT, another route proceeds via cross-feeding with bacteria that yield other metabolic end-products such as acetate and lactate (66). Acetate-producing bacteria (e.g., Bifidobacterium) and lactate-producing bacteria (e.g., Lactobacillus) are the main cross-feeding partners for butyrate producers in the gut, with butyrate as the main end product (67). In addition, the usage of Bifidobacterium spp. and/or Lactobacillus spp. as probiotics to induce remission or prevent relapse of quiescent UC has been documented (68, 69). Among the predominant genera within all incubations, Bifidobacterium and Lactobacillus were selectively stimulated in LB incubations. Overall, these results indicated that bacteria capable of producing butyrate or precursors of butyrate (e.g., lactate and acetate) were prone to be stimulated in LB subjects.

In contrast to LB incubations, members of the family Enterobacteriaceae and the genus Prevotella 9 showed higher relative abundance at 24 and 48 h in HB incubations irrespective of the DF prototypes. The bacteria belonging to the Enterobacteriaceae family have been reported to have a growth advantage over other bacteria in active UC, and are frequently described as inflammation-enhancing bacteria in IBD, with an overgrowth observed in active IBD (70). As for Prevotella 9 belonging to the phylum Bacteroidetes, a higher relative abundance of Bacteroidetes including Prevotella 9 was found in the mucosa of inflamed UC patients compared with the non-inflamed group (71). Within the same study, relieved inflammation with a reduced number of these bacteria was induced after treatment with 5-ASA. Thus, an
overgrowth of these potential inflammation-enhancing bacteria contributed to the exacerbation of UC. Interestingly, effective suppression of these inflammation-enhancing bacteria in the inocula derived from the faecal microbiota of HB subjects could not be achieved by DF prototypes.

Higher selective enrichment of *Bifidobacterium* in LB incubations was found with DF prototypes A and B. GKB, one of the two ingredients of DF prototypes A and B, has been shown to selectively stimulate *Bifidobacterium* spp. (72), and RS has been shown to be primarily degraded by certain *Bifidobacterium* species, most specifically *Bifidobacterium adolescentis* (73, 74). Moreover, in rats with colon inflammation, feeding an RS-containing diet reduced inflammation, increased faecal SCFA concentrations and the relative abundance of bacteria associated with RS utilisation including *Bifidobacterium* (75). Accordingly, *Bifidobacterium* showed higher growth in LB incubations in the presence of DF prototypes A and B, both containing RS. In contrast, higher selective enrichment of *Blautia* in LB incubations was found in DF prototypes C and D. As a genus of obligate anaerobic bacteria, *Blautia* plays an important role in maintaining gut environmental homeostasis and preventing inflammation by stimulating production of SCFAs and especially butyrate. Aside from this, some *Blautia* species are involved with butyrate production via cross-feeding. For instance, *Blautia hydrogenotrophica* could utilise hydrogen and carbon dioxide to produce acetate (76), which supports the growth of butyrogenic bacteria such as *Faecalibacterium prausnitzii* (77, 78). Therefore, *Blautia* spp. are generally considered as a potential next-generation probiotic (79). Compared to UC in remission, active UC patients had a lower level of *Blautia* in faeces, with the lowest level in the mucosal samples of severe UC compared with moderate and mild UC (80). Although DF intervention-induced changes in the *Blautia* abundance have not been reported in UC patients, increased *Blautia* was significantly associated with decreased faecal calprotectin levels (i.e., ameliorated mucosal inflammation) in UC patients in remission after taking the anti-inflammatory diet that was designed to increase DF intake (81). These results highlighted the anti-inflammatory role that *Blautia* may have. However, the stimulation of *Blautia* relies on the DF source. PHGG supplementation altered gut microbiota, featured with increased *Blautia* levels, in children with autism spectrum disorder (82), whilst RS intake led to decreased *Blautia* levels in normal body weight individuals (83). Accordingly, DF prototypes C and D that contain 40% PHGG selectively supported growth of *Blautia*
in LB incubations and thus had higher prebiotic potential in comparison to DF prototypes A and B that contain 50 and 70% RS, respectively.

In conclusion, DF could stimulate butyrate-producing capacity of inocula derived from faecal samples of UC patients, and more importantly, the stimulation was determined by DF mixture composition and faecal Bacteroidetes levels of UC patients. Specifically, DF prototypes C and D composed of over 80% AG and PHGG outperformed DF prototypes A and B composed of GKP and RS. DF prototype C was further selected as the most promising product due to higher tolerability and cost-effectiveness although similar prebiotic effects were shown for DF prototype D. Furthermore, UC patients with lower faecal Bacteroidetes levels (≤ 10%) are prone to have mucosal inflammation ameliorated by DF as a wider range of potential butyrate-producing bacteria (i.e., Subdoligranulum, Megasphaera, and Holdemanella) were selectively stimulated in LB incubations, while potential inflammation-enhancing bacteria (i.e., members of the family Enterobacteriaceae and genus Prevotella 9) selectively grew in HB incubations.

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Author contributions

Author contributions were as follows: conceptualisation, Z.L., B.J.M.W., and E.G.Z.; funding acquisition, Z.L., H.S., J.G., and E.G.Z.; resources, E.T., D.K., and B.J.M.W.; investigation, Z.L.; methodology, Z.L.; supervision, B.J.M.W., H.S., J.G., and E.G.Z.; writing original draft, Z.L. All authors have read and approved the manuscript.

Competing interests

The authors declare no competing interests.
Supplementary materials
Figure S1 Gas production, composition and SCFAs production during the incubation of UC faecal microbiota with different DF prototypes. Levels of (A) gas production, (B) headspace carbon dioxide and (C) hydrogen concentrations, culture liquid (D) butyrate, (E) acetate, (F) propionate, and (G) total SCFA concentrations during the incubation. Differences between each DF prototype were tested by t-test with FDR correction based on Benjamini–Hochberg procedure to correct for multiple testing. Significance is reported as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. Abbreviations: FDR: false discovery rate, SCFAs: short-chain fatty acids.
Figure S2 Headspace methane concentration during the incubation. Each dot represents the mean value of biological duplicates. Abbreviations: HB: high Bacteroidetes, LB: low Bacteroidetes.
Figure S3 Faecal microbial richness and diversity, and significantly different genera between HB and LB subjects. Data of (A) phylogenetic diversity, (B) inverse Simpson and (C) genera that showed significant difference in relative abundance between HB and LB subjects were visualised in boxplot. Mann-Whitney U test was performed to test for significance. Abbreviations: HB: high Bacteroidetes, LB: low Bacteroidetes.


References


Faecal *Bacteroidetes* Level Determined Response to Fibre in UC


Faecal Bacteroidetes Level Determined Response to Fibre in UC


Chapter 5


Chapter 5


Chapter 6

Prediction of Dietary Intervention Efficacy in Mild Ulcerative Colitis Patients Based on Faecal Microbiome Signatures (PREDUCTOME): Study Protocol for a Randomised Double-Blind Placebo-Controlled Parallel Trial

Zhuang Liu*, Sandra ten Bruggencate, Isolde Besseling-van der Vaart, Jacoline Gerritsen, Hauke Smidt, Ben J. M. Witteman, Erwin G. Zoetendal

* Corresponding author

Potential submission to Trials.
Abstract

**Background:** Ulcerative colitis (UC) is one type of inflammatory bowel disease (IBD) that causes chronic inflammation in the colon and rectum. Currently, there is no cure for UC and hence, interventions focus on symptom relief and maintenance of remission. However, UC patients respond differently to interventions (e.g., diet/faecal microbiota transplantation), but the reason for this individual specificity remains unknown. The investigators hypothesise that the baseline faecal microbiota composition determines the efficacy of an intervention, and that potential responders, i.e., patients showing symptom relief after intervention, can be predicted based on faecal microbiota composition. **Methods:** This four-arm randomised double-blind placebo-controlled parallel trial starts with a screening procedure in which around sixty adult mild UC patients will be assigned to be predicted responders ($n = 30$) or predicted non-responders ($n = 30$) based on faecal *Bacteroidetes* percentage. Afterwards predicted responders and non-responders will receive either prebiotics (mixture of acacia gum, partially hydrolysed guar gum, and resistant starch, 6 g/day) or a placebo (mixture of maltodextrin and corn starch, 6 g/day) for eight weeks of intervention, with $n = 15$ in each arm. The primary outcome is the response (mean Patient Simple Clinical Colitis Activity Index (P-SCCAI) score) at $T = 8$ weeks. Secondary outcomes are disease activity over time at $T = 0, 4, 8, 12, \text{and } 60$ weeks, mucosal inflammation (faecal calprotectin), gastro-intestinal complaints, stool consistency, stool frequency, faecal microbiota composition, faecal short-chain fatty acid concentrations, health-related quality of life, number of participants with increased or decreased medication use, and incidence of adverse events. **Discussion:** This clinical trial aims to validate the prediction that eight weeks of prebiotic intervention induces a higher response (lower mean P-SCCAI score) in adult mild UC patients with low faecal *Bacteroidetes* percentage (predicted responders), compared to those with high faecal *Bacteroidetes* percentage (predicted non-responders). **Trial registration:** NCT05579483 [ClinicalTrial.gov, registered on 13th of October, 2022]; NL79442.091.22 [METC Oost-Nederland, registered on 16th of August, 2022] **Keywords:** Inflammatory bowel disease; ulcerative colitis; gut microbiome; prebiotics; fibre; intervention; personalised nutrition
Administrative information

Note: the numbers in curly brackets in this protocol refer to SPIRIT checklist item numbers. The order of the items has been modified to group similar items (see [http://www.equator-network.org/reporting-guidelines/spirit-2013-statement-defining-standard-protocol-items-for-clinical-trials/](http://www.equator-network.org/reporting-guidelines/spirit-2013-statement-defining-standard-protocol-items-for-clinical-trials/)).

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Agrotechnology & Food Sciences Group  
P.O. Box 8033  
6700 EH Wageningen, The Netherlands |
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Introduction

Background and rationale (6a)

The incidence and prevalence of ulcerative colitis (UC) are increasing worldwide (1). It is a disease that causes inflammation extending from the rectum to the colon, and it is one of the two major types of inflammatory bowel disease (IBD) associated with abdominal pain, bloody diarrhoea, and urgency to defecate (2). Compared with the general population, patients with longstanding UC, regardless of clinical activity, have an increased risk for colorectal cancer (2, 3). Unfortunately, there is no cure for UC and hence, interventions focus on dampening the severity of symptoms and maintenance of remission. Besides many other known factors such as genetics and antibiotics exposure, diet also plays an important role in the development and maintenance of UC (4, 5). For instance, higher disease activity was observed in IBD patients consuming a diet with more pro-inflammatory ingredients (6). Moreover, besides medical or surgical treatments, about half of patients with UC have mild symptoms in which a dietary recommendation, including increasing dietary fibre intake, could help reduce inflammation (7-9).

Besides providing nourishment to the human body, diet also extensively affects the environment of the human gut, which harbours trillions of microbes, commonly referred to as the “microbiota”, whereas the term “microbiome” refers to the collection of microbes and all the genes and functionalities they encode and express (10). Dominated by two major bacterial phyla (i.e., Firmicutes and Bacteroidetes) (11), the human gut microbiome was found to be closely associated with overall human health (12, 13) and disease states including UC (14, 15). Generally, UC patients have a gut microbiome that is characterised by reduced microbial diversity, along with an increased level of Bacteroidetes and a decreased level of Firmicutes, and lower levels of butyrate compared with that in healthy subjects as revealed by numerous clinical studies (16-18). Targeting the gut microbiome, dietary modification such as increasing dietary fibre intake could be beneficial for maintaining periods of remission or improving clinical outcomes in patients with UC. Recently, a low-fat, high-fibre diet has been found to reduce inflammatory markers and alter the gut microbiome in patients with mild UC or UC in remission (19). Similarly, less abdominal pain and cramping was induced by a fibre-based prebiotic in UC patients.
Furthermore, short-chain fatty acids (SCFAs) such as butyrate are produced by the microbiome via breaking down dietary fibres, and these SCFAs are considered beneficial for gut health (21). With the addition of 60 g oat bran (corresponding to 20 g dietary fibre) to the daily diet for three months, higher faecal butyrate levels concomitant with less abdominal pain were induced in 22 UC patients (8).

However, the response to an intervention differs drastically between subjects, with some subjects entering remission (responders), whereas others fall or remain into relapse (non-responders). The reason(s) for this inconsistent response between subjects remains speculative, but may be the result of differences in the gut microbiome, given its intermediate role between diet and host (22). Nevertheless, despite the reported differences in gut microbiome between healthy subjects and UC patients, a consensus microbiome signature has not been observed. This could be due to the personalised character of the gut microbiome, as well as the different higher level order microbiota organisations, such as enterotypes (23) or alternative stable states (24) that have been suggested. In fact, strategies for microbiome-based stratification of patient groups have been widely employed to guide dietary intervention studies (25).

Looking from an ecological perspective, ecosystems are generally characterised by alternative stable states that are resilient to (minor) perturbations. Changing from one stable state into another stable state needs a strong perturbation in which a tipping point needs to be crossed (Fig. 1A). For example, both deserts and rainforests are stable states, and only a drastic perturbation, like deforestation, allows a rainforest to cross a tipping point to become a desert, and similarly, it will need a lot of effort to have it return again from a desert into a rainforest in which a tipping point needs to be crossed as well. We believe that similar ecological principles exist for the gut microbiome. A recently published review describes that specific microbiota compositions can be considered as alternative ecosystem states that are generally resilient to perturbations (26). These stable states exist in both microbiomes in healthy subjects as well as microbiomes associated with disease, for example in UC patients, and switching from one to the other needs strong perturbations (such as diseases/infections causing diarrhoea) or treatments (such as antibiotics use or prebiotic consumption) that result in crossing the tipping point. For healthy subjects, different alternative healthy states of the gut-microbiome have been identified (26). Similarly, we speculate that different alternative states also exist for UC patients. Indeed a recent study in a rat model for UC has indicated the existence
The principle of tipping points in ecosystems and its basis for the hypothesis of this study. (A) Alternative stable states exist in many ecosystems, and (minor) perturbations do not change the states. To achieve the switching between stable states, a strong perturbation that pushes the system through the tipping point is needed. (B) UC patients in remission share similar faecal microbiome signatures with healthy individuals, which is generally featured with low levels of Bacteroidetes and high levels of butyrate producers, while in active UC patients, two types of stable microbiome states exist. Type 1 is characterised by high Bacteroidetes and low butyrate producers, while type 2 is characterised by low Bacteroidetes and low butyrate producers. Prebiotic intervention fails in UC type 1 due to high resilience and insurmountable microbiome tipping point. In contrast, prebiotic intervention induces higher response in UC type 2 which shares an easier-to-cross tipping point with remission states. Abbreviation: UC: ulcerative colitis.

of health states and disease active states (27). Similarly, alternative intestinal ecosystem states have also been observed in paediatric UC patients, and it was found that these states are a discriminant of treatment success (28).

The *Bacteroidetes* phylum is one of the most abundant phyla in the human gut microbiome, and dramatic differences in relative abundance of this phylum have been observed in UC patients (16, 29). The association between *Bacteroidetes* species and UC activity has been previously identified (30). Remarkably, induction of sustained remission via faecal microbiota transplantation (FMT) was associated with a baseline microbiota that was more similar to that of healthy individuals, both characterised by a relatively low faecal *Bacteroidetes* percentage (5% in responders and 8% in healthy individuals on average), whereas non-responders had a relatively high baseline faecal *Bacteroidetes* percentage (average 19%) (16). Moreover, the induction of sustained remission was associated with an improved butyrate production capacity. Hence, we hypothesised that the gut microbiome of UC patients
with a low \textit{Bacteroidetes} percentage can be seen as an alternative state that is separated from a healthy state through a tipping point that is easier to be crossed by an intervention compared to a distinct alternative state characterised by a higher \textit{Bacteroidetes} percentage. We speculate that crossing this tipping point in subjects with low faecal \textit{Bacteroidetes} percentage can be reached by stimulating intestinal butyrate production capacity (\textbf{Fig. 1B}).

There is growing recognition that not an individual fibre but a mixture of fibres is more likely to promote the growth of butyrate producing bacteria via different cross-feeding routes (21, 31). To identify a potential intervention targeting the butyrate-producing capacity that could be used in an intervention study, we applied an \textit{in vitro} cultivation-based screening of different dietary fibre mixtures on the gut microbiome of UC patients to test our hypothesis (\textbf{Chapter 5}). This screening resulted in the identification of a fibre mixture (acacia gum, partially hydrolysed guar gum, and resistant starch) that most effectively stimulated butyrogenic activity in the microbiome with low \textit{Bacteroidetes} percentage, while butyrate production remained unaffected in those with high \textit{Bacteroidetes} percentage. Hence, we selected this fibre mixture (hereinafter referred to as prebiotics) for our intervention study (\textbf{Fig. 1B}).

\textbf{Objectives \{7\}}

\textbf{Primary Objective}

The primary objective is to validate the prediction that intervention with a specifically selected prebiotic mixture stimulates butyrate production and thereby induces a higher clinical response to the intervention (lower mean Patient Simple Clinical Colitis Activity Index (P-SCCAI) score) in mild UC patients with low faecal \textit{Bacteroidetes} percentage (predicted responders), compared to those with high faecal \textit{Bacteroidetes} percentage (predicted non-responders) at T = 8 weeks.

\textbf{Secondary Objectives}

The secondary objectives are to study the effects of an 8-week prebiotic intervention on disease activity over time (T = 0, 4, 8, 12 and 60 weeks), mucosal inflammation (faecal calprotectin levels), gastro-intestinal (GI) complaints, stool consistency, stool frequency, faecal microbiota composition, faecal SCFA
concentrations, health-related quality of life, medication use, and incidence of adverse events in mild UC patients.

**Trial design**

This study is a randomised, double-blind, placebo-controlled parallel trial in UC patients with mild severity (as shown in Fig. 2). UC severity will be determined by P-SCCAI score (32), and patients with a score of 3-5 points on a 19-point scale will be selected as study subjects with mild UC inflammatory activity (33, 34). The trial starts with a subject screening and selection procedure, during which around 180 mild UC patients will be screened and of which 60 patients will be selected based on their faecal *Bacteroidetes* percentage. Of these 60 patients, 30 will be chosen with a low faecal *Bacteroidetes* percentage ($\leq 10\%$) (predicted responders) and 30 with a high faecal *Bacteroidetes* percentage ($\geq 15\%$) (predicted non-responders). Within each group, patients will be randomly allocated 1:1 to the prebiotic or placebo intervention arm. Prebiotics (acacia gum, partially hydrolysed guar gum, and resistant starch) or placebo (maltodextrin and corn starch) will be consumed (6 g per day in two 3-g doses) during an 8-week intervention period. Faecal samples will be collected at baseline and at the end of the intervention ($T = 0$ and 8 weeks). Several questionnaires will be collected at baseline, in the middle and at the end of the intervention ($T = 0$, 4 and 8 weeks). All patients will be followed up via faecal sample and questionnaires at four weeks ($T = 12$ weeks) and one year ($T = 60$ weeks) post-intervention.
Figure 2 PREDUCTOME study: set-up and workflow. Trial set-up and workflow of the four-arm randomised double-blind placebo-controlled parallel trial comparing the response to a prebiotic and placebo intervention between predicted responders and predicted non-responders. It consists of the screening, intervention, and the follow-up periods, in which faecal samples and several questionnaires will be taken to evaluate the overall response. Green: predicted responders, red: predicted non-responders. Abbreviation: UC: ulcerative colitis.
Methods: Participants, interventions and outcomes

Study setting {9}

The trial will be conducted at the Laboratory of Microbiology, Wageningen University & Research, the Netherlands, that has all facilities to perform the proposed research. A call for the participation of this study will first be spread among the patient base (more than 1200 IBD patients) of Ziekenhuis Gelderse Vallei (ZGV) in Ede, the Netherlands. In case of under-recruitment, UC patients of Rijnstate, Arnhem, the Netherlands, will be included as well. In order to be eligible to participate in this study, a subject must meet all of the following inclusion criteria and none of the exclusion criteria.

Eligibility criteria {10}

Inclusion criteria

Patients must meet the following criteria to be eligible for the study.
- Subjects aged 18 to 65 years (previous studies have shown that the gut microbiome in adolescents and elderly can be different from that of adults (35, 36))
- Body Mass Index (BMI) between 18 and 30 kg/m² (self-reported)
- UC confirmed via previous endoscopy and histology
- Mild active UC as defined by P-SCCAI score of 3 to 5 points (range 0 to 19)
- Frequent relapse (at least one exacerbation in the last two years)
- No known allergy to any components of the study products (self-reported)
- Signed informed consent
- Stable UC medication defined as no switch to other medication or no dose change
- Mobile phone on which apps (used for questionnaires) can be downloaded (iOS version 9 and higher, Android version 4.4 and higher). Phones manufactured after 2013 are usually suitable
- Willing to maintain a stable dietary pattern during the study
Exclusion criteria

If patients meet any of the following criteria, they will not be eligible for the study.

- Any other underlying disease of the GI tract or previous bowel surgery, except cholecystectomy and appendectomy
- Pregnancy or intending to become pregnant during the whole study period
- Use of medication that can interfere with the study outcomes, as judged by the medical supervisor
- The need for routine antibiotic use during the intervention period
- Use of systemic antibiotics and proton pump inhibitors (except for omeprazole and pantoprazole with dosage < 20 mg), prebiotic supplements, probiotic supplements four weeks prior to the start of the study
- Currently participating in another intervention study
- Acquaintances of anyone within the research team

Who will take informed consent? {26a}

UC patients with mild activity will be screened for eligibility to participate in this study based on the abovementioned criteria. One or more information meetings will be organised prior to the study. In this meeting, the investigators will explain the background, objectives and study procedures. During this meeting, there will be time for the potential study subjects to ask questions. After the information meetings, subjects will have two weeks to decide whether they are interested in participation or not. Subjects who are willing to participate will receive the informed consent to sign.

Additional consent provisions for collection and use of participant data and biological specimens {26b}

Participants will give specific consent for use of their faecal samples and questionnaires data to answer the research questions of this study only. In case the collected material appears to be suitable for additional research, which includes research in relation to the health of the patients or other research activities that are not covered by this study, the medical ethics committee Oost-Nederland will need to be approached for evaluation, with previous approval from participants in the consent form.
Interventions

Explanation for the choice of comparators {6a}

Our research is based on the hypothesis that patients with a faecal microbiota composition that is characterised by a low percentage of the phylum *Bacteroidetes* will respond well to ingestion of prebiotics because this stimulates butyrate production and thus changes the microbiome to a more “healthy” situation for this patient population. Patients with a low faecal *Bacteroidetes* percentage are therefore defined in our study as “predicted responder” according to this hypothesis. On the other hand, there is a group that has a high percentage of the phylum *Bacteroidetes*, and is therefore not expected to be affected by neither prebiotic nor placebo consumption, resulting in no effect on gut health and the microbiome. In this study, this group is referred to as “predicted non-responder”. The determination of responder and non-responder is based on previous research (16).

In the present study we will classify the UC patients into potential responders and non-responders based on the percentage of *Bacteroidetes* in their faeces, and we want to investigate the success of an intervention with a specifically selected prebiotic mixture. To test this hypothesis, we need the following three comparisons: 1) Prebiotic intervention in predicted responders vs predicted non-responders to validate our hypothesis that our prebiotic intervention will only elicit a significant beneficial effect in subjects with a low faecal *Bacteroidetes* percentage; 2) Prebiotic vs placebo intervention in predicted responders to demonstrate that stimulation of butyrate production underlies the intervention response; 3) Prebiotic vs placebo intervention in predicted non-responders to validate that prebiotics do not elicit a beneficial response in subjects with high faecal *Bacteroidetes* percentage.

Intervention description {11a}

The prebiotic intervention is a specifically selected prebiotic mixture composed of three individual fibres (40% acacia gum, 20% partially hydrolysed guar gum, and 40% resistant starch) from Winclove Probiotics B.V. (Amsterdam, the Netherlands). The placebo is composed of food-grade corn starch and maltodextrin from Winclove Probiotics B.V. (Amsterdam, the Netherlands). The prebiotic and placebo powders are similar in colour, solubility, and taste. The dose of each individual fibre has been
selected to maximise the potential benefits but to limit potential GI side effects (e.g., abdominal pain, bloating). Additionally, our in vitro incubation study with faecal inocula from UC patients showed this product had the highest butyrate stimulating capacity in patients with low faecal Bacteroidetes percentage (Chapter 5).

Based on published studies on interventions on inducing clinical remission, an 8-week intervention should be sufficient for the prebiotic intervention to result in significant effects (7, 22, 37, 38). Hence, we will use an 8-week intervention period with four parallel arms: 1) predicted responders with prebiotics; 2) predicted responders with placebo; 3) predicted non-responders with prebiotics; 4) predicted non-responders with placebo. During the intervention period, all subjects will consume the respective supplement (3 g/sachet, two sachets per day, one in the morning and one in the evening). To avoid GI complaints due to a sudden intake of the prebiotic/placebo supplement, only one sachet will be consumed in the morning per day in the first week of the intervention period.

**Criteria for discontinuing or modifying allocated interventions {11b}**

Subjects can leave the study at any time for any reason if they wish to do so without providing a reason and without any consequences. The investigator can decide (upon consultation with the medical supervisor) to withdraw a subject from the study for urgent medical reasons, such as serious GI complaints, negative reactions upon supplement intake, or when study subjects have to start medication that interferes with outcomes during the study period. A subject will be withdrawn by the investigator from the study in the case of serious GI complaints or an exacerbation of UC reported by the subjects (e.g., continuous diarrhoea or constipation, bloating, abdominal pain).

**Strategies to improve adherence to interventions {11c}**

To minimise GI complaints due to a sudden intake of the prebiotic/placebo supplement, there will be a gradual increase in dosage: only one sachet will be consumed per day in the first week of the intervention period, followed by two sachets per day for the remainder of the intervention. In addition, subjects will be checked with respect to disease activity at \( T = 0, 4, 8, 12 \) and 60 weeks, so that any complaints will be reported in time. At the end of the intervention period (\( T = 8 \) weeks), subjects
will be asked to return everything including unused sachets to determine compliance to the study.

**Relevant concomitant care permitted or prohibited during the trial {11d}**

During the entire trial, study subjects will be asked to maintain their normal routines concerning their diet, medication use, and exercise pattern. Besides that, no concomitant care is prohibited.

**Provisions for post-trial care {30}**

The sponsor has a liability insurance which is in accordance with the legal requirements in the Netherlands (Article 7 of the Medical Research Involving Human Subjects Act (WMO)). This insurance provides coverage for damage to study subjects through injury or death caused by the study. The insurance applies to the damage that becomes apparent during the study or within four years after the end of the study.

**Outcomes {12}**

**Primary outcome**

The primary outcome is the response (mean P-SCCAI score) at T = 8 weeks. P-SCCAI is derived from the original SCCAI questionnaire by translating into patients’ comprehensible language, and has been validated to give substantial agreement with the original SCCAI (39). It refers to disease symptoms during the previous week. The mean P-SCCAI score for each arm will be determined and compared between each arm to determine the difference in response at T = 8 weeks.

**Secondary outcomes**

The secondary outcomes are the changes of disease activity, mucosal inflammation, GI complaints, stool consistency, stool frequency, faecal microbiota composition, faecal SCFA concentrations, health-related quality of life, medication use, and incidence of adverse events within each subject and between each arm over time. For all of these parameters, comparisons will be made within each subject and between each arm. Measurement time points and clinical relevance are listed below.
At $T = 0, 4, 8, 12, \text{ and } 60$ weeks, disease activity will be determined by P-SCCAI. Changes of P-SCCAI between each interval time point within each subject will be used for further analysis.

At $T = 0, 8, 12, \text{ and } 60$ weeks, mucosal inflammation will be determined by measuring faecal calprotectin levels. Faecal calprotectin has been used as one of the most extensively studied biomarkers in monitoring mucosal inflammation in IBD patients (40).

At $T = 0, 4, \text{ and } 8$ weeks, GI complaints will be assessed by the gastrointestinal symptom rating scale (GSRS) questionnaire. It provides GI complaints and tolerance information. The reliability and validity of the GSRS are well-documented, and norm values for a general population are available (41).

At $T = 0, 4, \text{ and } 8$ weeks, stool consistency will be assessed by study subjects using the Bristol stool form scale on a daily basis for 7 days. Average score within one day and fluctuation over time will be used for analysis.

At $T = 0, 4, \text{ and } 8$ weeks, stool frequency will be assessed by study subjects via counting the number of defecations on a daily basis for 7 days.

At $T = 0, 8, 12, \text{ and } 60$ weeks, faecal microbiota composition will be determined by 16S ribosomal RNA (rRNA) gene sequencing of faecal samples. Subsequent analyses of a variety of microbial ecological characteristics, including microbiota composition, α-diversity (diversity within the sample), β-diversity (diversity between the samples), dynamics of individual microbial taxa over time, and group differences will also be conducted.

At $T = 0, 8, 12, \text{ and } 60$ weeks, faecal SCFA concentrations will be measured. These reflect microbial activity in the gut.

At $T = 0, 4, \text{ and } 8$ weeks, health-related quality of life will be assessed by the short inflammatory bowel disease questionnaire (SIBDQ). Its reliability and responsiveness to change has been previously validated in UC (42).

At $T = 0, 4, 8, 12 \text{ and } 60$ weeks, information of medication use will be extracted from patient record and diary. It consists of the current medication use (e.g., aminosalicylates (5-ASA), corticosteroids, immunosuppressive agents, antimicrobial agents, and inhibitors of tumour necrosis factor-alpha (TNF-α)). Medication changes at week 4, 8, 12, and 60 provide information on disease development, with a decrease indicating improvement and an increase indicating worsening of UC.
At T = 0, 4, and 8 weeks, incidence of adverse events will be monitored by patient record and diary. In this diary, all relapse-relevant information, including the need for systemic steroids, hospitalisation, and surgery will be collected.

**Other outcomes**

At T = 0 (baseline), habitual dietary intake of the last month will be assessed by a validated food frequency questionnaire (FFQ). FFQ provides information regarding the usual foods consumed and the frequency of consumption. These data will be used for potential correlation analysis with other parameters as diet is considered an important modulator of the gut microbiome. In addition, it will also be used to monitor the dietary changes of subjects.

**Participant timeline {13}**

Fig. 3 shows the participant timeline.

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**Figure 3 PREDUCTOME study: participant timeline.** Stool consistency will be assessed daily on seven consecutive days using the Bristol stool form scale. Abbreviations: P-SCCAI: patient simple clinical colitis activity index, GSRS: gastrointestinal symptom rating scale, SIBDQ: short inflammatory bowel disease questionnaire, FFQ: food frequency questionnaire.
Sample size {14}

The primary objective of the trial is to validate the prediction that a higher response (lower mean P-SCCAI score) is induced at T = 8 weeks by the prebiotic intervention in mild UC patients with low faecal *Bacteroidetes* percentage (predicted responders), but not in those with high faecal *Bacteroidetes* percentage (predicted non-responders). According to an extensive study regarding UC disease activity conducted in the Netherlands, mild UC patients have a P-SCCAI score of 3.5 ± 0.5 (Mean ± standard deviation (SD)), and patients in remission score at 1.0 ± 0.33 (Mean ± SD) (6, 43). Based on two-sided statistical testing for unpaired continuous data, an α = 0.05 (chance of type-I error) and β = 0.10 (chance of type-II error), and assuming a 20% difference of mean P-SCCAI score between predicted responders with prebiotics and predicted non-responders with prebiotics, it was calculated that at least 11 subjects per arm are needed for the primary outcome P-SCCAI (µ1 = 3.5; SD1 = 0.5; µ2 = 2.8). According to previous trials on prebiotics in IBD patients, a 20% drop-out rate is commonly seen. Given that the intervention has a duration of 8 weeks, followed by a 1 year follow-up, a higher drop-out rate (30%) is expected. To compensate for a 30% drop-out rate, 15 subjects per arm are needed.

To reach inclusion of 30 predicted responders and 30 predicted non-responders, we made an estimate how many subjects we need to screen based on the earlier obtained microbiota composition of faeces samples from UC patients in the Ede area. Around 50% of these subjects were categorised as low *Bacteroidetes* subjects (*Bacteroidetes* ≤10%, predicted responders), while 17% of them were characterised as high *Bacteroidetes* subjects (*Bacteroidetes* ≥15%, predicted non-responders) (Chapter 5). To include sufficient participants in each arm we will need to screen around 180 mild UC patients.

Recruitment {15}

Patients will be recruited by nurses during their outpatient clinic visit at ZGV. There are more than 1200 IBD patients in the patients base of ZGV, with around half of all UC patients having mild activity. This guarantees that enough potential patients can be reached. Previous surveys performed at ZGV on dietary beliefs and behaviours showed that around 60% of IBD patients valued nutrition to be either more or equally
important compared to medication for their treatment (44). This further increases the likelihood to recruit enough patients.

**Assignment of interventions: allocation**

**Sequence generation {16a}**

Subjects in predicted responder and predicted non-responder groups will be randomly allocated 1:1 to the prebiotic or placebo arms by block randomisation using an online randomisation system available via password-protected access. The allocation sequence will be generated by a computer. To ensure blinding, the block sizes will not be disclosed.

**Concealment mechanism {16b}**

Generation of the allocation sequence will be performed by the product provider (i.e., Winclove B.V.) using the online randomisation system. The allocation sequence will only be shared with another researcher outside the research team who knows the responder/non-responder assignment but is not involved in further measurements/assessments in the study. In case of an emergency, the supplements can be unblinded after consultation of the principal investigator.

**Implementation {16c}**

Another researcher outside the research team will assign the subjects to the respective intervention arms in accordance with the allocation sequence.

**Assignment of interventions: Blinding**

**Who will be blinded {17a}**

Both investigators and study subjects will be blinded to the intervention group for the duration of the trial.

**Procedure for unblinding if needed {17b}**

After collection of all laboratory and questionnaire data, and performing all analyses of the baseline/intervention period (up to the 4-week follow-up; T = 12
weeks), the study will be unblinded for the investigators. At the end of the study (T = 60 weeks), study subjects will be informed of the kind of intervention they received (only when approved in the informed consent).

**Data collection and management**

**Plans for assessment and collection of outcomes {18a}**

Assessment will be performed by two major means:

**Questionnaires**

- P-SCCAI contains 13 items clustered into six domains: bowel frequency (during the day), bowel frequency (during the night), urgency of defecation, blood in stool, general well-being, and a number of defined extracolonic features of UC (i.e., arthritis, erythema nodosum, pyoderma angrenosum, uveitis). The score ranges from 0 to 19 with higher scores representing worse disease symptoms. With P-SCCAI score \( \leq 2 \) being regarded as clinical remission, and a decrease in the P-SCCAI score by more than two points from baseline being regarded as clinical response (33, 34, 45).

- GSRS contains 15 items clustered into five domains (reflux, abdominal pain, indigestion, diarrhoea and constipation) addressing different GI symptoms (46). It has a seven-point graded scale where 1 represents the absence of troublesome symptoms and 7 represents very troublesome symptoms.

- Score of Bristol stool form scale (seven-point scale from 1 = hard stool to 7 = diarrhoea) for each faeces will be collected (47).

- Average defecation per day and dynamic change will be calculated and collected as the stool frequency.

- SIBDQ is the shortened version of the original 32-item IBDQ with ten items that belong to four domains (bowel symptoms, emotional health, systemic systems and social function) (48). It has ten items and a seven-point graded scale where 1 represents very troublesome symptoms and 7 represents the absence of troublesome symptoms. Data from SIBDQ will be computed into scores, and the scores of all items will be pooled as the sum score, and the score of all items within each domain will be pooled as well.
- Type, dose, frequency and duration of each medication use will be extracted from patient record and diary. Number of subjects with increased or decreased medication use will be recorded.

- Adverse events will be extracted from patient record and diary. Incidence rate of each adverse event will be recorded.

- FFQ data will be collected via the FFQ-tool, a web-based interface tool (49).

- Subject demographics and characteristics (e.g., gender, age, BMI, disease duration, age at diagnosis, smoking habit, UC location, type of treatment) will be collected at baseline.

**Faecal samples**

After collection, faecal samples will be immediately frozen in the home freezers of the subjects, transported on dry ice (via courier) to the laboratory, and subsequently frozen at $-80\, ^{\circ}\mathrm{C}$ until further analyses as described below.

- Faecal *Bacteroidetes* percentage will be determined as follows: DNA will be isolated from an aliquot of the faecal samples and subsequently used for quantifying the 16S rRNA gene copy numbers of total bacteria and *Bacteroidetes* via quantitative polymerase chain reaction (qPCR). Based on the baseline microbiota from subjects in an FMT trial (16), in which healthy donors, sustained responding UC patients, and non-responding UC patients had an average percentage of 8%, 5%, and 19% *Bacteroidetes*, respectively; predicted responders are defined as UC patients having a percentage of *Bacteroidetes* $\leq 10\%$ in their faeces, while predicted non-responders are defined as UC patients with a percentage of *Bacteroidetes* $\geq 15\%$ in their faeces.

- Faecal microbiota composition will be determined as follows: DNA will be isolated from an aliquot of the faecal samples, followed by purification and PCR of the V4 region of the 16S rRNA gene with sample-specific barcode-tagged primers 515F (5'-GTGY CAGC MGCC GCGG TAA-3') (50) and 806R (5'-GGAC TACN VGGG TWTC TAAT-3') (51). After purifying PCR products, an equimolar mixture will be prepared and sent to Novogene (Cambridge, United Kingdom) for sequencing. Raw sequence data will be pre-processed using NG-Tax 2.0 with default settings (52, 53). Amplicon sequencing variant (ASV) picking and taxonomic assignments will be performed using the SILVA 138.1
database. The data processing and analysis can be found in section **Statistical methods**.

- Faecal SCFA concentrations will be measured by high-performance liquid chromatography (HPLC) (54).
- Faecal calprotectin levels will be measured by enzyme-linked immunosorbent assay (ELISA) methods, with > 50 mg/kg faeces as the cut-off for inflammation (55).

**Plans to promote participant retention and complete follow-up {18b}**

Telephone call reminders will be made by the coordinating investigator to subjects regarding each sample collection and questionnaire one day before sample/data collection.

**Data management {19}**

The handling of personal data will be done according to the General Data Protection Regulation (GDPR), which was enforced on the 25th of May 2018.

**Confidentiality {27}**

Before the start of the study, subjects will be assigned to a study code that will not change during the study. This code is linked to the name, address, date of birth, and telephone number of the subject in a password-protected file that is stored separately from the study data. Only members of the research team involved in the study logistics (e.g., collecting samples) can access this file. For all other purposes, the study code will be used for subject identification. Paper data such as informed consent will be stored in a cabinet with a lock separately from all other information. All research data will be stored for a period of 10 years after collection by the principle investigator. Thereafter, data and documents will be deleted and destroyed (e.g., by a shredder).
Plans for collection, laboratory evaluation and storage of biological specimens for genetic or molecular analysis in this trial/future use {33}

Faecal samples will be stored for 10 years after the study ends, defined as the last data collection point, and then disposed of according to biomedical waste procedures.

Statistical methods

Statistical methods for primary and secondary outcomes {20a}

Statistical analyses will be done using statistical programs embedded within R. The threshold for significance will be set at $p < 0.05$, with a false discovery rate (FDR) correction for 16S rRNA gene sequence data. In general, the continuous data will be presented as mean ± standard deviation or mean and interquartile range when skewed, and categorical data as counts and percentages. Normality will be checked by visual inspection and quantile-quantile (QQ) plots, followed by the Shapiro-Wilk test for testing if data show a normal distribution.

Primary outcome

To assess the response, data of mean P-SCCAI score within each arm at $T = 8$ weeks (after the intervention) will be obtained. Firstly, the mean P-SCCAI score will be compared between the predicted responders with prebiotics and predicted non-responders with prebiotics by using an independent samples $t$-test if normally distributed, otherwise a Mann-Whitney $U$ test when skewed. Secondly, the mean P-SCCAI score will be compared between prebiotic and placebo groups within predicted responders or predicted non-responders by using an independent samples $t$-test if normally distributed, otherwise a Mann-Whitney $U$ test when skewed.

Secondary outcomes

All the data will follow the same analysis procedure. First, comparisons will be made between groups as well as within subjects over time. For comparative analyses between baseline and intervention, the parametric paired sample $t$-test or the non-parametrical Kruskal-Wallis or Mann-Whitney $U$ test will be used, depending on the distribution of the samples. Differences between treatments before and after supplement intake will also be analysed by a generalised linear mixed-effects model (GLMM) for repeated measures, using “treatment” [prebiotic/placebo], “responding”
[responders/non-responders], “time point” [0, 4, 8, 12 and 60 weeks] and “treatment × responding × time point” as fixed effects and subject as random effect. Clinically relevant variables such as BMI, age, gender, and medication use will be taken into account.

For faecal microbiota composition, 16S rRNA gene sequencing provides sequence read count data. This data will be transformed to microbial relative abundance, and microbiota diversity indices (Shannon, Simpson, and Inverse Simpson) are calculated at amplicon sequence variant (ASV) level, as implemented in the picante (56) and phyloseq (57) packages in R, respectively. Mann-Whitney U test will be applied to determine whether diversity as well as the relative abundance of specific bacterial taxa, are significantly different between groups since the data is non-parametric. For microbiota analysis, p-values will be corrected for multiple comparisons using the Benjamini–Hochberg procedure. To correct for a false discovery rate, FDR adjusted p-values will be computed, so-called q-values (58). A corrected q-value of less than 0.2 will be considered significant. Paired tests will be used to compare the effects of prebiotics. Pairwise weighted UniFrac (59) and unweighted UniFrac (60) distance-based principal coordinates analysis (PCoA) will be used to visualise microbial community variation at ASV level. Permutational multivariate analysis of variance (PERMANOVA) will be used to test for significant differences between groups, as implemented in the vegan package (61).

**Other outcomes**

Data from FFQ will be analysed in the same manner as described above. Also, it will be used for Pearson r correlation coefficient calculation, or a Spearman rank when not normally distributed, with other parameters.

**Interim analyses {21b}**

Not applicable as there are no interim analyses planned.

**Methods for additional analyses (e.g., subgroup analyses) {20b}**

Not applicable as there are no additional analyses planned.
Methods in analysis to handle protocol non-adherence and any statistical methods to handle missing data {20c}

All drop-outs will be excluded from the analyses. For comparative analyses within the same subject over time, subjects with at least one missing data point will be excluded. For comparative analyses between arms, and all analyses within GLMM, missing data will be entered as NA, as this will not affect the outcome.

Plans to give access to the full protocol, participant level-data and statistical code {31c}

The full protocol approved by METC Oost-Nederland is available on ClinicalTrial.gov under identifier number NCT05579483. The handling of personal data will be done according to GDPR. Raw 16S rRNA gene sequence data will be released into the European Nucleotide Archive database after the manuscript is accepted for publication. Any dataset analysed during the current study can be made available from the corresponding author upon reasonable request.

Oversight and monitoring

Composition of the coordinating centre and trial steering committee {5d}

This trial is a monocentre study that is designed, coordinated and to be performed at the Laboratory of Microbiology, Wageningen University & Research. The coordinating centre consists of the principal investigator, coordinating investigator and medical supervisor. A monthly meeting will be scheduled with the research team. The principal investigator will supervise the progress of the trial including the recruitment of subjects, implementation of intervention and follow-ups, collection of samples and data, and data management. The coordinating investigator will be in charge of trial registration, trial coordination, trial execution, data and sample collection, data analyses, progress report and publication. The medical supervisor will be responsible for the identification of eligible subjects, making decisions regarding the continuation or termination of the study, and for monitoring adverse events.

There is no steering committee involved. The principle investigator will submit a summary of the progress of the trial to METC Oost-Nederland once a year.
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Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, adverse events, other problems, and amendments.

**Composition of the data monitoring committee, its role and reporting structure {21a}**

A data monitoring committee has not been appointed for this study in agreement with the decision from the local research ethics committee (i.e., METC Oost-Nederland), which did not recommend the creation of a data monitoring committee.

**Adverse event reporting and harms {22}**

All adverse events (AEs) reported spontaneously by the subject or observed by the medical supervisor or his staff will be recorded. If AEs occur, they are likely to relate to minor GI symptoms, such as bloating or flatulence or a minor change in defecation, especially when one uses prebiotics for the first time (62). Maltodextrin and corn starch as a placebo has been widely used in many clinical trials, and no AEs have been reported to date. In our case, UC patients are already used to GI complaints, hence they are expected to report less bloating and flatulence. These effects are expected to disappear within a few days.

No serious adverse events (SAEs) are expected from the administration of the prebiotics/placebo, as prebiotics have been shown to induce remission in UC patients. Subjects may potentially benefit from the dietary intervention with improved disease activity. Nevertheless, in case of suspected SAEs, the coordinating investigator will immediately notify the research team and the manufacturer of the product about the nature of the event, and the local research ethics committee. A decision on continuation or discontinuation of the trial will be made by the medical supervisor in agreement with the ethics committee.

**Frequency and plans for auditing trial conduct {23}**

The research team will meet once per month to monitor the progress of the trial.
Plans for communicating important protocol amendments to relevant parties (e.g., trial participants, ethical committees) {25}

Non-substantial changes (such as typing errors, administrative changes like changes in names, telephone numbers and other contact details of involved persons mentioned in the submitted study documentation) will not be notified to the ethics committee, but will be recorded and filed.

A “substantial amendment” is defined as an amendment to the terms of the METC application, or to the protocol or any other supporting documentation, that is likely to affect to a significant degree including: 1) the safety or physical or mental integrity of the subjects of the trial; 2) the scientific value of the trial; 3) the conduct or management of the trial; 4) or the quality or safety of any intervention used in the trial. All substantial amendments will be notified to the ethics committee by providing information including: 1) a brief description of the changes that are included in the amendment and the name of the documents that are modified; 2) an extract of the modified documents, where applicable, showing both the previous and new wording, where applicable; 3) the new version of the modified documents, where applicable, identified with updated number of version and date.

In case any of these amendments concern the subjects, changes will be notified to the subjects with additional consent if needed. In addition, all the information on ClinicalTrial.gov will be updated accordingly.

Dissemination plans {31a}

Subjects will be informed about the results of the study. Results will be presented in agreement with the METC publication statement. Results will be published by the investigators in scientific peer-reviewed papers and possibly also in professional journals. The authorship of the article shall be determined in appropriate consultations based on a considerable contribution to the set-up and execution of the study and active participation in publication. Outcomes of the study will be presented at conferences and meetings, on which data will be reported anonymously. None of the collaborating parties will withhold the public disclosure of study results. Collaborating parties may delay publication for up to three months after analysing the research results if it is applying for a patent or for other important
reasons. All collaborating parties are entitled to examine any form of public
disclosure prior to submission.

**Discussion**

**Intervention product safety**

The prebiotics used in the current study contain acacia gum (AG), partially
hydrolysed guar gum (PHGG), and resistant starch (RS). These are natural
ingredients and widely used in food industry. AG, also known as gum arabic, is
obtained from the stems and branches of *Acacia senegal* and *Acacia seyal* trees. AG
is a nonviscous, soluble, highly branched fibre, and its main components are
arabinose and galactose (63). It is a safe, natural, and plant-based food ingredient
used for many centuries. PHGG is a soluble fibre produced by controlled partial
enzymatic hydrolysis of guar gum (GG) which is derived from guar seeds, and it
consists of galactomannan, which is characterised by high viscosity. In the modern
food industry, GG is used as a food additive in various food products as thickener,
emulsion stabiliser and as fibre source (64, 65). RS refers to the fraction of starch
which is not hydrolysed in the small intestine, but is fermented by bacteria in the
colon. RS could be derived from many common foods, and the RS used in this study
consists of high amylose maize (corn) starch, which is natural and food-grade. Health
benefits and safety of RS as a food ingredient have been confirmed by animal and
human studies (66).

**Intervention product efficacy**

Previous studies which employed *in vitro* and animal models have
demonstrated beneficial effects of AG, PHGG, and RS on health of healthy individuals
or mice with colitis. In a mouse colitis model, rectal administration of AG (30 mg/kg)
showed significant effects in reducing inflammatory markers, and a stronger
therapeutic effect was observed when combining with the anti-inflammatory drug 5-
ASA (67). Another study with a mouse colitis model showed that production of SCFAs
including butyrate was stimulated in the colon when administrated with PHGG and
RS, concurrent with reduced intestinal inflammation and promoted proliferation of
butyrate producing bacteria (68). Using an *in vitro* colon model inoculated with
healthy human faecal material, AG showed potential prebiotic effects, as it promoted SCFA production, especially that of butyrate (69).

Many clinical studies have been performed to investigate prebiotic effects of AG, PHGG, or RS (e.g., modulating the gut microbiome and stimulating butyrate production) and their GI tolerance. AG is slowly fermented compared to other fermentable fibres (70), therefore it is well-tolerated, i.e., associated with less GI symptoms after consumption. In agreement with that, Cherbut et al. found that AG did not induce adverse GI symptoms even when consumed up to a daily dose of 30 g in healthy individuals (71). A study by Marzorati et al. also observed that combining AG with fructo-oligosaccharides (FOS, a well-researched prebiotic) can help to extend the time of fermentation of FOS in the colon (72). With regard to PHGG, no side effects were observed when consumed up to 20-40 g per day (73). Besides favouring butyrate-producing bacteria (74), PHGG also assists bowel function. In addition, increased stool frequency in constipated individuals and improved stool consistency and lower abdominal pain have previously been reported in constipated women (75) and irritable bowel syndrome (IBS) patients (76). Regarding RS, in general, doses of up to 30 g per day are well tolerated in healthy adults (77). In addition, a dose-response study indicated that a daily dose of 20-50 g of RS supplementation for one week resulted in beneficial changes in faecal microbiota and elevated faecal SCFA concentrations in healthy individuals (78).

**Strengths and limitations**

Our study has several strengths. First, the prebiotic mixture was selected among several candidates based on the tolerability and butyrate stimulation capacity. Second, the PREDUCTOME study is the first study in which literature- and *in vitro* study results-based prediction of response is conducted prior to the intervention. Third, this study covers not only the clinical outcomes (e.g., severity score, mucosal inflammation, stool consistency, stool frequency, quality of life, and medication use) but also the gut microbiome (e.g., gut microbiota and SCFA concentrations). Fourth, follow-up periods are added after intervention to monitor the maintenance of remission/response, which will provide additional evidence to evaluate our tipping point hypothesis.

We also acknowledge a few limitations. First, the definition of “responder” or “non-responder” is still not validated yet, thus further research is still needed to
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consolidate it. Second, the single-centre set up may only provide insights of response of UC patients around the Ede area. In case of confirmed hypotheses in our study, a multiple-centre study including different centres throughout the Netherlands, even Europe, is needed for further consolidating the general principle. Third, if succeed, only 25% of subjects will benefit from the intervention in this study, and the extra burden cannot be avoided for the majority. Fourth, a dose of 6 g a day might be overestimated even though it contains a mixture of fibres.

**Trial status**

The protocol (version 4, 26-07-2022) has been approved by the METC Oost-Nederland on the 16th of August in 2022 (Identifier: NL79442.091.22) and was registered on ClinicalTrials.gov on the 13th of October in 2022 (Identifier: NCT05579483). Study recruitment will start in December 2023, and is estimated to be completed around October 2024.

**Declarations**

**Acknowledgements**

The authors would like to thank Dr. Eleni Tsompanidou and Diana Kusuma from Winclove Probiotics B.V. for providing the candidate prebiotics for screening.

**Authors’ contributions {31b}**


Z.L. is the coordinating investigator, E.G.Z. is the principal investigator, and B.J.M.W. is the medical supervisor. All authors have read and approved the manuscript.

**Funding {4}**

This work is supported by Winclove Probiotics B.V. (Amsterdam, The Netherlands). Zhuang Liu acknowledges further financial support from the China Scholarship Council (File No. 201806850091).
Competing interests {28}

The authors declare no competing interests.

Ethics approval and consent to participate {24}

This protocol (version 4) has been approved by the METC Oost-Nederland on the 16th of August in 2022 (Identifier: NL79442.091.22). The trial will be conducted according to the principles of the Declaration of Helsinki (64th WMA Assembly, Fortaleza, Brazil, October 2013), and in accordance with the Medical Research Involving Human Subjects Act (WMO 1998). Written consent form to participate will be obtained from all participants.

Consent for publication {32}

By signing the consent form, all participants give the permission to use their anonymised data for publication in scientific peer-reviewed journals.

Availability of data and materials {29}

Data will be stored at secured hard drives of Wageningen University & Research, and will be locked with a password. All research data will be stored for a period of 10 years after collection. Faecal samples will be stored for 10 years after the study ends, defined as the last data collection point, and then disposed of according to biomedical waste procedures. Raw 16S rRNA gene sequence data will be released into the European Nucleotide Archive database after the manuscript is accepted for publication. Upon reasonable request, any dataset analysed during the current study can be provided.
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Chapter 7

General Discussion and Future Perspectives
The significance of the gut microbiome extends beyond its traditional association with digestion and gut health. Numerous studies have shown that systemic health effects also have associations with the gut microbiome (1). Consequently, an increasing number of studies have been conducted to unravel the complexities of the gut microbiome and explore its potential in health management (2). However, like unique fingerprints, substantial inter-individual variation has been observed in both healthy and aberrant gut microbiomes (3, 4). This poses challenges in developing universally effective approaches for gut microbiome management (5). Simultaneously, it highlights the need to explore novel avenues for the development of personalisation nutrition strategies based on the individualised characterisation of the gut microbiome (Chapter 2). The primary objectives of the research described in this thesis were to unravel the gut microbiome signatures that determine the response of subjects to specific dietary interventions and ultimately guide the development of gut microbiome-based stratification of dietary intervention strategies.

To achieve these objectives, complementary research methodologies encompassing both in vivo and in vitro approaches were employed in this thesis, utilising faecal samples obtained from individuals categorised as healthy, suffering from irritable bowel syndrome (IBS), and suffering from ulcerative colitis (UC). Specifically, Chapter 2 reviewed current gut microbiome-based stratification strategies and provided specific gut microbiome features associated with various clinical conditions, including IBS and UC. Chapter 3 described a study in which host and microbiota data of IBS patients from an intervention cohort and an independent observation cohort were compared and contrasted with the aim to identify baseline microbiota signatures of IBS subjects who achieved a clinical improvement in order to predict placebo response. Chapter 4 described an in vitro batch incubation approach with faecal microbiota collected from three healthy individuals at three different days, using either basal or rich culture medium with polydextrose as carbon source. This study aimed to investigate the impact of donor individuality, faeces collection date, and culture medium type on microbiota composition and metabolic activity during polydextrose degradation. With the same in vitro approach as described in Chapter 4, Chapter 5 focused on the effect of four different dietary fibre (DF) mixtures on the in vitro response and dynamics of microbiome of UC patients, aiming to select DFs that could stimulate intestinal butyrate production of
faecal inocula derived from UC patients. In addition, the impact of baseline microbiomes with low and high *Bacteroidetes* levels on DF efficacy to stimulate butyrate production during incubation was investigated. Based on observations from the previous chapters, Chapter 6 described a study protocol for a clinical trial in mild UC patients. This four-arm randomised double-blind placebo-controlled parallel trial aimed to predict the efficacy of a selected DF mixture (from Chapter 5, after minor composition modification) in mild UC patients based on their baseline faecal microbiome by stratifying them into predicted responders (low faecal *Bacteroidetes* levels) and predicted non-responders (high faecal *Bacteroidetes* levels).

**Profound inter-individual variation in healthy and aberrant gut microbiomes**

Inter-individual variation in healthy and aberrant gut microbiomes has been associated with a range of different factors such as genetic background, age, diet or environment (6). In addition to mother-to-infant connection, co-habitation has been identified as a significant factor contributing to the transmission of the gut microbiome, resulting in a notable sharing of strains (accounting for approximately 12% of the gut microbiome) among cohabiting individuals (7). Moreover, the variance of around half of the microbial taxa at species level occurring in human faecal samples was found to be significantly explained by cohabitation (8). In healthy individuals, there can be considerable diversity in the composition and function of the gut microbiome between different people. Healthy individuals can have different dominant microbial taxa, varying levels of microbial diversity, and distinct functional capacities within their gut microbiomes (4). In Chapter 4, it was also described that specific taxa can be observed in individual microbiomes as the presence of *Methanobrevibacter* and *Megasphaera* was only observed in one of the three subjects. Previous studies have already revealed that *Methanobrevibacter* is only detected in high numbers in the microbiome of approximately one-third of Caucasian individuals, both healthy as well as health-compromised (9, 10). This finding also matches with our findings in Chapter 5 in which *Methanobrevibacter* was only detected in four out of eleven UC patients. Similar observations have been described for several bacterial taxa (e.g., *Prevotella*) as well (9). Moreover, overall microbiota composition as well as the compositional dynamics during incubation were also...
found to be individual-specific. In agreement with our findings, An et al. used a similar *in vitro* approach as described in **Chapter 4** and found that subject individuality explained distinct portions of the overall microbiota composition variation during incubation (11). Another batch study conducted on partially hydrolysed guar gum revealed noteworthy variations in the production of short-chain fatty acids (SCFAs) as well as the microbiota composition between six healthy individuals (12, 13). In terms of the relative abundance of certain taxa in the gut microbiota, we stratified eleven UC patients into high or low *Bacteroidetes* groups based on the relative abundance of the *Bacteroidetes* phylum (ranging from 2% to 33% as determined by quantitative PCR) in their faeces, as described in **Chapter 5**. As expected, the microbiota composition differed drastically between subjects as well the dynamics during the incubation with DFs. This was also reflected in their metabolite production.

Inter-individuality also leads to cohort heterogeneity, even among individuals from the same geographical area and recruited based on similar criteria. In a previously conducted study on two metabolic syndrome cohorts in the Netherlands, pronounced cross-cohort variation in gut microbiome was observed (14). In **Chapter 3**, two cohorts of subjects with IBS that were recruited from the Wageningen area with the same criteria by the same research team also showed distinct gut microbiome differences. It is reasonable to attribute this heterogeneity to the inherent individual variations within the cohorts. Various factors such as the symptom severity or the time of participation of subjects could contribute to this variation. When looking into the IBS symptom severity, subjects in the cohort that received dietary intervention showed significantly higher baseline severity score than those who participated in the observation study. Specifically, more than half of the subjects in the intervention cohort had moderate symptoms based on the Rome IV criteria, whereas around half of the subjects in the observation cohort had mild symptoms. However, a previous analysis on the observation cohort did not support the association between gut microbiome and symptom severity, partially due to the large variability over time in symptom severity scores (15). Recruitment season might play a role in these variation as seasonal variations in diet have been shown to influence the composition of the gut microbiota (16). To this end it is interesting to note that subjects in the intervention cohort were recruited from May to July in 2021, while subjects in the observation cohort were recruited from September to December.
in 2018. Moreover, additional analyses revealed that age and habitual dietary fibre intake of subjects significantly explained the variation of microbiome characteristics in subjects from the intervention cohort (n = 63, **Fig. 1A**), while that was gender and habitual dietary fibre intake in subjects from the observation cohort (n = 55, **Fig. 1B**).

Previous research has revealed an association between age and the gut microbiome, with the gut microbiome of the elderly being more susceptible to a broad range of potentially confounding factors, including dietary choices (17). Moreover, the prevalence of women in cases of IBS has been consistently observed to be notably higher, indicating a predominant occurrence of IBS among women (18). Collectively, the profound inter-individual variation in gut microbiome characteristics still hampers the identification of a robust link between gut microbiome and health. Moreover, conducting comparative studies continues to pose challenges, even when subjects are recruited from the same area and technologies are identical, due to the presence of baseline variations in microbiome and host characteristics.

**Figure 1** Redundancy analysis (RDA) between metadata factors and microbiota composition at genus level of subjects in (A) the intervention cohort and (B) the observation cohort. RDA plot illustrating the relationship between metadata factors (red arrows) and relative abundance of microbial taxa at genus level from each subject (grey dots). The microbiome data were centred log-ratio transformed. Arrows indicate the direction and magnitude of the effect of factors. Permutation tests (n = 999) were used to determine the significance of variance explained by each metadata factor. Only significant factors that showed a p-value below 0.1 are shown. Data from Chapter 3.
Stable and resistant gut microbiomes within a month

In Chapter 1, the gut microbiome is described as an ecosystem. Within this ecosystem, researchers have discovered alternative stable (maintaining a consistent composition and function over time), resistant (withstanding external disturbances or perturbations), and resilient (restoring its equilibrium state after undergoing perturbations) states such as enterotypes with different dominant bacterial taxa (19). In Chapter 4, principal coordinates analysis (PCoA) plots demonstrated that microbiomes collected from healthy subjects at different time points cluster per individual. The fluctuation of microbiota over a short (i.e., one day) or longer period (i.e., one month) was thus minimal, and it also had limited contribution to the variation of either microbiota composition or SCFA production during in vitro batch incubation. Similarly, in Chapter 3, we investigated microbiota composition dynamics of 55 IBS patients after a 4-week period of observation. It is noteworthy that irrespective of their fluctuation in symptom severity, the gut microbiota remained stable over time. More remarkably, within the same study, another 63 IBS patients who received dietary supplement treatments, the microbiota showed strong compositional resistance to those treatments within the same time period. These results collectively indicated the stability of both healthy and aberrant gut microbiomes. Although resistance of aberrant gut microbiomes has not yet been fully understood (20), it may explain why certain disease states or health conditions are difficult to improve.

Different states of the gut microbiome have been shown to be resistant to minor perturbations (9), and in order to transit from one state to another requires a significant perturbation, which involved crossing what is referred to as a tipping point (21). In the gut microbiome of healthy individuals, it was found that short term perturbations (four days to three weeks) were unlikely to induce alterations in terms of microbial diversity (22). In the case of UC, it has been observed that a 2-week intervention of DF did not lead to changes in the gut microbiome (23). However, studies reviewed by Zhang et al. have shown that DF interventions lasting for four or eight weeks have demonstrated positive treatment outcomes in UC patients (24). Similarly, other studies have also reported favourable results in UC patients receiving an 8-week DF intervention (25, 26). Therefore, I speculate that a longer intervention period, such as eight weeks, is necessary to perturbate the aberrant gut microbiomes
and push them into other alternative states that are close to healthy ones. Based on this understanding, Chapter 6 adopts an 8-week intervention strategy using the DF mixture selected in Chapter 5. Additionally, there will be a 4-week follow-up and a 1-year follow-up to assess the resilience of the aberrant gut microbiomes. These measures will allow us to monitor the long-term effects of the intervention.

**Dietary fibre drives gut microbiome variation**

Diet has shown its efficacy to modify the composition of the gut microbiota (6). Among different dietary components, DF could reach the colon and stimulate the growth of specific microorganisms. Therefore, DF has gained increasing attention to manage both healthy and aberrant gut microbiomes (27, 28). Polydextrose (PDX) is a type of soluble fibre, and it is often added to food products to increase their fibre content and provide potential health benefits (29). For gut microbiomes in healthy individuals, in Chapter 4, we found that microbiota composition variation explained by PDX supplementation during in vitro batch incubation had increased from 0% at 0 h to 20% after 24 h of incubation. Moreover, PDX supplementation increased the total amount of bacteria and selectively stimulated the growth of certain bacteria, but in a medium-dependent manner. Specifically, *Bifidobacterium* thrived in the medium simulating the proximal colon, whereas *Bacteroides* flourished in the medium resembling the distal colon. The degradation of DF is a collaborative effort among a group of bacteria that exhibit distinct preferences for environmental conditions (30, 31), such as pH, as well as the energy source (32). Thus our findings serve as a reminder during the selection of DF to exercise caution and choose the cultivation medium carefully, considering its selective support for bacteria. As the gut is a complex ecosystem with varying pH levels, nutrients availability along its different segments, a combination of region-specific media allows for a more targeted analysis of DF degradation and its impact on the gut microbiome.

Nonetheless, these results confirmed the potential for DF in managing and redirecting microbiomes. In addition, ~ 50% of the variation in SCFA production was explained by PDX supplementation at 24 h, reinforcing the main role of DF in SCFA production. With respect to aberrant gut microbiomes, using the same in vitro approach, the supplementation of DFs also induced shifts in terms of composition and production of SCFAs with the microbiome derived from UC patients, albeit in a DF mixture composition-dependent pattern (Chapter 5). Within a confined and
controlled environment, like the *in vitro* incubation vials, it is easy to observe clear microbiome alterations in terms of composition and activity. However, I acknowledge that *in vitro* simulations will not fully replicate the dynamic and diverse conditions present in the human gut, let alone the lack of the complex interactions between the gut microbiome and the host (33). In fact, when DF is supplemented to subjects in *in vivo* study settings, it often does not lead to consistent observations or outcomes. The variability in responses to DF supplementation can be attributed to several factors (34). First, individuals have different baseline diets and gut microbiomes, which can influence how they respond to DF interventions (Chapter 2). Secondly, the type and dose of DF used in supplementation can vary across studies, leading to divergent outcomes. Additionally, the duration of intervention plays a role, as shorter-term studies may not capture the full effects of fibre on the gut microbiome and associated health outcomes as already indicated earlier in this chapter. Furthermore, interactions with other dietary factors and individual lifestyle habits can further contribute to the lack of consistent observations. Lastly, the heterogeneity in study designs, including sample sizes, participant demographics, and methodologies used to measure outcomes, can introduce variations and make it challenging to establish consistent conclusions. Therefore, while DF supplementation holds potential benefits for gut health, the lack of consistency in observations highlights the need for transparency and standardisation of study designs and experimental approaches on one hand, as well as on the other hand more personalised approaches that consider individual factors and optimise DF interventions for improved outcomes (Chapter 2). In this thesis, I demonstrated the effect from a variety of those above mentioned factors including 1) heterogeneity of individuals, 2) habitual dietary intake of individuals, 3) dose and type of DF, and 4) duration of intervention.

**Heterogeneity of individuals and consequences for intervention trial design**

The responses of the gut microbiome to different DFs show remarkable conservation within individuals, indicating a high level of individual consistency (35). For the gut microbiomes in healthy individuals, in Chapter 4, we partitioned the contribution of different factors to microbiota composition and dynamics during its incubation with PDX within 24 h. Among examined factors, donor individuality was confirmed to be an important contributor to the observed variation in microbiota
composition. For the aberrant gut microbiomes, subjects who respond to a certain treatment have a distinct gut microbiome signature, which is also dependent on their clinical condition. For example, in Chapter 3, IBS patients in the intervention cohort showed distinct outcomes, with only 44% of subjects achieving a clinical improvement. Intriguingly, those subjects shared specific baseline host and microbiota features including high symptom severity and lower relative abundances of two genera belonging to the Lachnospiraceae family. Similarly, although not with a DF intervention, UC patients who gained sustained remission from faecal microbiota transplantation (FMT) had relatively low abundance of the Bacteroidetes phylum in their faeces during active disease (36). Furthermore, as described in Chapter 5, UC patients who had low relative abundance of the Bacteroidetes phylum (~4%) also showed distinct microbiota composition at genus level compared to those with high Bacteroidetes (~14%). Moreover, the dynamics of microbiota composition and metabolic activity exerted a Bacteroidetes-dependent manner, with a wider range of potential butyrate-producing bacteria (i.e., Subdoligranulum, Megasphaera, and Holdemanella) being selectively stimulated in low Bacteroidetes incubations, whereas potential inflammation-enhancing bacteria (i.e., members of the family Enterobacteriaceae and genus Prevotella 9) were selectively stimulated in high Bacteroidetes incubations. The design of the clinical trial described in Chapter 6 takes this inter-individuality into account with a set-up dedicated to microbiome-based subject stratification. To this end, we would only include patients with mild symptoms and stratify them into predicted responders with low faecal Bacteroidetes levels and predicted non-responders with high faecal Bacteroidetes levels. I also acknowledge that this design strategy might oversimplify the correlation between microbiota composition and responsiveness, however, I consider this an important first step towards improved efficacy of dietary interventions. Furthermore, in recent years, machine learning (ML), as a subfield of artificial intelligence (AI), has shown high potential to discover possibly hidden patterns in gut microbiome characteristics, which helped with disease diagnoses (37) or prediction of treatment efficacy (38). Therefore, in future trial design efforts, baseline microbiota-based stratification of subjects should integrate AI tools such as ML algorithms to deal with data complexity and microbiome variation in different intervention studies.
Habitual dietary intake of individuals

Individuals on a Western diet, which is defined by high dietary intake of saturated fats and sucrose and low intake of DF, have shown aberrant gut microbiomes and increased occurrence of diseases (39). Healthy individuals with deficient habitual DF intake were found to be more likely to show microbiome responses to different DFs as revealed by Holmes and colleagues (35). The authors further emphasised that individuals who normally consumed high levels of DF should not seek for supplemental DF intake. Similarly with respect to aberrant gut microbiomes, habitual DF intake of IBS patients was found to be associated with gut microbiota variation of subjects in two cohorts in Chapter 3 (Fig.1). Specific dietary components, such as onions and cabbage have shown to be triggers associated with IBS symptoms in a majority of patients with IBS (40, 41). Therefore, future research needs to increase the emphasis on the habitual dietary intake prior to the interventions. With the observed association between habitual DF intake and gut microbiome in Chapter 3, future research might look into how baseline habitual DF intake affects the treatment efficacy. To this end, in Chapter 3, we trained an ML model with the dataset of baseline habitual DF intake, gender and microbiota of subjects in the observation cohort data as input. Remarkably, this model robustly predicted the outcome in the placebo group with an accuracy of 92%. Collectively, habitual DF intake of subjects determined the response of the microbiome, which affects the outcome of treatments.

Dose and type of dietary fibre

Both the overall dose and types of DFs consumed are important when considering the impact on gut microbiome. For instance, Deehan et al. conducted a study demonstrating that chemically modified resistant starches, despite having only subtle structural variations, can elicit distinct and highly specific impacts on the gut microbiome, resulting in alterations in the production of SCFAs (42). Notably, in that study, the dominant effects observed within each treatment group were remarkably consistent and dose-dependent, reaching a plateau at a dosage of 35 g. Likewise, Puhlmann et al. also discovered that the influence of DF on the overall composition of the gut microbiota of subjects at risk for type 2 diabetes was reliant on the dosage of administration (43). However, a high amount of DF supplementation might not be
advantageous for subjects who experience symptoms such as bloating and flatulence when consuming higher DF loads. A meta-analysis study revealed that a daily dose of 5 g DF was sufficient to induce changes in the gut microbiome, particularly by promoting the growth of bifidobacteria (22). Therefore, for UC patients in Chapter 6, we opted for a DF mixture that minimises gas production and avoids triggering bloating symptoms, administering it at a dosage of 6 g per day.

There is an extensive variety of different DFs, which differ in structure, physicochemical characteristics and physiological effects (44). A wide variety of microbial enzymes have been discovered that play a role in breaking down DFs. The breakdown of a particular type of DF often involves several enzymatic processes involved with numerous microbes, leading to the production of SCFAs (45). Moreover, different species of microbe have the capacity to degrade specific DF types (46). Given the considerable inter-individual variation in gut microbiome as described in previous sections, it is reasonable to speculate that the distinct response of the gut microbiome to DFs among individuals may be partially attributed to the specific types of DFs. For instance, although the general benefits of DF in IBS treatment have been documented, the type of different DFs determined their effectiveness in IBS symptom relieve. Specifically, soluble DF has demonstrated treatment efficacy in IBS, whereas insoluble DFs in some cases worsened the outcomes (47, 48). Likewise, in one IBS study, significant improvements in symptoms and alterations in gut microbiota composition (e.g., increased relative abundances of two genera belonging to the Lachnospiraceae family) were observed after following a diet rich in soluble DF for a period of four weeks (49). In UC, not all DFs are equal in terms of treatment efficacy and microbiome modulation due to their complex structure and diverse subtypes (50). Having a diversity of DFs relates to a highly diverse and potentially more stable microbiome (45). In the in vitro batch incubation experiments described in Chapter 5, four DF mixtures that were composed of two to three different individual DF sources were shown to result in DF mixture composition-dependent pattern of microbiota composition as well as the concentrations of SCFAs and gaseous metabolites. Among those DF mixtures, the mixture containing 40% acacia gum, 40% partially hydrolysed guar gum and 20% resistant starch induced the highest stimulation of SCFA production, with the lowest gas production. To ensure the cost-effectiveness of this potentially marketable product, modifications were made to the composition of the DF components. The
proportion of resistant starch, which is more economical, was increased to 40%, while the percentage of partially hydrolysed guar gum, which is more expensive, was reduced to 20%. As a result, a combination of 40% acacia gum, 20% partially hydrolysed guar gum, and 40% resistant starch was chosen as the intervention treatment in Chapter 6.

**Duration of intervention**

Many studies have investigated the effects of DF intervention duration on the gut microbiome of healthy individuals. For example, a two week DF intervention at a dose of 40-50 g per day induced compositional changes of the gut microbiota featured with increased relative abundances of carbohydrate utilising bacteria (51). However, these alterations might be transient. In another randomised double-blind cross-over study in which healthy subjects consumed 16 g of DFs (fructooligosaccharides (FOS) or galactooligosaccharides (GOS)) per day for two weeks, an altered microbiota characterised by increased relative abundance of *Bifidobacterium* was observed (52). After a 4-week washout period, the gut microbiota returned to its initial state before the intervention, indicating that the microbiota alteration induced by the DF were transient. This also suggests that without ongoing consumption of these specific DFs, the observed microbial alterations dissipate within a relatively short period, demonstrating the resilience of the gut microbiomes in healthy individuals towards perturbations. Therefore, maintaining a continuous DF intake may be necessary to sustain the beneficial effects on the gut microbiome. In other words, a powerful perturbation by sufficient DF intake duration is needed to push the gut microbiome over a ‘tipping-point’ to reach another stable state as described in Chapter 1. To further consolidate this idea, in Chapter 6, after an 8-week intervention, two follow-up assessments on gut microbiome will be conducted. A 4-week intervention duration is commonly used in IBS studies (53, 54), with observed gut microbiome alterations. However, in Chapter 3, a 4-week intervention with DF or other suppletions did not induce significant gut microbiome alterations, including those who achieved a clinical improvement. I speculate that this is partly due to the resistance of the gut microbiome. On the other hand, improved outcomes were not concomitant with alterations in gut microbiota composition, which requires further research towards the link between both aspects in the situation of IBS.
Considerable research has demonstrated the benefits of DF on the management of UC as documented by Yusuf et al. (55) and Zhang et al. (24). Generally, interventions lasting for four or eight weeks have demonstrated positive treatment outcomes. For instance, an intervention involving germinated barley foodstuff (GBF), which is rich in DF, significantly reduced clinical activity and increased the abundance of *Bifidobacterium* and *Eubacterium limosum* in eighteen patients with mildly to moderately active UC over a 4-week period, without any adverse effects (56). Faghfoori and colleagues conducted two additional studies on GBF, extending the intervention duration to eight weeks (25, 26). Although they did not specifically investigate the gut microbiome, they also observed a significant reduction in inflammation and clinical symptoms in UC patients.

It should also be noted that our current understanding of the impact of intervention duration on efficacy and the gut microbiome is limited. Ideally, the intervention duration should be sufficient to ensure biological efficacy. However, there are important considerations to take into account. On one hand, shorter interventions lasting 1-4 weeks have been reported to yield significantly higher placebo response rates from IBS patients compared to trials lasting more than eight weeks (57). On the other hand, prolonged interventions over several months have resulted in higher dropout rates and reduced compliance (58). Therefore, in order to enhance the likelihood of a successful intervention, we implemented an 8-week DF intervention in the trial design described in Chapter 6. In addition to the 8-week intervention, we have planned two follow-up assessments to examine the gut microbiome and the remission status of the subjects. These follow-ups will provide valuable insights into the long-term effects of the intervention and help us assess its sustained impact.

**Future perspectives**

The field of gut microbiome research is expected to play a significant role in personalising dietary interventions and optimising health outcomes. Advancements in high-throughput sequencing technologies and bioinformatics will facilitate the characterisation of the gut microbiome at a more detailed level, allowing for a more comprehensive understanding of its functional capabilities. Furthermore, advances in AI and ML algorithms are likely to enhance our ability to analyse complex gut microbiome data and derive meaningful insights (59). By integrating gut microbiome
profiles with other health-related data, such as clinical parameters and lifestyle factors, sophisticated predictive models can be developed to generate highly personalised dietary recommendations (37, 60).

Overall, the future of gut microbiome-based stratification of dietary intervention strategies holds great promise. On top of the in vitro DF pre-selection approach that helps identifying most promising DFs and patient stratification as described in this thesis, combining advanced omics technologies, sampling devices from different gut sections, and continuous measurements of health parameters could substantially improve our understanding of gut microbiome functionality. By integrating these data with AI-driven analysis, researchers may be able to identify personalised dietary interventions that can optimise health outcomes by leveraging an individual’s unique gut microbiome profile.
References


General Discussion and Future Perspectives


Summary
Emerging evidence has suggested that the gut microbiome plays an essential role in human health and well-being. In Chapter 1, I provided a general introduction to the gut microbiome, including its composition, functionality and roles in various clinical conditions. Nutritional intervention has been recognised as a robust and attainable modulation strategy targeting the gut microbiome with the ambition to prevent or treat disorders/diseases and eventually improve human health and thereby quality of life. For example, short-chain fatty acids (SCFAs) such as butyrate are produced by gut bacteria when they break down food components, especially dietary fibre (DF), and these SCFAs are considered beneficial for our health. However, due to the considerable inter-individual variability between subjects, especially in the gut microbiome, as described in Chapter 1, universal dietary recommendations demonstrated to have different effects in different subjects, with only a fraction of subjects responding positively. The positive side of this individual effect is that it offers the possibility to use the gut microbiome as a pool of potential biomarkers for distinguishing “responders” and “non-responders” to specific treatments, which subsequently can be used to classify subjects with the ambition to increase prevention or treatment efficacy. Therefore, Chapter 2 outlines the need for human gut microbiome stratification with currently available options including 16S rRNA gene-, metagenome-, and metabolite-based stratification strategies. Moreover, specific gut microbiome features in irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) were summarised, and a 5-step strategic approach to apply microbiome-based stratification to obtain improvements in dietary intervention efficacy was proposed.

The mechanisms underlying IBS clinical improvement remain unclear, but dietary intervention studies often observe high clinical improvement rates in placebo groups (35-40%). In Chapter 3, we used data from an IBS intervention cohort and an independent observation cohort to identify subjects who experienced clinical improvement. Improvements were assessed using the IBS Severity Scoring System (IBS-SSS) over a four-week period. Although we observed cohort-specific host- and microbiome characteristics at baseline, in both cohorts, subjects who achieved a clinical improvement were characterised by a higher IBS-SSS score and a lower relative abundance of genera belonging to the Lachnospiraceae family. To perform cross-cohort prediction of clinical improvement, a machine learning (ML) approach
was applied using host- and microbiota-derived data as input. Although the predictive power of the model drastically dropped due to cohort heterogeneity, the model trained on the observation cohort datasets correctly predicted whether or not IBS subjects will show clinical improvement for 12 out of 13 subjects of the placebo group in the intervention cohort. This study highlighted the potential of ML in identifying subjects who may or may not achieve a clinical improvement from to placebo treatment, which can be instrumental in setting up IBS clinical studies.

In addition to the insights from subject stratification that allows for selection of subjects that are likely to respond to dietary intervention, the selection of effective DF also determines the intervention efficacy. The faecal batch culture (FBC) model is often chosen to assist in DF selection by mimicking colonic fermentation. However, FBC is susceptible to various confounding factors such as the individuality of donor faeces, date of faeces collection, and choice of cultivation medium. To examine the impact of these factors, an FBC experiment was performed. FBCs were inoculated with faecal microbiota from three healthy donors sampled on three different days, using either basal or rich culture medium with the DF polydextrose (PDX) as a carbon source (Chapter 4). The results revealed that both donor individuality and medium type have major impacts on the composition and activity of PDX degrading consortia, whereas the impact of faeces collection date was limited. Independent of the medium, two genera (i.e., Blautia, Fusicatenibacter) belonging to the Lachnospiraceae family were stimulated by PDX supplementation, indicating their potential role in PDX degradation. Of interest is that bifidobacterial growth was more stimulated in rich medium with PDX as a carbon source, whereas growth of members of the Bacteroidetes was more stimulated in basal medium with PDX as carbon source. Based on these findings, it was recommended that variations in medium and donor samples should be taken into account when planning and interpreting in vitro incubation studies. This will allow for a more accurate selection of DFs for intervention studies and ultimately improve their efficacy.

Patients with ulcerative colitis (UC), one of the chronic IBDs, have an aberrant gut microbiome characterised by a deficiency in butyrate production, a lower level of the Firmicutes phylum, but a higher level of the Bacteroidetes phylum as revealed by previous studies. In recent studies, it has been observed that sustained remission in UC patients who underwent faecal microbiota transplantation, was associated with an increased butyrate-production capacity. Importantly, sustained remission was only
observed in patients with relatively low relative abundances of *Bacteroidetes* (less than 10%) in their faeces during active disease. Thus, we hypothesised that the gut microbiota of individuals with UC with a lower level of *Bacteroidetes* would exhibit a higher butyrate-producing capacity that could be stimulated by specific DFs and ultimately reduce mucosal inflammation. To test this hypothesis, we tested four different DF prototypes (containing various DF mixtures) in an FBC setting with inocula derived from faecal samples of high or low *Bacteroidetes* UC patients (Chapter 5). Based on the conclusions described in Chapter 4, the experimental setup was chosen and inocula were incubated in rich medium. The results revealed that butyrate production was stimulated by all four DF prototypes, in which a mixture of acacia gum, partially hydrolysed guar gum and resistant starch showed the highest stimulation. It was noteworthy that incubations with inocula derived from faecal samples with low level of *Bacteroidetes* selectively enriched a wider range of potential butyrate-producing bacteria (i.e., *Subdoligranulum, Megasphaera*, and *Holdemianella*) in addition to *Bifidobacterium* and *Lactobacillus*. On the other hand, potential inflammation-inducing bacteria (e.g., members of the family *Enterobacteriaceae* and genus *Prevotella*) were selectively enriched in incubations with inocula derived from faecal samples with high levels of *Bacteroidetes*.

Based on the observations in Chapter 5, a research protocol was proposed in Chapter 6 for a dietary intervention study, the so-called PREDUCTOME study, using a double-blind placebo-controlled parallel design in patients with mild UC. The aim of this study is to achieve prediction of dietary intervention efficacy in mild UC patients based on faecal microbiome signatures. In brief, mild UC patients will be assigned to either a predicted responder or predicted non-responder group based on their faecal *Bacteroidetes* levels, and will undergo an 8-week dietary intervention in which they will either consume a prebiotic (selected DF mixture from Chapter 5 with minor composition modification) or placebo. This study has been approved by the medical ethics committee and is still in the early stages of logistic planning at the time of writing of this thesis. Once completed, this study will provide further evidence regarding the effectiveness of subject stratification (Chapter 5) and the FBC pre-screening (Chapter 4 & 5) approaches. Furthermore, it will offer valuable insights into the efficacy of selected prebiotics and enable us to test our hypothesis on the feasibility of using *Bacteroidetes* as a biomarker to enhance the success rate of interventions in patients with mild UC.
Finally, in Chapter 7, all the findings from this thesis were discussed, along with a summary of the remaining challenges and future perspectives in this field of research.
Appendices
## Co-author Affiliations

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Prokopis, your unwavering assistance has been a guiding light. Whether it’s clarifications on NG-tax or unravelling the complexities of Galaxy, you’ve always been there with a clear and patient explanation. I am sincerely appreciative of your support. Anna, your willingness to share your experiences whenever challenges arose has been invaluable. Your insights have often provided new perspectives that helped navigate through obstacles for many people. Federica, your innate sweetness and kindness radiate through your interactions with everyone. Thank you for opening your space for our enjoyable hot pot gatherings. Evy, your presence in the office has been a true delight. The thoughtfulness you put into the balloons and decorations for my birthday was heart-warming and memorable. Lieke, the discovery that we both work at 6027 was a testament to the smallness of our world and the unexpected connections it holds. To each of you, I extend my gratitude for contributing to the mosaic of experiences that this office has offered. You’ve made this space more than just a workplace; you’ve transformed it into a community filled with camaraderie and shared moments.

To my other colleagues from MIB, Detmer and Clara, the discussions we’ve shared during the MolEco meetings have been truly insightful. Our exchanges have added depth to my understanding, and I am grateful for our interactions. To my exceptional gut group mates (Marie-Luise, Marina, Martha), our collaborative efforts in organising the online symposium during the challenging times of COVID were a
remarkable achievement. Each one of us played a crucial role, and I commend us all for a job well done. Marie-Luise, your warm and engaging personality is a gift that allows you to connect effortlessly with anyone. Your mutual support throughout this journey has meant a lot to me, and I extend my heartfelt gratitude. Marina, your hospitality in inviting us for a BBQ at your place created cherished memories. I wish you all the success and happiness in your endeavours. Martha, I enjoyed our talk the canteen, sometimes a bit over break ;). Your ever-present smile radiates positivity, and I thank you for being a constant source of sweetness in our workplace. Kelly, our shared passion for food has added an extra layer of enjoyment to our conversations. Sharing some recipes of Asian cuisine has been a delightful experience, and I look forward to the day when I can try your handmade noodles. Diana, your academic diligence and attention to detail when reviewing our manuscript have not gone unnoticed. Your thoroughness in identifying both significant and minor errors is greatly appreciated. I am grateful for your contributions. Sofia and Louis, our discussions on MLD have been intellectually stimulating. I wish you both the best as you approach the completion of your PhDs. Gwen, your initiative in organising volleyball brought joy and a healthy dose of competition to our days. It was a pleasure to be a part of it. Baris, your infectious laughter has left an indelible mark. Your positivity is contagious, and I encourage you to keep spreading it. Pavlo, our bouldering escapade was an adventure. Thank you for sharing your skills and making it an enjoyable experience. Iris, your patience in answering my questions has been instrumental in my growth. I extend my best wishes to you as you pursue success in WholeFiber. Maryse, the Dutch lunch you organised was a delightful experience, even if I struggle to recall every word I learned. Your efforts were appreciated. Nancy, your knack for injecting humour into conversations is a constant delight. Carolina, our lunch talks have been enriching, and I value the insights we’ve shared. Ruth, it was so nice to catch up with you in Switzerland last year. Wish you all the best in Nestle. Michelle, congratulations on your new-born baby! Your joy is shared among us, and I wish you all the happiness in this new chapter. Menia, may success follow you in your postdoc journey. Janneke, make the most of your time in Denmark. Emmy, Valentina A, Kate, Reiner, may the successful completion of your PhDs open new doors of opportunity. I would also like to thank Sharon, Jannie, Carrie, Marjet, Sudarshan, and many other colleagues for being there with me during the PhD journey.
To my Chinese group colleagues. Ran, you were the first PhD I had the pleasure of meeting in MIB, and your assistance during my early days here was invaluable. I extend my best wishes for your journey in Shanghai. Ying Z, our numerous conversations and exchanges during your time in MIB have been enlightening and engaging. Your insights have been impactful, and I wouldn’t be surprised if I soon address you as a professor. Best of luck on your path. Caifang, your inherent kindness shines through in all your interactions. May your postdoc venture in Hong Kong bring you the success you truly deserve. Taojun, the memories and experiences we shared during our time in Erwin’s group hold a special place. I wish you a future filled with great accomplishments and happiness in US. Yang, our journeys at MIB began together, and over the years, we’ve shared both moments of joy and those of challenges. I have every confidence that your next chapter will be just as bright. Good luck! Chen, our shared moments on the basketball court and afternoon walks have been some of the most cherished. The fact that you’re uniting with Ying L in the US brings me immense happiness. Yifan, Jie, and Peng, I want to express my gratitude for sharing your invaluable experiences in the Netherlands. Your insights have been immensely helpful. Dailing, your positive attitude is truly inspiring. Keep that spirit alive, for you have the capacity to achieve great things. You’ve got this! Tuo, our trip to Haarlem was an enjoyable escapade, and I’ll always remember it as my first painting in years. May your future be filled with equally delightful experiences. To each of you, thank you for being a part of my journey at MIB. The camaraderie and shared moments have made this experience truly special.

To my wonderful students. Berna, you hold the distinction of being my first BSc student, and I’m truly grateful for the dedication you poured into the review paper. Your contributions were invaluable, and I appreciate your commitment to the project. Daria, your relentless work ethic and the abundance of innovative ideas you brought to the table have left a lasting impression on me. Despite the challenges posed by COVID, your determination carried you through to the completion of your thesis. Your resilience is nothing short of remarkable, and I’m proud of your accomplishments. Esther, big congratulations on achieving your degree and getting a job. Your unwavering dedication and your down-to-earth nature have not only been a privilege to witness but also a true source of inspiration. Guiding you and watching
your growth has been an incredible journey. I stand proud of all that you have achieved.

To my friends in the Netherlands. **Marije**, we have known each other since I moved to the corridor 15A in 2019. You are so kind to people around you. Although I had to move out after one year, we still manage to have some tea time together to catch up every now and then. I wish you success with your study and let’s keep in touch. **Ayoub**, I am so glad to know you by Marije. Every time I stay with you guys, I feel very relaxed and want to talk about anything interesting. I wish you all the best! Hey **Edwin** (foodie/TikToker/Influencer), what is the recent food trend in NL? You are a kind person and a good friend! Thank you for letting us joining your food tour. May your reach on social media continue to soar, and here’s to reaching 1 million followers soon! Hello **Amy**, Amy, how are your adventures unfolding across South America? Your adventurous spirit is truly inspiring, as you fearlessly explore the corners of the world. I wish you endless joy and fulfilment in your pursuits. Hi **Sandra**, you are such a kind and patient person. I wish you and **Jurriaan** have a beautiful weeding! Hey, **Xintong** and **Derk**, our escape room time was so fun and hot. Thank you for brainstorming together.

To my Chinese friends. **Li**, you are such a good and warm-hearted friend. Navigating NL with your little car was a memorable adventure, and your kindness made it all the more enjoyable. **Xueyuan** and **Mu**, you two are like my big sister and brother. Your hospitality and the joy we shared at your place have etched precious memories in my heart. **Zhan, Zulin, Danlei, Lintianxiang, Kangni**, how my life in Wageningen will be like without your companion? We spent half time sharing cooking and the other half playing Mahjong, which often stretched until the early hours. Food is a big part of our life, but Mahjong is indispensable. **Qiuhuizi** and **Zulin**, my fellow squash enthusiasts, you made up most of my sports time in de Bongerd. Of course, there is also my food specialty ‘oil spill noodles’ that you like the most. **Shutong, Yutong, Ruolei, Shenyu**, I had good memories about our unforgettable trip to Norway, alongside **Zulin**. Each moment we shared, the laughter we echoed, and the beauty we discovered together will be remembered. Distance hasn’t dimmed the camaraderie between my companions from NJAU – **Yanan, Zhenxiang**, and **Yin**. Our enduring connection perseveres despite the span of time zones.
I also would like to express my heartfelt gratitude to Prof. Zhu, my supervisor during my MSc. Your introduction to the captivating realm of microbiomes transcended the confines of research, expanding the horizons of my knowledge. I appreciate very much of your invaluable guidance. Dr Shen, my supervisor during my BSc. Your mentorship has been a cornerstone of my growth, enabling me to gather substantial insights.

My dearest Wing Kie, my Wing. I am so lucky and grateful to have you in my life. From the very moment our paths intertwined, we’ve engaged in daily conversations and shared countless moments, creating an unbreakable bond. Gradually, you became a major part of my life. Our shared adventures have painted vibrant memories spanning across Germany, Austria, Switzerland, Italy, Croatia, Malta, and Portugal. Each destination holds a unique chapter of laughter, discovery, and connection between us. Our treasured 'sweet weekend' file stands as a testament to our meticulous planning and the excitement we infuse into every journey we embark upon together. Love you, dear! Not only have you become an indispensable part of my life, but your parents have also enveloped me in their warmth, becoming an extended family that I hold dear to my heart. To Uncle and Auntie, thank you for your hospitality, constantly nurturing me with the flavours of Cantonese cuisine, a taste of home that resonates deeply within me.

最后，我最爱的家人们! 爸爸和妈妈，语言无法形容我有多么思念你们! 你们坚定的支持一直是我生活中的精神支柱。与你们分隔在异国他乡, 让我更加觉得那些在一起生活的时光是多么地值得珍惜。多少个夜晚, 我都会在梦里见到你们为家庭忙碌的背影。爸爸, 你每次朴实的教诲构成了我基本的待人之道。妈妈, 我还是无法在厨房复刻你能做出的好多味道。我迫不及待想回到家, 与你们一起共享久违的家宴。我爱你! 亲爱的大姐, 秋亚, 在我出国的这些年, 我很感激你为这个家庭的付出。作为家中的长女, 你尽到了责任。每个周末, 我们的 线上‘家庭会议’成了我们之间联系的桥梁。我爱你, 祝你(们)幸福! 我另一位亲爱的姐姐刘洁和姐夫段康, 感谢你们对爸妈的照顾。你们付出的爱将我们紧密地联系在一起。祝福你们生活美满! 还有我两个可爱的外甥女, 段羽萱和段熙睿, 你们的到来为我们的生活带来了太多的欢声笑语。你们可爱又幼稚的问题总是能带给我一丝温暖。希望你们在继续探索世界的同 时,不要失去那份好奇心。等我们下次见面, 不要忘记给舅舅一个久违的亲亲。

With gratefulness and love,

Zhuang Liu
Wageningen, September 2023
About the Author

huang Liu was born in the Year of Rooster in Henan, China.

In September 2011, after finishing high school, he studied Animal Science in Nanjing Agricultural University (NJAU) as he has always been an animal enthusiast. By the end of his BSc, he got fascinated by the bacteria in the gut during a lecture given by Prof. Weiyun Zhu. Later on, he resolved to dedicate his BSc thesis to the development of strategies aimed at reducing methane emissions through microbiota-targeted interventions under the supervision of Prof. Weiyun Zhu and Dr Junshi Shen at the Laboratory of Gastrointestinal Microbiology in NJAU.

In September 2015, advancing his pursuit, he seamlessly transitioned into an MSc program within the same laboratory, where he has dedicated himself to exploring the link between gut microbiota and nitrogen nutrient utilisation in the large intestine of
growing pigs. His immersion extended beyond academic boundaries, as he actively participated in international symposium and academic events, where he first got acquainted with Dr Erwin G. Zoetendal.

In June 2018, his academic excellence was rewarded with the NOVUS International Graduate Scholarship. This accolade granted him the opportunity to undertake an internship in the headquarter of NOVUS Int. located in St. Louis, United States. During the internship, he visited the leading institute and university in the field of animal production, and finished a project on the interplay between feed acidic additives and gut health of broilers.

In October 2018, Zhuang embarked on his journey as a PhD candidate at the Laboratory of Microbiology in WUR. Engaged in a dynamic collaboration with Winclove Probiotics B.V., his work unfolded under the supervision of Prof. Hauke Smidt, Dr Erwin G. Zoetendal and Dr Jacoline Gerritsen. This project seamlessly integrated in vitro and in vivo methodologies, showcasing the profound potential of gut microbiome-based approaches for stratifying dietary interventions—a pivotal theme expounded upon within this thesis.

In XX 2023, a captivating journey anticipates its unfolding...
Appendices

List of Publications


2. Zhuang Liu*, Maartje van den Belt, Kelly Klomp, Diana M. Hendrickx, Ben J. M. Witteman, Taojun Wang, Nicole J. W. de Wit, Hauke Smidt, Erwin G. Zoetendal. Placebo-Induced Clinical Improvement in Irritable Bowel Syndrome Can Be Predicted From Host and Microbiota Observational Data. (Manuscript in preparation)


* Corresponding author

† Shared authorship
## Overview of Completed Training Activities

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<th>Discipline specific activities</th>
<th>Organising institute</th>
<th>Year</th>
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<tr>
<td>Annual gut day 2018</td>
<td>WUR</td>
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<td>The Intestinal Microbiome and Diet in Human and Animal Health</td>
<td>VLAG</td>
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<td>Summer School on microbial community modelling</td>
<td>KU Leuven</td>
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<td>Introduction to R</td>
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<td>Applied statistics</td>
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<td>AIO/Postdoc meeting</td>
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About the Cover

‘A person relies on their attire’.

In the realm of books, a captivating and visually appealing cover has the power to captivate readers and ignite their passion for reading. With this notion firmly in mind, Zhuang embarked on a journey of self-learning basic illustration through online resources. His goal? To adorn his PhD work with a splendid attire, a cover that would entice and intrigue anyone who laid eyes upon it.

Despite decades of technological advancements, obtaining a clear ‘image’ of the microbiome remains elusive. The front cover showcases a captivating image of a black hole at its centre, surrounded by a luminous microbiome. The orbiting red and green nebulae symbolise the signals that could potentially be derived from the microbiome. The thesis title word Microbiome features two distinctive fingerprints filling in the letter ‘o’. One fingerprint 🟢 belongs to Zhuang, while the other 🟠 belongs to Wing Kie, Zhuang’s partner. In the bottom right corner, the author’s name is elegantly inscribed in both traditional Chinese characters and English. The family name is emphasised using the distinctive format of a traditional Chinese seal, adding an air of prestige and authenticity.

Similar to fingerprints, the microbiome is unique to each individual. The back cover showcases a captivating array of 35 distinct fingerprints, each infused with a vibrant spectrum of colours. These exquisite patterns are derived from the two original and unparalleled fingerprints found on the front cover.

The concept of incorporating fingerprints on the book cover drew inspiration from the design of the cover of Sapiens: A Brief History of Humankind by Yuval Noah Harari. Likewise, the notion of including the depiction of a black hole was influenced by Christopher Nolan’s film Interstellar.

The cover was crafted by the author, Zhuang Liu, who personally designed it. The creative process involved utilising the advanced capabilities of Adobe Illustrator 2023 to bring the vision to life.
Colophon

The research described in this thesis was conducted as a collaborative PhD research project between MIB-WUR and Winclove Probiotics B.V.. Chapter 3 was conducted within the IBSQUtrition consortium (TKI-16012). Zhuang Liu was supported by a fellowship from China Scholarship Council (File No. 201806850091) and Winclove probiotics B.V..

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Cover design by Zhuang Liu.

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Towards Gut Microbiome-Based Stratification of Dietary Intervention Strategies

ZHUANG LIU

2023