THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Effects of FODMAPs and gluten on irritable bowel syndrome - from self-reported symptoms to molecular profiling

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Dedication

To my lovely family and friends

- past, present, and future

“I have no special talent. I am only passionately curious”

- Albert Einstein

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ABSTRACT

Irritable bowel syndrome (IBS) is a complex disorder of gut-brain interactions. The diagnosis of IBS is based on subjective reporting of abdominal pain and altered bowel habits in the absence of any clinical alterations of the gut or other pathological conditions. Dietary regimens for symptom management include a low fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs) diet and a gluten-free diet. However, scientific evidence supporting these dietary recommendations for managing IBS symptoms is weak: trials have been non-blinded and underpowered. While mechanistic understanding and objective markers of response remain scarce. Therefore, the aim of this thesis was to conduct a large double-blind study to investigate the effect of FODMAPs and gluten on symptomatic and molecular data including 16S rRNA analysis of the gut microbiota and metabolomics analyses, both at a group and subgroup (differential response) level. The resulting data served also to assess the accuracy of the Bristol Stool Form Scale (BSFS) used in IBS subtype diagnosis, and thus overcome the lack of objective evaluation of IBS symptoms.

Trial data revealed that gluten caused no symptoms and FODMAPs triggered only modest symptoms of IBS, albeit with large inter-individual differences. Subjective reporting according to the BSFS conformed only modestly with stool water content in IBS, warranting caution towards IBS subtyping. FODMAPs increased saccharolytic microbial genera, phenolic-derived metabolites and 3-indolepropionate, but decreased bile acids. The genera *Agathobacter*, *Anaerostipes*, *Fusicatenibacter*, and *Bifidobacterium* correlated with increased plasma concentrations of phenolic-derived metabolites and 3-indolepropionate, i.e, metabolites related to decreased risk of incident type 2 diabetes and inflammation. Indeed, among FODMAP-related metabolites, only weak correlations to IBS symptoms were detected, as in the case of 3-indolepropionate to abdominal pain and interference with quality of life, warranting further investigation. Gluten displayed a modest effect on metabolites involved in lipid metabolism, including carnitine derivates, an acyl-CoA derivate, a medium-chain fatty acid, and an unknown lipid, but with no interpretable link to health.

No molecular markers of a differential response were found, despite a comprehensive exploration with multiple analytical approaches. This could be explained by the absence of baseline variables, such as other omics layers or psychological factors, that could have determined the difference.

In summary, the results indicate that gluten does not cause IBS symptoms. Moreover, the minor effect of FODMAPs on IBS symptoms must be weighed against their potential beneficial health effects. While the complexity of IBS likely explains the absence of molecular evidence for differential responses, such data analytical approach has potential where clear benefits of dietary interventions exist. Finally, the use of BSFS should include training for self-assessment, as a tool for subtyping IBS.

Keywords: irritable bowel syndrome, randomized clinical trial, double-blind, FODMAPs, gluten, Bristol Stool Form Scale, fecal consistency, stool water content, microbiota, short-chain fatty acids, metabolomics, personalized nutrition, differential responses, metabotyping

This thesis is based on the work covered in the following manuscripts/papers, referred to by Roman numerals in the text:

I. **Nordin E**, Brunius C, Landberg R, Hellström PM. Fermentable oligo-, di-, monosaccharides, and polyols (FODMAPs), but not gluten, elicit modest symptoms of irritable bowel syndrome: a double-blind, placebo-controlled, randomized three-way crossover trial. *Am J Clin Nutr* 115, 344–352 (2022).

II. **Nordin E**, Brunius C, Landberg R, Hellström PM. FODMAPs – Do they really affect IBS symptoms? (submitted)

III. **Nordin E**, Hellström PM, Brunius C, Landberg R. Modest conformity between self-reporting of Bristol stool form and fecal consistency measured by stool water content in irritable bowel syndrome, a FODMAP and gluten trial. *Am J Gastroenterol* 2022 1;117(10):1668–1674 (2022).

IV. **Nordin E**, Hellström PM, Vuong E, Ribbenstedt A, Brunius C, Landberg R. IBS randomized crossover challenge study: FODMAPs alter bile acids, tryptophan and phenolic-derived metabolites, while gluten modifies lipid metabolism (submitted)

V. **Nordin E**, Hellström PM, Dicksved J, Pelve E, Landberg R, Brunius C. FODMAPs not gluten, alter the microbiota and associate with the metabolome in irritable bowel syndrome (submitted)

VI. **Nordin E**, Landberg R, Hellström PM, Brunius C. Exploration of differential responses to FODMAPs and gluten in people with irritable bowel syndrome- a double-blind randomized cross-over intervention study (submitted)

Published papers not included in the thesis

Nordin E[#], Steffensen SK[#], Laursen BB, Andersson S-O, Johansson J-E, Åman P, Hallmans G, Borre M, Stærk D, Hanhineva K, Fomsgaard IS[¤], Landberg R.[¤] An inverse association between plasma benzoxazinoid metabolites and PSA after rye intake in men with prostate cancer revealed with a new method. *Sci Rep* 12**,** 5260 (2022).

# Contributed equally, corresponding author

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I – Elise Nordin (EN) wrote the protocol and the ethical application, developed the intervention foods and conducted the trial, analyzed and interpreted the data, wrote the first draft of the manuscript and revised the manuscript together with co-authors.

II – EN wrote the commentary and revised the manuscript together with co-authors.

III – EN participated in designing the study, analyzed and interpreted the data, wrote the first draft of the manuscript, and revised the manuscript together with co-authors.

IV-VI – EN carried out laboratory work involving metabolomics and analysis of short-chain fatty acids, pre-processed metabolomics data, analyzed and interpreted the data, wrote the first draft of the manuscript, and revised the manuscript together with co-authors.

ANOVA	Analysis of Variance
BMI	Body mass index
BSFS	Bristol Stool Form Scale
CR	Classification rate
FDR	False discovery rate
FODMAPs	Fermentable oligosaccharides, disaccharides, monosaccharides, and polyols
FOS	Fructo-oligosaccharides
GOS	Galacto-oligosaccharides
IBS	Irritable bowel syndrome
IBS-C	Irritable bowel syndrome with constipation predominance
IBS-D	Irritable bowel syndrome with diarrhea predominance
IBS-M	Irritable bowel syndrome with mixture of constipation and diarrhea
IBS-SSS	Irritable bowel syndrome -severity scoring system
LC	Liquid chromatography
m/z	Mass-to-charge ratio
MS	Mass spectrometry
PARAFAC	Application of Parallel Factor Analysis
qTOF	Quadrupole time-of-flight
RF	Random Forest
rRNA	ribosomal ribonucleic acid
RT	Retention time
SCFAs	Short-chain fatty acids
SF-36v2	Short Form 36 version 2
UHPLC	Ultra High-Performance Liquid Chromatography

TABLE OF CONTENT

1. INTRODUCTION

Irritable bowel syndrome (IBS) is a disorder of gut-brain interaction. It is characterized by abdominal pain and altered bowel habits, but no biochemical markers for its diagnosis exist.[1] According to the recent Rome IV criteria, the prevalence of IBS in the western world is 3-5 %.[2] IBS is subtyped according to a predominance of constipation (IBS-C), diarrhea (IBS-D), or a mixture of the two (IBS-M), based on subjective reporting.[1] The prevalence of IBS subtypes varies depending on population type, geographical area, and IBS criteria.[3] Individuals with IBS experience lower quality of life compared to the general population.[4,5] As IBS commands abundant medical resources but reduces work productivity, it puts a substantial burden on the healthcare system and society.[6,7] The etiology of IBS remain to be elucidated, although it is believed to arise from alterations in the gut-brain axis, stress and psychological factors, visceral hypersensitivity, abnormal gastrointestinal motility, intestinal barrier function, low-grade inflammation, as well as gut microbiota composition and function.[8–11] IBS is a complex condition and integrated medical care is needed, including lifestyle and dietary adaptations, as well as pharmacological- and behavioral treatments.[12]

Dietary adaptations through elimination effectively diminish IBS symptoms. The two most common interventions include a diet low in fermentable oligosaccharides-, disaccharides-, monosaccharides, and polyols (FODMAPs) and a gluten-free diet. A low FODMAP diet reduces IBS symptoms, however, although a recent systematic review and meta-analysis concluded that the evidence was weak due to small sample size in trials and lack of blinding.[13] Similarly, there is insufficient evidence to recommend a gluten-free diet in IBS, owing to inconsistent results from clinical trials.[13] Nevertheless, a gluten-free diet is still commonly followed by individuals with IBS.[13] Even if dietary regimens have been shown to be effective in mitigating IBS, large, high-quality double blind trials, as well as studies aimed at mechanistic understanding and identification of objective markers of response to diets remain scarce.

Gut microbiota composition constitutes an important determinant of health status of the host and dietary regimens can modify the intestinal microbiota.[14] The function and activity of gut microbiota is reflected in the host metabolome, which may be key to understanding the role of microorganisms in health and disease.[15] The metabolome is the final manifestation of the interaction between the gene expression cascade and the environment, thereby representing the omics readout closest to the actual phenotype. Integrative multiomics analysis, i.e. combining several different biological layers, is considered an even more comprehensive approach to study biological processes, e.g. connecting molecules, diet, and IBS symptoms.[15]

In summary, dietary regimens are believed to alleviate IBS symptoms and a large proportion of individuals with IBS follow a low FODMAP and/or gluten-free diet. However, these dietary regimens are supported by limited scientific evidence,[13] creating the need for large, and double-blind trials, as well as mechanistic studies linking diets to IBS symptoms.[16] The personalization of dietary interventions requires fundamental knowledge of the factors underlying response/non-response. Integrative multiomics approaches offer promising tools to overcome these challenges. Furthermore, since IBS subtyping is based on subjective reporting, tools to diagnose IBS must be thoroughly evaluated.

2. OBJECTIVES

The aim of the thesis was to study the effects of FODMAPs and gluten vs inert control on IBS using symptomatic and molecular data such as fecal microbiota, short-chain fatty acids (SCFAs) in feces and plasma and the untargeted plasma metabolome. The aim was further to explore differential responses at individual and group (metabotype) level. Moreover, accuracy of the Bristol Stool Form Scale (BSFS) used in IBS subtyping was evaluated.

Specific objectives for IBS investigations using symptomatic and physicochemical data:

- The primary objective in this thesis was to assess the effects of FODMAP and gluten provocations on IBS symptoms (**I**) and to critically evaluate the efficacy of a low FODMAP diet against the methodological drawbacks in clinical trials (**II**).
- A secondary objective was to examine concordance of self-assessed stool consistency with objectively measured water content, given the reliance of stool consistency upon IBS subtyping. In addition, to investigate if stool consistency or water content were affected by the FODMAP or gluten interventions (**III**).

Specific objectives examining the molecular effects of FODMAPs and gluten in IBS:

- To evaluate effects of week-long provocation FODMAPs and gluten on fecal microbiota composition, fecal and plasma SCFAs, plasma metabolome, and further association to IBS symptoms (**IV** & **V**).
- To explore whether differential IBS responses to the FODMAP and gluten provocations could be identified from baseline clinical and molecular phenotype data (microbiota, SCFAs, the untargeted metabolome) both at an individual as well as at an intermediate group (metabotype) level, i.e. potentially revealing diagnostic markers already at baseline (**VI**).
- Similarly, to explore whether differential IBS responses could be accurately predicted from metabolomics data from a rapid provocation test containing both FODMAPs and gluten, thus potentially providing a rapid diagnosis within hours (**VI**).

3. BACKGROUND

3.1. Irritable bowel syndrome

The diagnosis of IBS is based on reported symptoms and exclusion of medical conditions (clinical findings and laboratory tests). According to the Rome IV criteria, IBS is diagnosed if reoccurring abdominal pain is reported at least once per week during the last three months, and symptoms persist for at least six months. The abdominal pain must relate to at least two of the following: bowel emptying, bowel frequency, and changed stool consistency.[17]

The condition is considered chronic, but symptoms often fluctuate over time.[18] The severity of the disease is usually measured by the IBS-severity score system (IBS-SSS),[19] a questionnaire that quantifies severity of abdominal pain, frequency of abdominal pain, abdominal distension, dissatisfaction with bowel habits, and interference with quality of life on a visual analog scale of 0 to 100. A composite score (total IBS-SSS score) is measured from 0 to 500. IBS subtyping into IBS-C, IBS-D, and IBS-M[17] is done with the BSFS,[1] a seven-point Likert scale,[20] which defines stool consistency as hard (BSFS 1-2), normal (BSFS 3-5) and loose (BSFS 6-7) (**Figure 1**).

Epidemiological markers of IBS are non-specific, and no differences in socioeconomic factors[21] or body mass index (BMI)[22] have been reported. Instead, a few risk factors for IBS have been established, including gastrointestinal infection,[23] gender (greater prevalence in women), age , (slightly less severe symptoms with increasing age[21]), as well as depression and anxiety.[24] Solid evidence points out abuse in youth and intimate partner violence as triggers of IBS.[25–27] Whereas IBS is not related to mortality,[21] affected individuals experience greatly reduced quality of life and are willing to accept considerable risks from medication to gain symptom relief.[28]

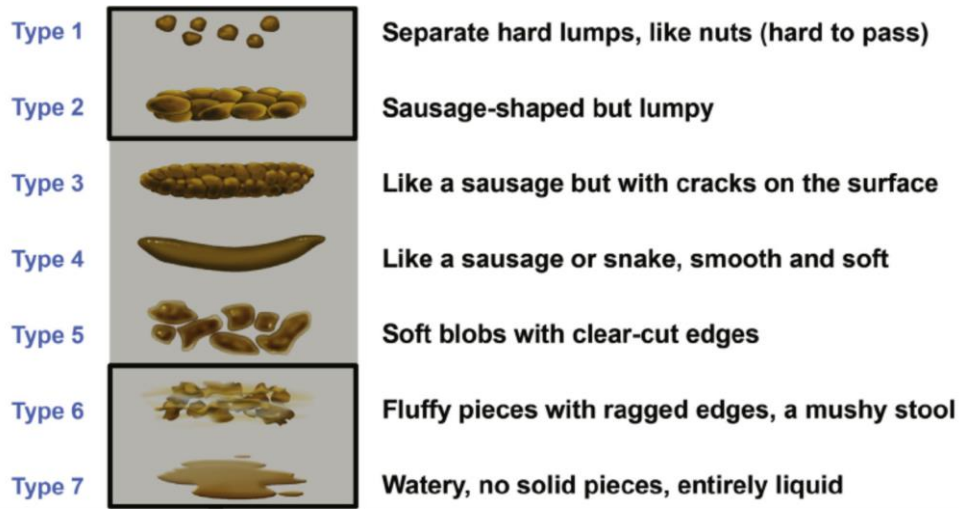


Figure 1. Consistency of feces according to the BSFS Likert-scale (1-7) The image is reproduced from [Bowel Disorders, Lacy BE, Mearin F, Chang L, et al., 150, 1393-1407.e5, 2016] with permission from The Rome Foundation.

3.2. Pathophysiology of IBS

The etiology of IBS remains poorly understood, although its pathogenesis is believed to relate to gut-brain interactions, stress and other psychological factors, visceral hypersensitivity, abnormal gastrointestinal motility, intestinal inflammation, abnormal gastrointestinal immune function, and altered gut microbiota.[29–31] However, results are inconclusive and further research is warranted.

The bidirectional communication in gut-brain interactions comprises the central nervous system, the autonomic nervous system, the enteric nervous system, and the hypothalamic-pituitary-adrenal pathway. The gastrointestinal tract sends signals to the brain, and vice versa, regulating motility, immune function, and secretion.[32–35] Such communication is important for the regulation of food intake, and control of bowel habits.[36] Stress and psychological conditions are risk factors for IBS and can have a major impact on gut-brain communication.[37,38]

Abdominal pain is a central symptom in IBS, believed to relate to disturbance in the gut-brain axis causing visceral hypersensitivity. Individuals with IBS are more sensitive to pain stimuli in the visceral organs caused by sensitization of nerve pathways related to the gastrointestinal tract.[30] The condition is considered typical of IBS as it has been observed more often (33-90%)[39,40] in individuals with IBS compared to healthy subjects.[30,41] However, it is not an accurate marker. Gastrointestinal dysmotility (abnormal frequency, irregular bowel contractions, altered transit time) is another characteristic symptom in IBS, [42] regulated by the gut-brain axis.[43,44] Consistent motility abnormalities have not been found in IBS,[44] and transit time is only of minor or no importance for IBS symptoms.[42] Hence, dysmotility is not a clear marker of the syndrome.

The mechanisms underlying gut-brain communication involve neuro-immuno-endocrine mediators.[45–47] Several studies suggest disturbance in intestinal barrier function in subgroups of IBS patients, causing a leaky gut that enables the passage of potentially pathogenic substances.[48] In line with this, inflammatory markers such as neutrophils, T-lymphocytes, and mast cells in the intestinal mucosa and cytokines, appear elevated in IBS.[49] Notably, immune mediators, especially those released by mast cells can activate or sensitize pain-transmitting nerves.[50]

The involvement of gut microbiota in IBS is gaining prominence,[51,52] particularly because microbial metabolites could serve as signaling molecules in gut-brain communication.[53] A systematic review concluded that individuals with IBS had an altered gut microbiota composition, characterized by an abundance of *Proteobacteria*, *Lactobacillaceae*, *Bacteroides* and decrease of *uncultured Clostridiales I*, *Faecalibacterium*, and *Bifidobacterium*.[54] However, a subsequent study found no significant differences in gut microbiota composition between IBS subjects and healthy controls.[55] In fact, some of the differences between such groups could at least in part, ascribed to by the diet[56] Dysbiosis of the gut microbiota has been considered a trigger of IBS symptoms and manipulation of the gut microbiota has been proposed as a possible treatment via prebiotics,[57] probiotics,[58] and fecal transplantation.[59]. Still evidence of strong and coherent IBS symptom improvements is lacking for these therapies.[57–59] It is debated if small intestinal bacterial overgrowth is a cause of IBS since specific antibiotics can be efficient in some people with IBS. However, the diagnostic breath tests usually applied

show low sensitivity and specificity, why novel molecular techniques are needed for further evaluation.[60]

3.3. Treatment of IBS

IBS treatment aims at reducing symptoms. Due to the complexity of IBS, optimal treatment requires a holistic approach.[12] As a primary strategy, lifestyle and dietary advice are given. If unsuccessful, other approaches such as pharmacology and behavioral therapy are suggested.[61]

Use of dietary adaptation as a first line therapy is supported by the large percentage of individuals with IBS who reportedly associate symptoms with specific foods, prompting them to develop strategies to avoid dietary triggers.[62,63] Primarily, dietary advice for IBS focuses on 'when' and 'how' to eat and limit certain foods, such as onions and beans, which may cause symptoms.[64,65] If this is insufficient, a low FODMAP diet is normally implemented.[66] Other dietary strategies involve targeted prebiotics,[57] probiotics,[67] and a gluten-free diet.[13]

3.3.1. A low FODMAP diet

In a low FODMAP diet, the intake of fermentable carbohydrates, such as fructose, lactose, fructo- and galacto-oligosaccharides (FOS/GOS), mannitol, and sorbitol are restricted.[68] The FODMAP hypothesis was proposed in 2005, based on the rationale that rapid fermentation of FODMAPs caused bacterial overgrowth and production of metabolites, such as SCFAs, which at high concentrations may lead to increased intestinal permeability.[68,69] Excessive consumption of fructose and fructan can lead to abdominal discomfort, bloating, and altered mobility, all of which are common characteristics in IBS.[70] Provocation studies with fructose and sorbitol have shown that there was no difference in malabsorption between healthy subjects and individuals with IBS but symptoms evolve more readily in individuals with IBS.[71,72] Therefore, removal of fructose from the diet was suggested to reduce IBS symptoms.[70] However, this study did not include a placebo treatment.[71,72] Indeed, reduced intake of individual FODMAPs and particularly their outright elimination can have beneficial effects on IBS symptoms. However global restriction of FODMAPs is considered even more efficient.[65,70] Notwithstanding shaky scientific evidence derived from small sample size and lack of blinding, a low FODMAPs diet remains the dietary therapy of choice for IBS.[13,68,73]

FODMAP-rich foods are considered healthy as they consist mostly of vegetables and fruits.[14] To avoid any nutritional deficiencies and alterations in gut microbiota arising from the absence of certain nutrients,[74,75] it is recommended that a low FODMAP diet is prescribed by a dietician,[14] allowing for restrictions to occur in a stepwise manner and determine individual tolerance.[76]

The dose-response of FODMAP intake in IBS remains yet unknown. Trials with widely different intake of FODMAPs report similar reduction of IBS symptoms.[77,78] Despite the lack of a clear dose-response relationship, cut-off levels for the intake of FODMAPs have been suggested[68] based on experience and results from clinical trials (**Table 1**).[79]

Table 1. Cut off levels for the intake of FODMAPs per serving of food.

FODMAP	Gram per serving of individual food (g)
Oligosaccharide (e.g. nuts, legumes)	<0.3
Oligosaccharide (e.g. vegetables, fruits)	<0.2
Polyols (mannitol or sorbitol)	<0.2
Total polyols	<0.4
Excess fructose	<0.4
Lactose	<1.0

Abbreviation: FODMAPs, fermentable oligosaccharides, disaccharides, monosaccharides, and polyols

Akin to other dietary fiber components, FODMAPs are not absorbed upon ingestion. Instead, they pass through the gastrointestinal tract and exert an osmotic load on the gut and are rapidly fermented by the gut microbiota in the colon, thereby resulting in gas production with ensuing abdominal distention and pain (**Figure 2**).[80] Luminal distension is the accepted explanation for symptom induction by FODMAPs,[81] however, microbial dysbiosis, colonic barrier dysfunction, activation of mast cells, or visceral hypersensitivity could be complementary mechanisms. As the above studies have been carried out mostly *in vitro* or in rodents, clinical trials in humans are required to validate these results.[34,81]

Fructose is found in several fruits and honey[68] and is absorbed though GLUT2 transport which requires presence of glucose.[82] In situations where fructose is present in excess of glucose, absorption instead occurs predominantly via GLUT5, which has a lower capacity that is also highly variable between individuals.[83] Therefore, when its concentration exceeds that of glucose, fructose is considered a FODMAP.[84]

Lactose is a milk disaccharide normally cleaved into glucose and galactose by lactase in the small intestine.[68] However, lactase activity varies across population. In Sweden, as in most Nordic countries, lactose intolerance is only 7 %,[85] whereas the global average is about 68 %.[85] Differences in tolerance are driven by selective pressure related to the availability of lactose-rich foods.[86] In lactose intolerant individuals, lactose is poorly absorbed and becomes fermented by gut microbiota. Hence, it is also considered a FODMAP.[68]

Fructans are fructose-based polysaccharides found in many vegetables and fruits, such as wheat, onion, and garlic.[68] Fructans can have different chain lengths, with shorter ones fermenting more rapidly. Polymers with a degree of polymerization < 10 are referred to as FOS; whereas those with degree of polymerization > 10 are referred to as inulin.[87] The human body lacks hydrolases for cleaving both FOS and GOS; the latter are chains of galactose units ending with a glucose, found in legumes. Polyols, commonly found in fruits and vegetables,[68] are slowly absorbed in the gut via passive diffusion, leaving a substantial portion unabsorbed.[88] Poorly absorbed carbohydrates can be problematic in individuals with IBS but also in healthy people, as their excess may cause gastrointestinal symptoms.[89]

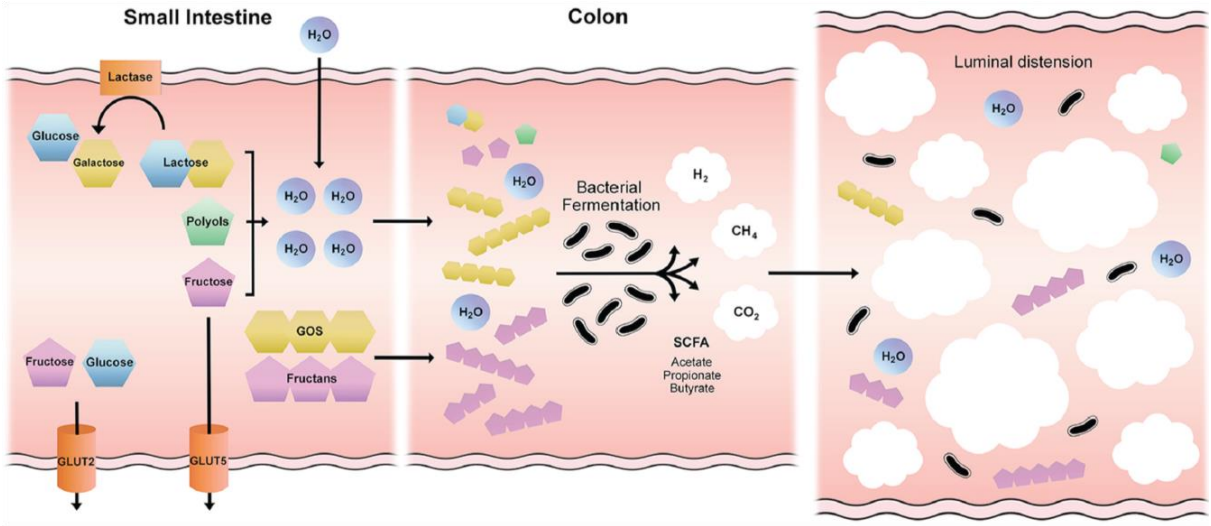


Figure 2. Potential mechanisms of FODMAP intake in the gastrointestinal tract. Lactose is cleaved by lactase. Fructose is absorbed by GLUT2 and GLUT5 transporters. Unabsorbed fructose and lactose increase intestinal water content. Unabsorbed fructose, lactose, FOS, GOS, and polyols are transported to the colon and fermented by bacteria, causing luminal distension. These mechanisms are assumed to cause IBS symptoms. The image is reproduced from [The low FODMAP diet: recent advances in understanding its mechanisms and efficacy in IBS, Heidi M Staudacher, Kevin Whelan, 66, 1517–1527, 2017] with permission from BMJ Publishing Group Ltd.
Abbreviations: FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides

3.3.2. Gluten-free diet

Non-celiac gluten/wheat sensitivity is a condition characterized by intestinal and extraintestinal symptoms (fatigue, headache, skin rash, anxiety, and depression) triggered by wheat intake, despite absence of wheat allergy and coeliac disease.[90] Mechanisms are unclear and it is debated whether IBS and non-celiac gluten/wheat sensitivity are distinct diseases or linked disorders, as they share many similar traits.[91] In fact, even the actual existence of non-celiac gluten/wheat sensitivity is questioned, owing to the absence of objective markers and lack of coherence in clinical trials.[90,92]

Gluten is a major inducer of symptoms after ingesting wheat-containing products.[90] However, trials linking gluten to IBS, either in the form of provocation studies or as elimination tests,[13] have yielded mixed results. In one clinical trial, individuals with IBS were provoked with 16 g gluten, which increased symptom severity.[93] However, when the same research group performed a follow-up study and first put participants on a low-FODMAP diet, symptoms decreased[106] and no effect arose following gluten provocation.[94] Therefore, symptom induction in the first trial was assumed to be related to FODMAP intake rather than gluten.

Whether substances other than gluten present in wheat cause IBS symptoms remains to be determined. In *in vitro* and *in vivo* animal models, low-molecular weight α-amylase/trypsin inhibitors and lectin agglutins have been shown to induce the innate immune response, thereby augmenting intestinal permeability.[95–97] However, these observations need to be confirmed in humans.[90] Moreover, gluten and fibers co-exist in wheat products, highlighting the difficulty of isolating the effects of gluten from those of dietary fiber or antinutrients.[90] A systematic review and meta-analysis concluded that results from clinical trials were inconsistent and that there was insufficient evidence to recommend a gluten-free diet for individuals with IBS.[13]

3.3.3. Other treatments

In addition to dietary treatment, pharmacological or behavioral therapies can be undertaken. Such treatments are outside the scope of this thesis and will not be described in detail. In brief, the choice of drug is based on IBS symptoms, such as abdominal pain, abdominal distention or altered bowel habits, and sometimes a combination of pharmacological agents is necessary.[98]

Moreover, anxiety, psychological distress, and somatization (the physical expression of stress and emotions) are often related to IBS symptoms.[99] Psychiatric treatments have been shown to successfully reduce IBS symptoms and are currently part of an integrated approach to IBS.[12] A systematic review concluded that cognitive behavioral therapy was effective in IBS,[100] when administered either face to face or remotely. Also gut-directed hypnotherapy, mindfulness, meditation, and yoga have shown some effectiveness.[12,101,102]

3.4. Gut microbiota

Microbiota (bacteria, archaea, viruses, and fungi) coexist with humans, most of them in the intestine.[103] Epidemiological and omics studies, as well as cell and animal work, point to gut microbiota as mediators of environmental stimuli.[103] While dietary interventions can modify gut microbiota,[103,104] at least over the short time,[105] it is less clear what constitutes a healthy gut microbiota, owing to substantial variation between individuals.[106] Rather than seeing gut microbiota as healthy vs unhealthy, the beneficial effects of the microbiota composition seem to depend on a multitude of factors of the host and the gut microbiota, including duration of colonization, age of the host, and environmental factors.[106–108] Gut microbiota are commonly analyzed by sequencing of the 16S ribosomal ribonucleic acid (rRNA) gene which is present in all bacteria and archaea. For taxonomic identification, the sequences are compared to a reference database,[109] using either operational taxonomic units,[110] or amplicon sequence variant[109]. The former consists of similar DNA sequences clustered into a consensus sequence; whereas the latter represent single DNA sequences and have become the most accurate approach for classifying different taxa.[109] One limitation of 16S rRNA sequencing is its inability to provide accurate information below the genus level. Moreover, it does not yield information regarding microbiota activity or function.[111] In the last years, shot-gun metagenomics, whereby the entire DNA is sequenced, has become increasingly popular, contributing both taxonomic and functional insights.[124] The 16S rRNA method is, however, less expensive and requires less advanced bioinformatics operations compared to metagenomics. In this thesis, the 16S rRNA method was used to evaluate the bacterial microbiota composition.

3.4.1. Effect of FODMAP and gluten on gut microbiota

The effect of FODMAPs on gut microbiota composition is inconsistent between clinical trials. The most robust findings point to a reduction in *Bifidobacterium* abundance following a low FODMAP diet and, conversely, an increase following a FODMAP-rich diet.[112,113] The effect of gluten on gut microbiota remains poorly investigated, but one study found no effect of a gluten-rich diet in healthy subjects.[114]

3.5. The metabolome

The metabolome is constituted by all small molecular metabolites (<1500 Da, although definitions vary) present within biological samples such as cells, biofluids, tissues or organisms.[115] Metabolites are the end products derived from the interaction of gene cascades with the exposome, i.e., the sum of all life course exposures (gut microbiota, diet, and lifestyle factors)[115–117] (**Figure 3**). The metabolome is therefore widely considered as the omics level closest to the phenotype and the best representation of the molecular phenotype in health and disease.[118]

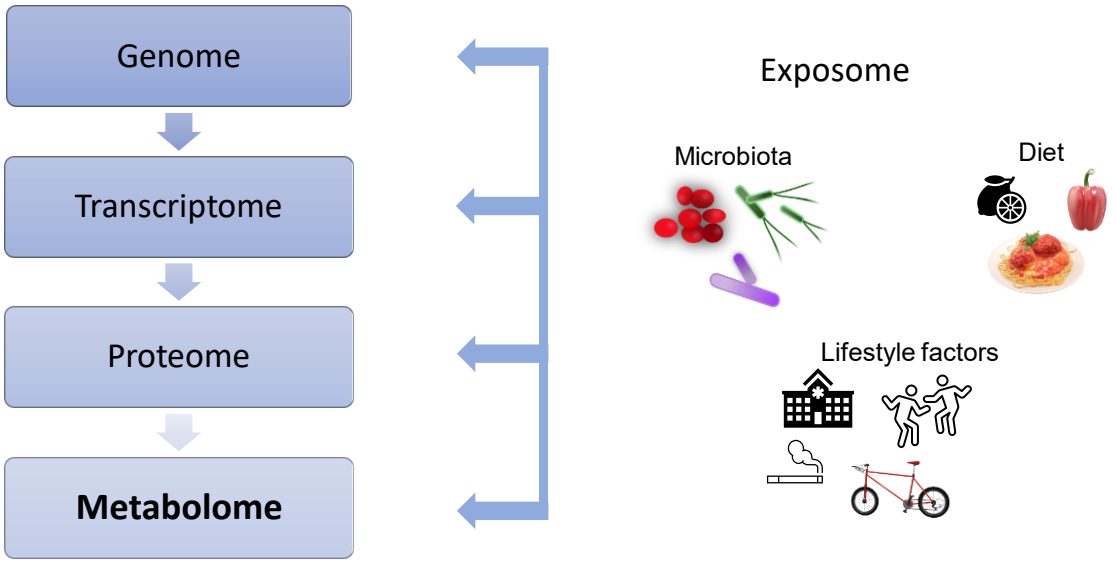


Figure 3. The metabolome is constituted by all low-molecular weight metabolites in a biological system and sums up the downstream products arising from interactions between gene cascades and the exposome, i.e., the sum of all life course exposures (gut microbiota, diet, and lifestyle factors).

3.5.1. Microbial-derived metabolites

Microbial metabolites are produced or converted by microorganisms and are considered a key factor in host-microbiota crosstalk. These metabolites include signaling molecules involved in gut-brain communication and metabolic reactions.[117,119] Microbial metabolites such as bile acids, tryptophan metabolites, and SCFAs are considered important for gut-brain signaling and IBS symptoms.[46,120–124] A deeper understanding of gut microbial-host interactions and microbial metabolites may reveal how IBS symptoms develop.[120]

3.5.1.1. Effect of FODMAP and gluten on SCFAs concentration

SCFAs are side products from microbial fermentation of indigestible foods.[122] Acetate, propionate, and butyrate are the main SCFAs derived from the fermentation of dietary fibers;[125] whereas isobutyrate and isovalerate are derived from the fermentation of proteins.[126] It has been hypothesized that dietary FODMAPs could alter SCFAs,[113] which has been verified in some *in vitro* and *in vivo* animal studies.[127,128] However, in human trials reduced intake of FODMAPs has only a minor or even no effect on SCFA levels in feces;[113,129] while conflicting results have been reported with gluten.[114,130] SCFAs are thought to be related to IBS pathogenesis due to their neuro-immuno-endocrine regulation.[120,122,123]

3.6. Metabolomics

Metabolomics, the study of the metabolome, has emerged as a promising tool to gain mechanistic insights on dietary intake or disease.[131,132] Metabolomics encompasses two approaches: targeted metabolomics for absolute quantification of a known subset of metabolites, usually based on a predetermined hypothesis,[133] and untargeted metabolomics, which measures all metabolites in a sample, normally without previous knowledge as to their involvement in the research question. Untargeted metabolomics has enormous potential for the generation of new hypotheses, biomarker discovery, and mechanistic understanding of biological processes.[134]

Mass spectrometry (MS) is a common technique used for metabolomics as it provides high sensitivity and, therefore, wide coverage of the metabolome. MS is normally coupled to an upstream separation technique such as, liquid chromatography (LC), which benefits from easy sample preparation and versatility.[135] LC-MS has become the main tool for untargeted metabolomics. Ultra High-Performance Liquid Chromatography (UHPLC) is the most commonly applied form of LC. The small particle size (normally < 2 μm) of LC columns ensures a large surface area, thereby enhancing analyte separation. A mobile phase is delivered as a gradient to enable the separation of differently charged molecules. Separation can be enhanced by applying different combinations of liquid and mobile phase. The two most common are reversed-phase chromatography which uses a hydrophobic column and a polar mobile phase to primarily separate molecules of low polarity; and hydrophilic interaction liquid chromatography, which uses a hydrophilic column and an even more hydrophilic mobile phase to elute molecules of high polarity. In LC-MS, the analyte must be ionized, as the spectrometer measures the mass-to-charge ratio (m/z). Soft ionization techniques, such as electrospray ionization, are commonly applied to reduce fragmentation of the analyte. The m/z (or MS1) can be measured using several types of detectors, with quadrupoles and time-of-flight (qTOF) being among the most common.[136] Reversed phase- and hydrophilic interaction liquid chromatography are used in combination with both positive and negative ionization mode, because unknown molecules may be more easily ionized as either negative or positive ions. In this work, quadrupoles were used for targeted analysis of SCFAs and qTOF was used for untargeted metabolomics.

When features of interest are discovered in LC-MS data, they are generally identified by matching retention time (RT), m/z, and fragmentation pattern (or MS2) to standards or databases. There are several databases available, but only a limited number of metabolites are registered. The absence of a universally accepted standard on how to perform identification, means there is a need to harmonize identification and reporting.[137] There are, however, different standard schemes for reporting the level of identification certainty and in this thesis, the 5-grade Schymanski's scale[138] was used. Level 1 corresponds to a match between RT, m/z, and MS2 data with an authentic standard, whereas level 2 corresponds to a match between MS2 and a library spectrum. Level 3 implies evidence of a putative structure, but insufficient data for an exact determination. Level 4 corresponds to a known molecular formula, with no further information. Finally, level 5 reports the exact mass (MS1). In general, identification of metabolites is challenging and resource-intensive, wherefore it is justly considered a major bottleneck in metabolomics.[139]

3.6.1. Pre-processing of untargeted metabolomics data

Raw MS data are highly complex and can be seen as a three-dimensional topographical map with RT, m/z, and intensity. To transform this complex 3D data into actionable, tabular format, complex pre-processing is needed.[140] Manual pre-processing is not feasible due to the high number of peaks present in the raw instrument data. Hence, algorithms have been developed to process the data with an increased degree of automation. The preprocessing workflow for metabolomics data used in this thesis is presented in **Figure 4**: Automated peak picking is performed using a dedicated algorithm.[141] In LC, retention is not entirely stable between injections and time drift normally occur both within and between batches, thus requiring adjustments to be made.[142,143] Based on RT and m/z, peaks are grouped across samples with the purpose to extract common features.[144] The peak picking algorithm will fail to identify peaks in some samples, requiring peak filling and frequently also imputation.[145] Moreover, a metabolite can be represented by several features in the data, including isotopes, adducts and fragments. Feature clustering is thus normally performed, to avoid over-representation of metabolites in the dataset which can create artifacts in downstream data analysis.[146]

LC-MS instrumentation is rapidly developing, yet challenges with LC-MS reproducibility in metabolomics remain[140] and automated algorithms will not be able to deliver perfect results. Thus, to obtain high-quality data for downstream analysis, it is imperative that sufficient attention is spent to optimize parameters for these algorithms, which is a demanding and time-consuming task.

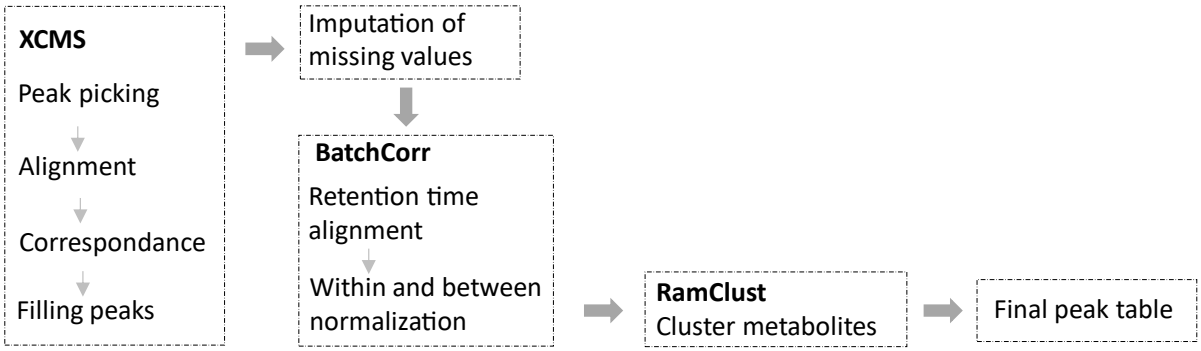


Figure 4. Workflow describing preprocessing of metabolomics data in this thesis.

3.6.2. Statistical considerations in the analysis of metabolomics data

Pre-processed metabolomics data normally consist of thousands of metabolite features. Machine learning is an effective way to identify and select the features carrying the most information in relation to the research question. Machine learning algorithms do not require multiple statistical tests and are insensitive to collinearity. In addition, some machine learning algorithms are also insensitive to data distribution (in particular tree-based methods). However, because the number of variables usually exceeds the number of samples, there is a risk of overfitting, which leads to overconfident predictions and biased conclusions.[140,147] To mitigate issues with overfitting, validation is required, such as cross validation.[148,149] Machine learning is best suited for investigating predictive associations between predictors (e.g. omics variables) and targets (e.g. exposures or outcomes). However, given the difficulty of adjusting for covariates is not easily achieved, these methods are normally not as effective in establishing

causality and quantifying independent associations. Moreover, visualization and interpretation are challenging for several machine learning methods.[140,147]

Instead of analyzing multiple variables simultaneously, a common approach is to analyze one variable at a time, using univariate analysis. Considering that metabolomics normally comprises numerous variables, adjustment for multiple testing is normally applied to limit false positive discovery, although this has the unintended consequence of augmenting the false negative rate. A successful approach combines machine learning with univariate modelling[150,151] to select the most predictive variables and avoid issues with multiple testing. After selecting the variables of interest, univariate methods can be applied and adjustments for covariates and quantification become easier.

3.6.3. The effect of FODMAPs and gluten on the metabolome

Few studies have investigated the effects of FODMAPs on the metabolome. McIntosh et al[152] found that a low FODMAP diet led to reduction in histamine and increase in p-hydroxybenzoic acid and azelaic acid, with reversed effect with a high FODMAP diet. Nybacka et al[153] found a decrease in plasma glucose and polyols in urine after a low FODMAP diet. Rossi et al[154] found that baseline fecal organic volatile acids could classify response to the FODMAP diet with high precision. To my knowledge, there has been no untargeted metabolomics study for gluten, except for one trial reporting minor distinctions in the metabolome between individuals with IBS and healthy controls after a gluten-free diet, but lacks reporting of specific metabolites.[155]

3.7. Multiomics, IBS, and FODMAPs

Combining multiple omics layers has been shown to improve our mechanistic understanding of biological processes[15] and pathological changes.[156] Combined analysis of gut microbiota and metabolomics has been suggested as a means to provide further insights on the pathophysiology of IBS.[157] However, only few studies on IBS and FODMAPs have used multiomics.[152] One study found that *Porphyromonadaceae* spp. were associated with urinary histamine levels; whereas another study did not find any association among gut microbiota, cytokines, SCFAs, and IBS symptoms.[158] There have been no similar studies for IBS and gluten.

3.8. Differential IBS-response to dietary intervention

The diverse physiological and metabolic responses to diet among individuals suggest the need for personalized nutrition strategies to improve the efficacy of dietary treatment. Personalization of prevention and treatment strategies could elicit individual health benefits and reduce health care expenditure.[159–161] The response to low FODMAP and gluten-free diets differs substantially between individuals with IBS.[90,162] Hence, there is a potential for more targeted, personalized interventions.[90,163–165]

Several IBS and FODMAP studies have attempted to group individuals into differential responders aiming to explain the symptomatic response to the intervention. Such response and non-response groups have been linked to gut microbiota,[166–171] colonic methane and SCFA production,[172] intake of FODMAP at baseline,[173] hydrogen production,[174] fecal volatile organic compounds,[154] metabolite patterns,[153] and psychological and nutritional factors.[171] Conversely,

other studies have failed to identify a rationale for differential responses.[175,176] Although subgroups have been identified in several studies, results are not entirely coherent. As an example, increased abundance of saccharolytic genera has been observed both in response and non-response to a low FODMAP diet.[166–168] Concerning gluten, one study found a difference in the metabolome between response and non-response to a gluten-free and a gluten-containing diet.[155]

Differential responses in IBS studies have been subjected to a variety of analytical approaches, including univariate[170,171,173–175] and machine learning[153–155,166–169,176] methods. Machine learning regression methods have previously been used in IBS, however without sufficient safeguards against overfitting, e.g., by implementing double cross-validation.[148] Another successful approach to analyze response and non-response,[177] which has not been applied in IBS studies, is based on grouping individuals into similar metabolic phenotypes.[178] These metabotypes share similarities in metabolism and metabolic regulation within a group but differ between groups.[179,180] As there is no analytical framework for analyzing response and non-response to dietary interventions in IBS, use of robust analytical tools is needed for future studies.

In summary, there are no accepted factors predicting responses to dietary interventions in IBS,[1,181] despite the strong demand for predictors of response to both FODMAPs[163–165] and gluten.[90]

4. METHODS

4.1. Hypotheses and research strategies

The hypothesis driving the work in this thesis presumed that FODMAPs caused a marked increase in IBS symptoms, while the effect of gluten was mild or non-existent. Given the observed inaccuracy between self-reported fecal consistency and stool-water content in IBS,[182] only a modest correlation between reported BSFS score and the corresponding objectively measured water content was expected. Furthermore, the FODMAP intervention was expected to affect the gut microbiota and plasma metabolome, as well as associate with IBS symptoms; whereas no or minor effects were expected for gluten. Finally, microbiota and metabolite profiles reflecting differential responses to the interventions were anticipated, either at an individual or intermediate (metabotype) level. Such expectations were supported by wide inter-individual variability of IBS symptoms reported in previous FODMAP and gluten trials,[90,162] and the power of a large dataset that included microbiota, SCFAs, metabolomics, and clinical parameters.

To investigate these hypotheses, a large double-blind, crossover clinical trial was set up, and the effect of high intake of FODMAPs, gluten, and placebo was investigated in subjects with IBS. Fecal and plasma samples, as well as clinical data were collected for analysis, resulting in five original scientific manuscripts and a viewpoint included in this thesis.

4.2. Study Design

A double-blind, placebo-controlled, randomized, three-way crossover trial that included 110 participants with IBS was performed. Each subject participated in the study for seven weeks, with weekly visits to the clinic. Before the start of the trial, each participant met with a dietician specialized in IBS and received instructions on how to exclude gluten from the diet and consume minimal amounts of FODMAPs. To help participants adjust to the diet and stick to it throughout the study, they were given recipes, a list of foods low in gluten and FODMAPs, and an app to scan food labels in the store (Belly Balance Sverige AB, Stockholm, Sweden). Additionally, the participants could contact the dieticians and study personnel for further support any time during the trial.

After one week on a low-impact diet, participants came to the clinic and underwent a rapid provocation test, using a cake containing 17.3 g gluten and 50 g FODMAPs. Blood samples were collected at -10, 0, 10, 20, 30, 90, 150, and 240 min (details are presented in sections 4.4 and 4.6), followed by an additional week of low-impact diet. At the onset of the third week, one-week interventions of FODMAPs, gluten, and placebo were initiated, with one-week washout in between. Study participants were randomized in three sequences (CBA, ACB, and BAC) carried out in blocks of 12, with A = FODMAPs, B = gluten, and C = placebo. The daily amount of FODMAPs and gluten during intervention weeks was equal to the content in the cake consumed during the rapid provocation test. At each visit to the clinic, blood samples, anthropometric measurements, and the most recent fecal samples were collected, together with questionnaires asking participants about the previous week. The study design is presented in **Figure 5**. At enrollment, subjects were diagnosed following the Rome IV criteria and subtyped into IBS-C, IBS-D, and IBS-M[17]. The study was conducted from autumn 2018 to spring 2019 in a clinical facility in Uppsala, Sweden. The study protocol was approved by the Ethics Review Board, Uppsala (2018/159) and the trial was registered at www.clinicaltrials.gov under number NCT03653689. The clinical trial was performed in accordance with the Helsinki Declaration regarding ethical principles for medical research involving human subjects. A workflow chart tracking participants during the study is presented in **Figure 6**.

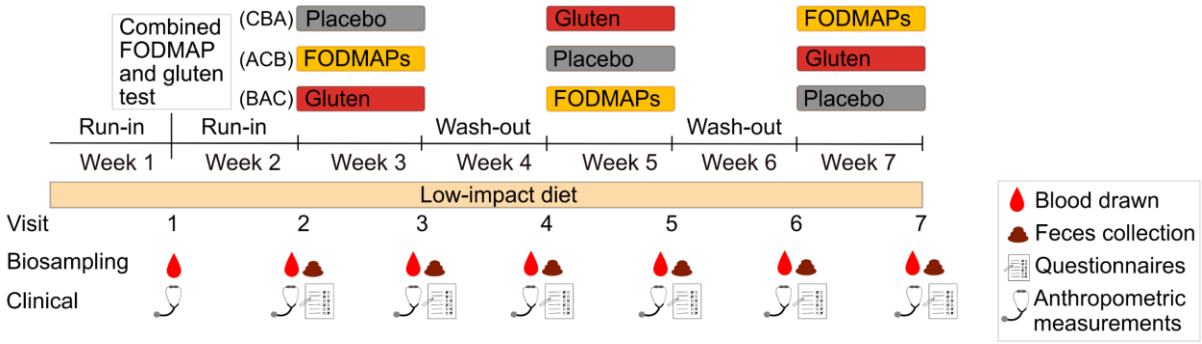


Figure 5. Study design of the three-way double-blind, placebo-controlled, randomized crossover trial of FODMAPs, gluten, and placebo in subjects with irritable bowel syndrome. Participants were randomized into the sequences CBA, ACB, and BAC (A = FODMAPs, B = gluten, and C = placebo).

Inclusion criteria were: women and men, age 18–70 years, BMI 18.5–38 kg/m^2, moderate to severe IBS (IBS-SSS > 175), systolic/diastolic blood pressure ≤160/≤105 mmHg, transglutaminase immunoglobulin A <7 U/ml, hemoglobin 120–160 g/l, thyroid-stimulating hormone <4 mU/l, and C-reactive protein <5 mg/l. Exclusion criteria were: celiac disease, *Helicobacter pylori* infection during the preceding 6 months, functional dyspepsia or other functional or inflammatory gastrointestinal disease, previous bariatric or abdominal surgery other than appendectomy, previous or ongoing cancer treatment, weight reduction treatment, >10 kg body weight change in the preceding year, unstable medication from 14 days prior to inclusion or during the study, refusal to give informed consent, probiotic or antibiotic medication within the last four weeks, reluctance to consume rice porridge daily during three separate weeks, blood donation or participation in other intervention trials within 30 days prior to screening or any time during the study, pregnancy or lactation, history of drug or alcohol abuse, smoking, and inability to understand the Swedish language.

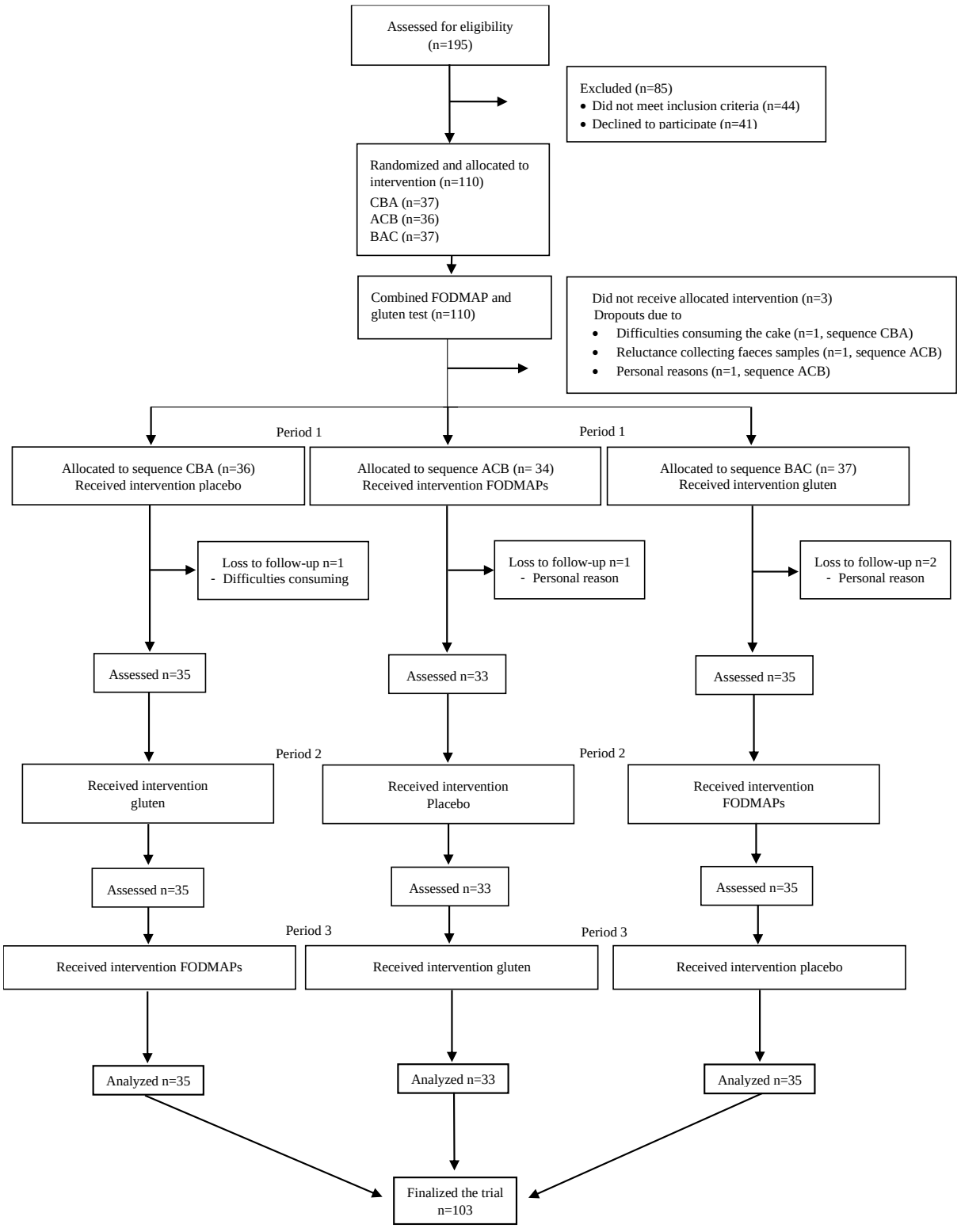


Figure 6. Workflow chart tracking participants in the three-way double-blind, placebo-controlled, randomized crossover trial of FODMAPs, gluten, and placebo in 103 subjects with irritable bowel syndrome.

4.3. Sample size estimation of the study

The sample size had to enable the detection of differential responses to the interventions. To overcome the lack of consensus for proper power calculations in machine learning-based analysis of omics-data and the shortage of omics-studies in this field, previous experience with omics data suggested that approximately 100 completers would be sufficient to identify three subgroups of approximately equal size. Therefore, to allow for dropouts, the study included 110 participants.

When evaluating the effect of the intervention on IBS symptoms and other clinical parameters (**I**), a post hoc power calculation was conducted to confirm sufficient sample size. The parameters for sample size calculation were set as follows: within-individual standard deviation = 111.6 (obtained from our trial), power = 0.8, and level of significance in a 2-sided test = 0.05/3(3 = number of comparisons, Bonferroni correction). The samples size needed for the observed data, accounting for 20% drop out, was 64 participants. Therefore, with 103 participants finishing the trial, the study was well powered, and its sample size was much higher than in most other trials.

4.4. Intervention foods

Combined exposure to FODMAPs and gluten was served as a cake; whereas single dietary interventions were served as rice porridge, with the daily dose divided into three servings. The FODMAP intervention was based on 150% of the daily intake by the Australian population[183] (the data available at the time of the design of the study) except for lactose and gluten, which were based on 150% of the intake by the Swedish population.[184] The content of intervention foods is presented in **Table 2**, while their nutritional composition is listed in **Table 3**. Rice porridge was used as a vehicle because of its neutral taste and palatability.

Table 2. Content of intervention foods.

		Daily rice porridge intake		
	Cake (g)	**FODMAPs (g)**	**Gluten (g)**	**Placebo (g)**
Fructose	19.5	19.5	0	0
Lactose	15.7	15.7	0	0
FOS	7.0	7.0	0	0
GOS	1.5	1.5	0	0
Sorbitol	4.5	4.5	0	0
Mannitol	1.8	1.8	0	0
Gluten	17.3	0	17.3	0
Cocoa	4.0	0	0	0
Sucrose	0	0	0	18.0
Icing sugar	0	0	24.0	0
Rice flakes	0	78.0	78.0	78.0

Abbreviations: FODMAPs, fermentable oligosaccharides, disaccharides, monosaccharides, and polyols; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides.

Table 3. Nutritional composition of intervention foods.

	Cake		Rice porridge with (per 100 g)			Daily intake of rice porridge (3 servings) with		
	per 100 g	per serving	FODMAPs	Gluten	Placebo	FODMAPs	Gluten	Placebo
Energy (kcal)	349.1	275.4	397.6	401.2	397.6	492.7	472.9	372.7
Protein (g)	22.9	18.1	4.7	18.1	5.9	5.8	21.3	5.5
Ash (g)	0.6	0.5	0.3	0.4	0.4	0.4	0.5	0.4
Fat (g)	2.5	2.0	0.5	1.7	0.7	0.6	1.9	0.7
TC (g)	58.7	46.3	93.7	78.5	91.8	116.1	92.5	86.1
Fructose (g)	24.5	19.3	17.0	< 0.04	< 0.04	21.1	< 0.04	< 0.04
Lactose (g)	18.3	14.4	12.2	< 0.04	< 0.04	15.1	< 0.04	< 0.04
FOS (g)	8.7	6.9	4.7	0.4	0.3	5.8	0.5	0.2
GOS (g)	2.4	1.9	1.5	< 0.03	< 0.03	1.9	< 0.03	< 0.03
Sorbitol (g)	5.2	4.1	3.3	< 0.04	< 0.04	4.1	< 0.04	< 0.04
Mannitol (g)	2.1	1.7	1.4	< 0.04	< 0.04	1.7	< 0.04	< 0.04
DF (g)	1.6	1.2	0.9	1.3	1.1	1.1	1.6	1.0

Abbreviations: DF, dietary fiber; FODMAPs, fermentable oligosaccharides, disaccharides, monosaccharides, and polyols; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides; TC, total carbohydrates.

4.5. Blinding

Generally, blinding is challenging in nutritional studies. Because most studies analyzing the effects of FODMAP on IBS have been conducted as elimination trials, they have not been blinded or only single-blinded.[13] In this trial, the participants were exposed to powders of FODMAPs and gluten. The provocation design thus made it possible to utilize a double-blind study design, where neither study participants nor clinical staff were aware of treatment allocation.

4.6. Outcome assessment

4.6.1. Anthropometric measures and questionnaires

Waist circumference, blood pressure, pulse, and weight were recorded at each visit to the clinic. In addition, participants handed in questionnaires reflecting their status during the previous week. They included:

- IBS-SSS, a questionnaire to define the severity of IBS symptoms (see section 3.1).
- Short Form 36 version 2 (SF-36v2), a validated questionnaire[185] evaluating health and wellbeing in individuals based on physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional, mental health, mental component score, and physical component score.
- BSFS, a seven-point Likert scale to assess stool consistency (see section 3.1).
- A food frequency questionnaire used in the Swedish Mammography cohort,[186] Baeckes physical activity questionnaire[187], and a demographic questionnaire summarizing dietary habits, physical activity, and basic demographic information (e.g., age, sex, ethnicity, educational and income level), all of which were collected at baseline.

4.6.2. Fecal microbiota

Participants collected fecal samples as close to the next visit to the clinic as possible, stored them at -20°C at home until arrival to the clinic. There, the samples were stored at -20°C for up to one week, and then transferred to -80°C in the study biobank where they were kept until completion of the trial. In total, 621 samples were analyzed by 16S rRNA gene amplicon sequencing at the Swedish University of Agricultural Sciences, Uppsala. A detailed description is provided in **V**. Briefly, DNA was extracted from fecal samples, the V3-V4 region of 16S rRNA genes was amplified with primers and sequencing libraries were generated. Amplicon sequence variants were compared to reference databases. In addition, they were used to assess alpha diversity and perform compositional analysis when merged at genus level. Henceforth fecal microbiota will be referred to simply 'microbiota'.

4.6.3. Short-chain fatty acids

The SCFAs formate, acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, and the SCFA analogues succinate, and caproate were measured both in feces and plasma samples. Formate was only quantified in plasma. Henceforth all these molecules are referred to as SCFAs in the manuscript. SCFAs were analyzed as described by Han et al,[188] with addition of a quenching step (manuscript in preparation), as described in **V**. For each batch, feces, plasma samples, blanks, and quality control samples were prepared and mixed with derivatizing reagents, followed by shaking and centrifugation. Thereafter the reaction was quenched using quinic acid, followed again by shaking and centrifugation. Finally, an internal standard was added to each sample. Samples were analyzed on a quadrupole ion trap triple-quadrupole mass spectrometer with an atmospheric pressure chemical ionization source, operated in the negative-ion mode. The mobile phase consisted of water and acetonitrile delivered in a gradient. Samples were randomized in a constrained fashion, such that samples from the same individual were randomized within the same batch. Multiple-reaction monitoring was used to detect analytes, using one transition for quantification and another as qualifier.

4.6.4. Untargeted metabolomics

Untargeted metabolomics was performed on 623 plasma samples from the intervention weeks and 874 samples from the provocation test, analyzed as two separate data sets on a UHPLC-QTOF-MS system. Details of the procedure are described in **IV**. Briefly, 30 µl plasma was mixed together with 200 µl cold acetonitrile in a 96-deep-well microplate, shaken, centrifuged, and filtered. Study-specific quality control samples were prepared by pooling aliquots from each plasma sample in the first analytical batch. Both study-specific and long-term quality control samples from an independent population were included in each batch. For the intervention weeks, three-level constrained randomization was performed to i) assure that samples from the same participant were analyzed in the same batch and in direct proximity during the injection sequence; ii) randomize the order of interventions within individual participants; and iii) randomize the order of interventions and their preceding washout week within respect to individual and intervention order. For the provocation test, two-level constrained randomization was performed by ensuring that samples from the same participant were adherent to each other and by randomizing the order of time points.

The mobile phase consisted of gradients of water, methanol, and 0.04 % formic acid. Samples were analyzed by reverse-phase chromatography and the metabolites were ionized by electrospray ionization, both in positive and negative mode. Tandem MS2 data acquisition was performed on study-specific quality control samples and selected test samples under collision energies of 10, 20, and 40 V.

Preprocessing of metabolomics data followed the procedure presented in section 3.6.1 Annotation of metabolites was reported according to Schymanski's scale[138] (see section 3.6). In brief, annotation was performed by matching RT, m/z, and fragmentation patterns against online databases and authentic standards. If no MS2 could be generated, MS1 comparisons were made to the human metabolome database[189]. A cosine similarity score was calculated for samples in comparisons to facilitate matching with online database/authentic standard. Further details are provided in **IV**. The workflow describing the generation of plasma metabolome data is illustrated in **Figure 7**. Henceforth the plasma metabolome will be referred simply as 'metabolome'.

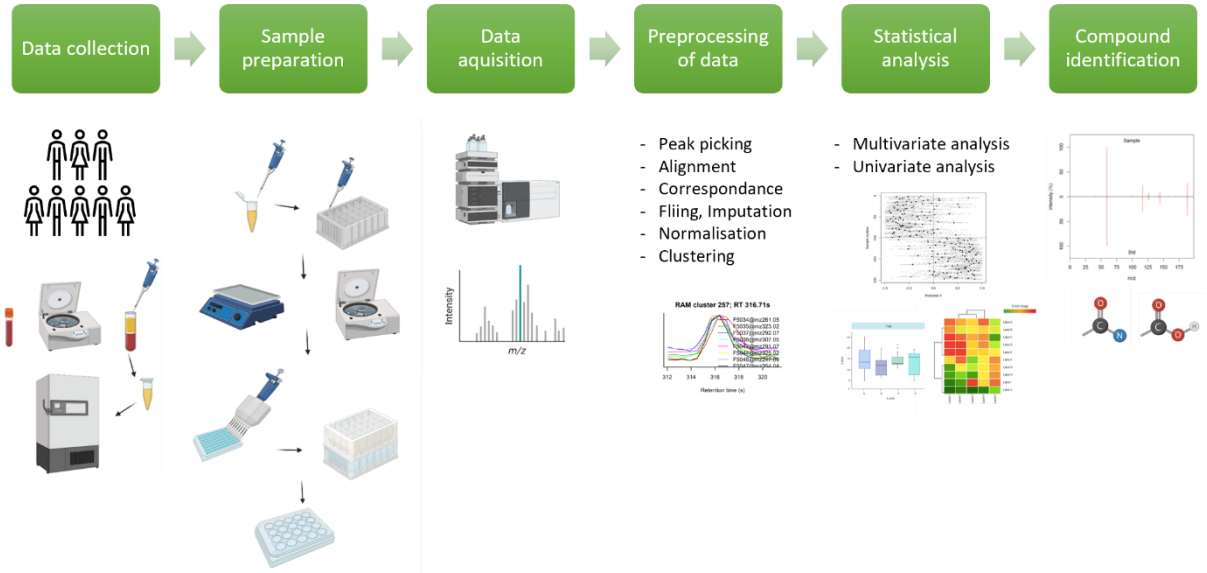


Figure 7. Workflow describing the generation of plasma metabolome data. At the trial site, blood samples were collected, centrifuged, and plasma samples were immediately frozen. Prior to untargeted metabolomics analysis, proteins were precipitated from plasma and analyzed by LC-MS. Metabolomic data were pre-processed via algorithms for peak picking, alignment, correspondence, peak filling, imputation, normalization, and clustering of peaks belonging to the same feature (see section 3.6.1). Thereafter, data analysis was applied followed by identification of specific metabolites. Images created by the author and with BioRender.com.
Abbreviation: LC-MS, Liquid Chromatography Mass Spectrometry

4.7. Data analysis

Linear mixed models were used to assess the relationship between IBS symptoms, water content in feces, microbiota, SCFAs or metabolites and reported BSFS levels, interventions and subtypes. In studies involving repeated measurements, data collected from a single subject are not independent. Therefore, both within-subject and between-subject variance need to be properly managed in linear mixed models.[190] A further challenge with crossover trials is the risk of a carryover effect. Because negative responses to food intake in IBS tend to come and

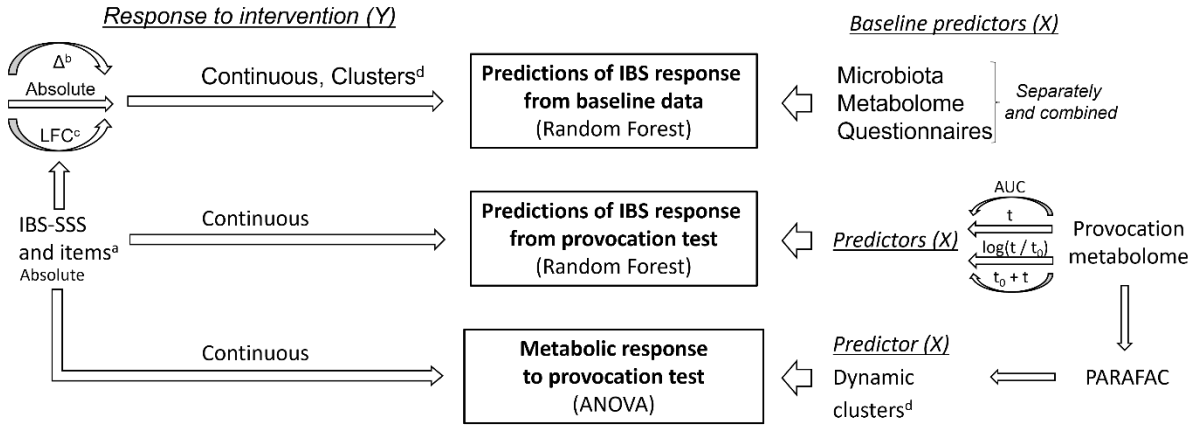
go swiftly,[191–193] a single week of washout between interventions was considered sufficient. Testing for carryover is not recommended due to loss of power.[194] Instead, the study design must be carefully considered before initiating the trial. Mixed models benefit from a balanced analysis, do not require adjustment for confounders, and are free from artefacts arising from such adjustments. In all linear models, normality and homoscedasticity were visually inspected by Q-Q and residual plots.

Partial Spearman correlation analysis was conducted to investigate any correlations between water content and BSFS, interventions, microbiota, SCFAs, the metabolome and IBS symptoms. To discover the most discriminative bacterial genera and metabolites, the MUVR algorithm[149] (a Random Forest (RF) algorithm with unbiased variable selection in a repeated double cross-validation) was used, followed by permutation tests ($p < 0.05$) to assure that the outcome was not due to overfitting. RF modelling is a machine learning algorithm which is based on multiple decision trees, where each tree aims to find the best split in data. By introducing independence between trees, each tree becomes a less effective predictor. However, by combining multiple trees, the overall predictions tend to both improve and become more robust. RF has the advantage of not requiring specific variable distributions or linearity between predictors and response, not requiring scaling or transformation and is therefore less sensitive to variable preprocessing schemes.[195] In prediction modelling, cross-validation is a method which uses segmentation of data for training and testing with the aim to minimize overfitting. However, overfitting still occurs,[148] and repeated double cross validation can instead be used for more robust modelling.[148,149]

A comprehensive methodological approach was applied to associate a differential response to the interventions based on baseline predictors, such as microbiota, SCFAs, the metabolome, and questionnaire data (separately and in combination), as well as data collected during the provocation test (**Figure 8)**. RF modelling of IBS-SSS (response) as a function of baseline data (predictors) seeks out predictive molecular explanatory models for differential response to treatment. The use of regression with IBS-SSS values identifies such responses at a continuous scale (individual responses). Clustering of IBS-SSS responses yields potential associations at group level, which could correspond to metabotypes. Unsupervised clustering was performed, without anticipating cut-off limits. Parallel factor analysis (PARAFAC)[196] is an extension of principal component analysis, but applied to multidimensional data. Here, PARAFAC captured metabolite dynamics during the 4 h of the provocation test. This approach is for general metabotyping and does not require a response variable. Its advantage lies in relating unsupervised findings of clusters or components to measurable differences in response or health trajectory. Here, components and scores were clustered and analyzed by ANOVA as dependent variables to IBS-SSS items, for subsequent evaluation of a potential link to IBS symptoms.

In **I**, the Bonferroni method was used to adjust for multiple comparisons, given that the nature of the analysis was confirmatory. In the other manuscripts, primary and other outcomes were adjusted using the Benjamini-Hochberg false discovery rate method. Adjusted p-values (q) <0.05 were considered statistically significant. Explorative analyses (i.e, microbiota, SCFAs, and untargeted metabolome analysis) were not adjusted. Further details of the statistical tests

used in **I** and **III**–**VI** are listed in **Table 4** and in the respective manuscripts. All analyses were performed in R software (version 3.6.1–4.0.0).



[a] IBS-SSS items
Severity of abdominal pain, frequency of abdominal pain, abdominal distension, dissatisfaction with bowel habits, interference with quality of life, total IBS-SSS score
[b] Δ = Difference in IBS-SSS (or items) from baseline
[c] LFC = log fold-change in IBS-SSS (or items) from baseline
[d] Clusters obtained from k-means and hierarchical clustering, 2-4 cluster, none/scaled data, ≥ 8 per cluster, down sampling

Figure 8. Machine learning RF algorithm used to investigate differential responses to treatment (response) in relation to molecular data (predictors). At a continuous scale (i.e., individual responses), RF regression with IBS-SSS values (response) was used. To evaluate potential associations at group level, representing metabotypes, RF classification using clustered IBS-SSS responses (response) was used. Baseline microbiota, metabolome, and clinical data (e.g., dietary intake, physical activity, and demographic data) were modelled separately and combined as predictors. Finally, unsupervised PARAFAC analysis of molecular data was performed to capture the dynamics of the metabolome during the 4 h of the provocation test. PARAFAC component scores were clustered and related to IBS response using ANOVA.

Abbreviations: ANOVA, analysis of variance; AUC, Area under the curve; IBS-SSS, Irritable bowel syndrome severity scoring system; LFC, log fold change; PARAFAC, Application of Parallel Factor Analysis; RF, Random Forest; t, timepoint -10 min (-10, 0, 10, 20, 30, 90, 150, 240 min)

Table 4. Statistical analysis performed in **I** and **III-VI**.

Title	Statistical procedure	Statistical test	Covariate	Multiple testing
Fermentable oligo-, di-, monosaccharides, and polyols (FODMAPs), but not gluten, elicit modest symptoms of irritable bowel syndrome: a double-blind, placebo-controlled, randomized three-way crossover trial (**I**)	Mixed models	Independent variables: Intervention, participant ID, period. Dependent variables: Questionnaire items, anthropometric measures		Bonferroni
	McNemar's test	Test between interventions for >50 (or >100) IBS-SSS pointsrelative to respective wash out		
Modest conformity between self-reporting of Bristol stool form and fecal consistency measured by stool water content in irritable bowel syndrome, a FODMAP and gluten trial (**III**)	Mixed models	Independent variables: BSFS levels or IBS subtypes or intervention, participant ID, period. Dependent variable: Fecal water content		FDR
	Partial Spearman correlation	BSFS score and water content	Participant ID	FDR
	Generalized estimating equations (correlation structure = exchangeable, family = binomial)	Proportion of subjects with reported BSFS categories with ≤68.5% and ≥78% water content overall and per IBS subtype		FDR
	Intraclass correlation	Measure agreement in reported water content within individuals		
	Krippendorff's alpha (ordinal data)	Measure agreement in reported BSFS within individuals		
IBS randomized crossover challenge study: FODMAPs alter bile acids, tryptophan and phenolic-derived metabolites, while gluten modifies lipid metabolism (**IV**)	MUVR, permutation tests	Predictor data (X) = metabolome, response (Y) = Intervention or subtype Classification Permutation test		
	Mixed models	Independent variables: Intervention, participant ID, period. Dependent variable: Metabolite		
	Partial Spearman correlation	End of intervention metabolome and questionnaires	Age, sex	

Title	Statistical procedure	Statistical test	Covariate	Multiple testing
FODMAPs not gluten, alter the microbiota and associate with the metabolome in irritable bowel syndrome (**V**)	MUVR, permutation tests	Predictor data (X) = metabolome, microbiota response (Y) = Intervention or subtype Classification Permutation test		
	Mixed models	Independent variables: Intervention, participant ID, period. Dependent variable: microbiota, SCFAs		
	Partial Spearman correlation	End of intervention metabolome, microbiota, SCFAs and questionnaires	Age, sex	
Exploration of differential responses to FODMAPs and gluten in people with irritable bowel syndrome- a double-blind randomized cross-over intervention study (**VI**)	MUVR	Predictor data (X) = microbiota, and/or SCFAs, and/or metabolome and/or variables at baseline[a], Response (Y) = Intervention response (IBS-SSS and its individual items) Regression and classification		
	Fisher t test, ANOVA	Clusters from models with CR >0.6 and $p_{permutation}$<0.05 were further analyzed univariately against IBS symptoms		FDR
	PARAFAC, (Details described in **IV**)	PARAFAC model on time series metabolomics data from the provocation test		
	ANOVA	Each component score clustered and univariately analyzed against IBS-SSS score		FDR

Abbreviations: ANOVA, analysis of variance; BSFS, Bristol Stool Form Scale; CR, Classification rate; FDR, False discovery rate; FODMAPs, fermentable oligosaccharides-, disaccharides-, monosaccharides, and polyols; IBS-SSS, Irritable bowel syndrome Severity Scoring System; ID, Identity; MUVR, Random Forest algorithm with unbiased variable selection in a repeated double cross-validation[149] run as regression and classification; PARAFAC, Application of Parallel Factor Analysis; SCFAs; Short-chain fatty acids

[a] Data from the screening occasion: IBS severity and subtype, the questionnaires IBS-SSS, SF-36v2, the demographic questionnaire, Baecke's physical activity questionnaire, FFQ, and anthropometric measurements.

5. RESULTS AND DISCUSSION

5.1. Baseline characteristics, diet and compliance

A detailed description of baseline characteristics is available in **I**. Briefly, the study aimed to recruit an equal number of women and men but due to difficulties in recruiting men, only 14 of 110 participants were male. Among the 103 completers, 90 were women and 13 were men; their average age was 46 ± 15 years and BMI was 24 ± 4 kg/m^2. The study also aimed to assess an equal number of IBS subtypes; hence, participants were classified into IBS-C (n = 29), IBS-D (n = 35), and IBS-M (n = 39).

The participants were highly motivated to follow the study instructions, as reflected by the low dropout rate (6%), and were observed by the same personnel throughout the study. Also, the low IBS-SSS scores during the washout periods suggested high compliance throughout the study. As there are no objective markers for FODMAP and gluten intake, compliance was based on self-reporting. The FODMAP intervention had a clear effect on the microbiota and metabolomics profiles (**IV** and **V**), further confirming good compliance. A challenge when supplementing foods is dietary confounding, which leads to food displacement.[197] However, a properly designed double-blind study ensures that substitutions are similar for all interventions, and outcome variations reflect the different interventions.

5.2. Clinical effects of the FODMAP, gluten and placebo interventions

FODMAPs, but not gluten, caused modest symptoms in IBS subjects (**I**). The total IBS score for FODMAPs was higher (mean ± standard error of the mean = 240 ± 9) compared to placebo (198 ± 9; p = 0.0006) and gluten (208 ± 9; p = 0.013), with no observable difference between gluten and placebo (**Table 5**). A clinically meaningful effect requires a change of 50 points in the IBS-SSS score.[19] Hence, the difference between FODMAPs and placebo was only modest and the proportion of subjects increasing by >50 or >100 points in the interventions compared to the preceding washout week was similar for both treatments (**Figure 9**). Abdominal distension increased in FODMAP compared to both placebo and gluten interventions, while the frequency of abdominal pain was higher with FODMAP than placebo. There were no observable differences between the interventions with respect to severity of abdominal pain, dissatisfaction with bowel habits or interference with quality of life (**Table 5**). Similarly, no observable differences between interventions were reported for bowel habits, wellbeing measured by the SF-36v2 questionnaire or anthropometric measurements. Some studies have reported an effect on bowel habits and wellbeing for FODMAPs[152,173,183,198–200] and gluten;[93,201,202] whereas others have not.[94,158,173,203,204] We also observed large inter-individual variability in response to the interventions. Thus, although only a modest treatment effect was observed, we could at this point not rule out underlying mechanisms for differential responses.

Table 5. Total IBS-SSS score and subdivided items after the FODMAP, gluten, and placebo interventions. Results are presented as estimated marginal means [standard error] and (95% confidence interval).

	FODMAPs	Gluten	Placebo	Overall p-value	FODMAPs-Placebo	FODMAPs-Gluten	Gluten-Placebo
Total IBS-SSS score	240 [9] (222, 257)	208 [9] (190, 226)	198 [9] (180, 215)	0.0023	42 [11] (20, 64) p = 0.0006	32 [11] (10, 54) p = 0.013	10 [11] (-11, 31) p = 1.0
Severity of abdominal pain	35 [2] (31, 40)	34 [2] (29, 38)	32 [2] (27, 36)	1.0			
Frequency of abdominal pain	58 [4] (51, 65)	49 [4] (42, 55)	44 [3] (37, 51)	0.012	14 [4] (6, 22) p = 0.0020	9 [4] (1, 17) p = 0.072	5 [4] (3, 13) p = 0.74
Abdominal distension	45 [2] (40, 49)	37 [2] (33, 42)	32 [2] (28, 37)	0.0003	13 [3] (7, 19) p < 0.0001	8 [3] (2, 14) p = 0.023	5 [3] (-1, 11) p = 0.25
Dissatisfaction with bowel habits	56 [2] (52, 60)	52 [2] (48, 56)	50 [2] (46, 54)	0.51			
Interference with quality of life	55 [2] (51, 59)	50 [2] (46, 54)	52 [2] (47, 56)	0.29			

Abbreviations: FODMAPs, fermentable oligosaccharides, disaccharides, monosaccharides, and polyols; IBS-SSS, Irritable bowel syndrome severity scoring system

A remarkable finding of the trial was that IBS symptoms at baseline (screening) were considerably worse compared with all interventions (**Figure 10**). This could, e.g., relate to increased awareness of dietary choices and a healthier lifestyle during the trial. Furthermore, it could also relate to a psychological attention effect due to regular visits to health care facilities, which is known to improve health and wellbeing.[205] In fact, both dietary and behavioural therapies are effective therapies towards IBS.[12]

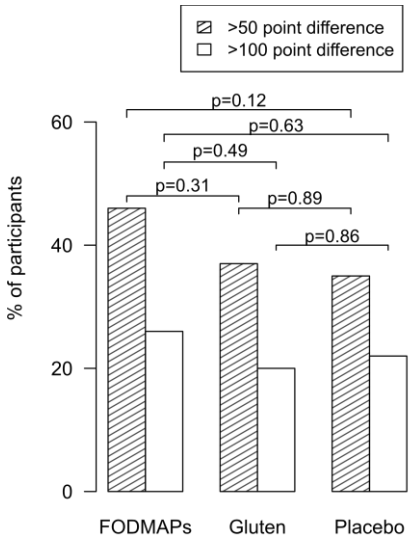


Figure 9. Proportion of participants increasing by >50 and >100 total IBS-SSS points after the FODMAP, gluten and placebo interventions.

Even though provocation tests were done with high doses of gluten, no systematic effect on IBS symptoms was observed. Some studies have reported that gluten worsened IBS symptoms,[93,202] while others found no effect.[94,204] The present study, combined with the results from a systematic review and meta-analysis,[13] suggests that the overall effect of gluten on IBS symptoms is an overestimate.

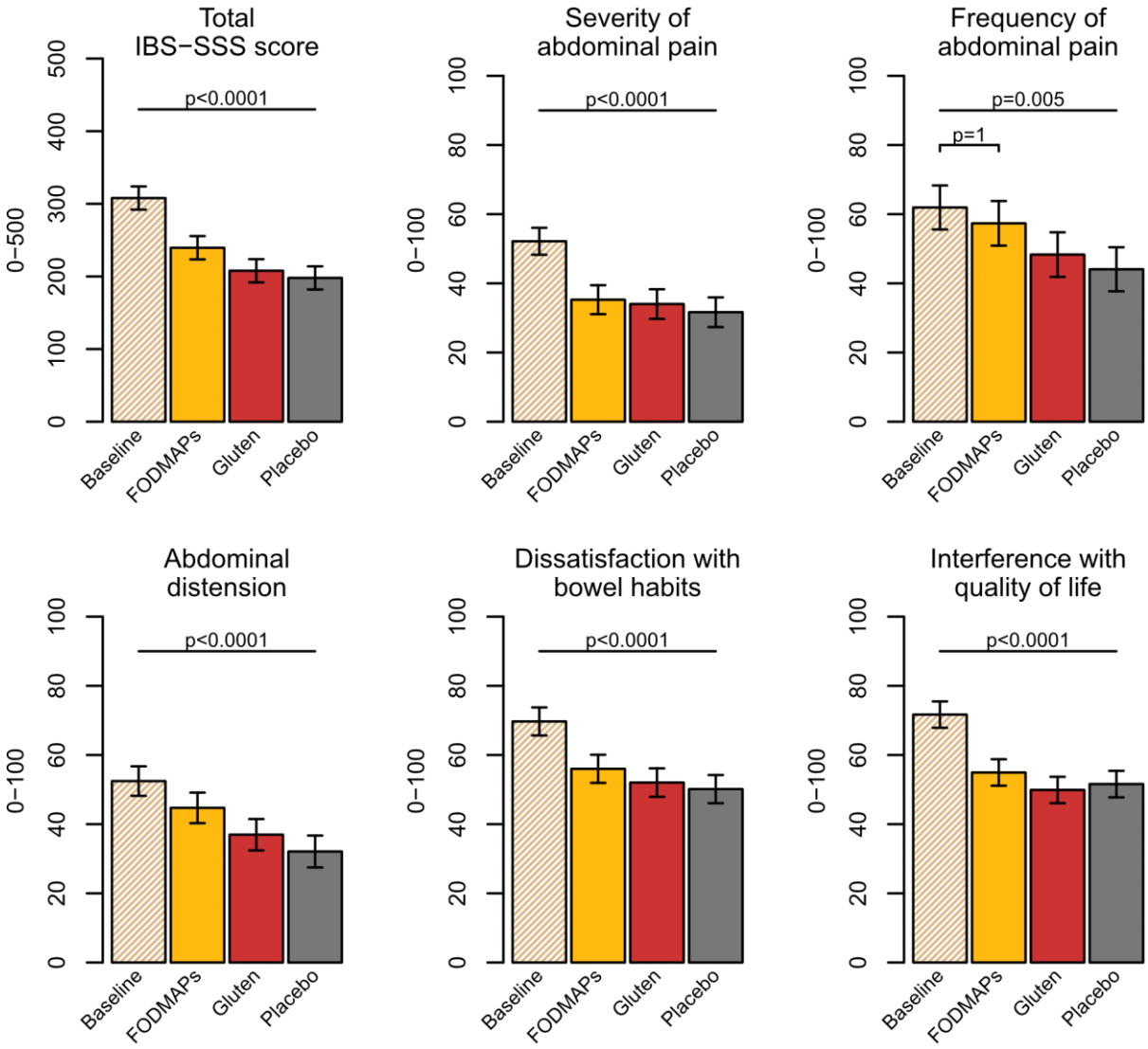


Figure 10. IBS-SSS scores at baseline and after the FODMAP, gluten, and placebo interventions. Data are presented as estimated marginal means, confidence interval (%) and p-value from a type III test. The only significant pairwise comparison between baseline and the interventions was for FODMAPs in frequency of abdominal pain (p = 1). Both type III test and pairwise comparisons were adjusted with Bonferroni correction.
Abbreviations; IBS-SSS, Irritable bowel syndrome Severity Scoring System

5.2.1. BSFS and stool water content

Concordance between subjectively reported fecal consistency based on BSFS and objectively measured stool water content was analyzed (**III**). BSFS is the recommended tool to distinguish different IBS subtypes[1]. Surprisingly, BSFS has not been evaluated against an objective marker in IBS patients. In this trial, BSFS scores and stool water content correlated only modestly ($r = 0.36$, $p < 0.0001$, **Figure 11**). A marginally higher correlation was previously found between reported BSFS scores and stool water content in healthy individuals and those with lactose intolerance: $r = -0.44$ for BSFS vs. stool dry matter[206] and $r = 0.49$ for BSFS vs. water content.[207]

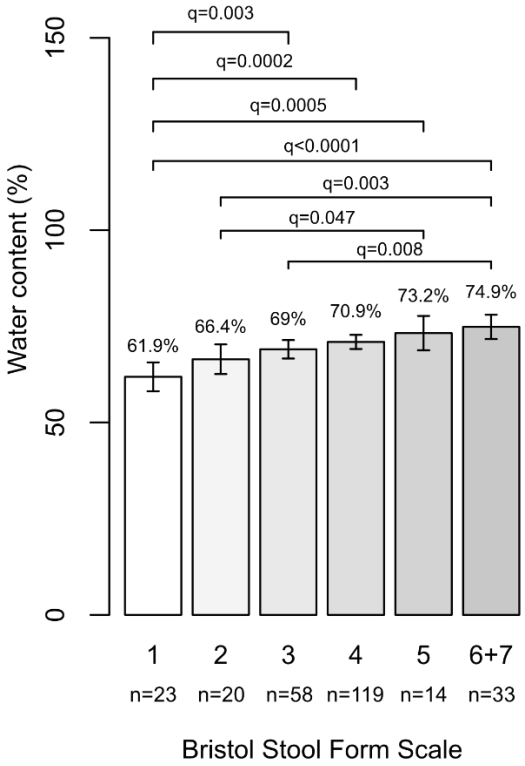


Figure 11. Measured water content for each reported Bristol Stool Form Scale score (BSFS). BSFS 1-2 = hard stool, BSFS 3-5 = normal stool, BSFS 6-7 = loose stool. Results are presented as estimated marginal means ± standard error with pairwise comparisons adjusted by the false discovery rate method.

As expected, subjects with IBS-C (32%) reported hard stool more often than those with IBS-D (12%) or IBS-M (10%) ($q \leq 0.02$). The association for IBS-D (18%) was less clear, with only a tendency of reporting loose stool more often compared to IBS-C (3%) ($q = 0.09$). A similar trend was found for water content (**Figure 12**). Predictably, 77% of those who reported hard stool had a water content ≤68.5%, which is synonymous with constipation. Among those who reported loose stool, only 52% had a water content ≥78%, corresponding to diarrhea. These results are in accordance with those of Halmos et al.,[182] who observed greater accuracy in reporting hard stool consistency by IBS-C individuals than loose consistency by IBS-D individuals.

Individuals with IBS may have more difficulty in accurately reporting fecal consistency due to simultaneous experience of symptoms, such as pain and urgency.[207] In agreement with these observations, IBS patients have reported difficulties in interpreting the BSFS.[208] However,

correct IBS subtyping is vitally important, as distinct subtypes will benefit from different treatment regimes.[209] Also, incorrect subtyping could mislead the interpretation of results in clinical trials. Training individuals towards a correct usage of the BSFS augments precision and accuracy.[20,207,210] Despite repeatedly collecting and reporting BSFS in the present trial, correlation between BSFS and water content did not improve over time, indicating that repeated self-reporting does not constitute successful training. Instead, more controlled training is needed.

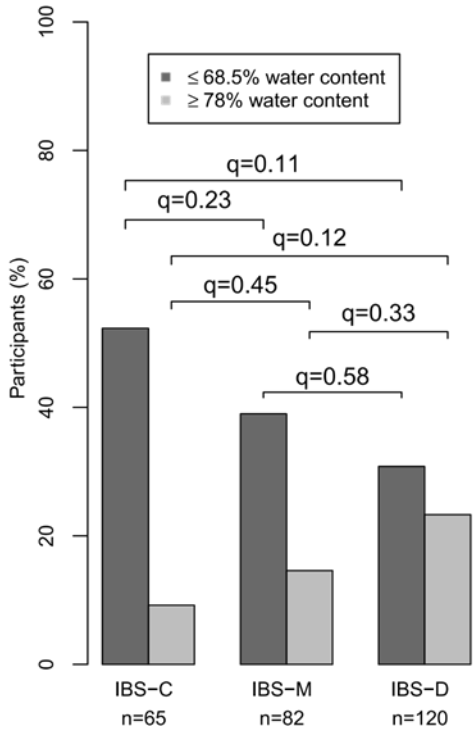


Figure 12. Water content in stool samples from respective IBS subtype, IBS-C, IBS-M, and IBS-D.
Abbreviations: IBS-C/M/C, Irritable bowel syndrome with constipation/mixed/diarrhea predominance

FODMAPs are believed to increase intestinal water content due to osmosis.[80] Here, no differences in stool water content were detected between interventions, which is in accordance with previous findings.[182] Analogously, as described in **I**, there were no differences in reported BSFS between interventions, although results from similar studies may vary.[93,94,173,198,200,201,203,204,211] Inconsistency between reported BSFS in trials may partly relate to the study design, as most studies lack sufficient blinding, which can affect consistency.

There was a tendency for dissatisfaction with bowel habits to increase as BSFS was further away from normal consistency (BSFS 4). No similar associations were found for other IBS items. Consequently, there was no evidence supporting a link between reported BSFS and IBS symptoms, in accordance with previous findings.[42]

5.1. Effect of interventions on microbiota

None of the interventions had a significant effect on the alpha diversity measures of richness, Shannon's diversity index, Simpson's index or inverse Simpson's index. In supervised

analysis, FODMAP intake affected microbiota composition (classification rate ~0.90 for all pairwise FODMAP-related models, $p_{permutation} \leq 0.0001$), although no such effect was observed for gluten (classification rate ≤ 0.64, $p \geq 0.11$) (**V**). No microbial genera appeared to discriminate the interventions between IBS subtypes.

Several genera were affected by the FODMAP intervention compared to control: *Anaerostipes, Bifidobacterium, Faecalibacterium, Fusicatenibacter, Agathobacter, Paraprevotella,* and *Oxalobacter* increased, whereas *Lachnoclostridium, Roseburia, [Ruminoccocus]torques, Lachnospiraceae NK4A136, Lachnospiraceae NA, Hungatella, Eisenbergiella, Negativibacillus, Coprostanoligen NA,* and *Flavinofractor* decreased (**Figure 13**). FODMAPs favored the presence of genera with saccharolytic capacity, which could be expected by their propensity to increase after fiber intake. So far, *Bifidobacterium* is the only genus that has shown a consistent increase upon FODMAP treatment in IBS.[113] *Bifidobacterium* prevents pathogens and harmful bacteria from colonizing the gut mucosa, modulates systemic immune responses, and enables the bioconversion of a number of dietary compounds into bioactive molecules, although further work is needed to improve the solidity of the evidence.[212] Other health-promoting genera that showed an increase following FODMAP intervention included *Anaerostipes*, which is associated with improved renal function,[213] as well as *Faecalibacterium* and *Fusicatenibacter*, which possess anti-inflammatory properties.[214,215] Interestingly *[Ruminococcus] torques*, which has previously been associated with increased gut permeability and inflammation,[216,217] showed a decrease with FODMAPs, confirming earlier results obtained with a high intake of rye, which is rich in dietary fiber.[218] This finding indicates that FODMAPs, which are constituted of rapidly fermented fibers, promote a healthier microbiota composition.

A few of the FODMAP-affected genera showed a weak association with IBS symptoms ($r < 0.25$), **Figure 14** . *Bifidobacterium* correlated with pain linked to bowel emptying, even though this genus had not been reported to associate with IBS symptoms.[152,158,203] Other genera displayed no obvious pattern of correlations, suggesting that these associations were likely spurious. Finally, genera such as *Roseburia*, *Lachnoclostridium*, and *Lachnospiraceae NA*, correlated with IBS symptoms ($r \leq 0.25$), but they were not affected by FODMAPs.

The absence of any effect by the gluten intervention was expected, because similar findings had been observed in previous trials.[219] Moreover, carbohydrates are the preferred substrate for the fermentation of microbiota and protein fermentation occurs only once carbohydrates are depleted.[220] While participants consumed a high amount of gluten as part of the intervention, they simultaneously consumed also carbohydrates.

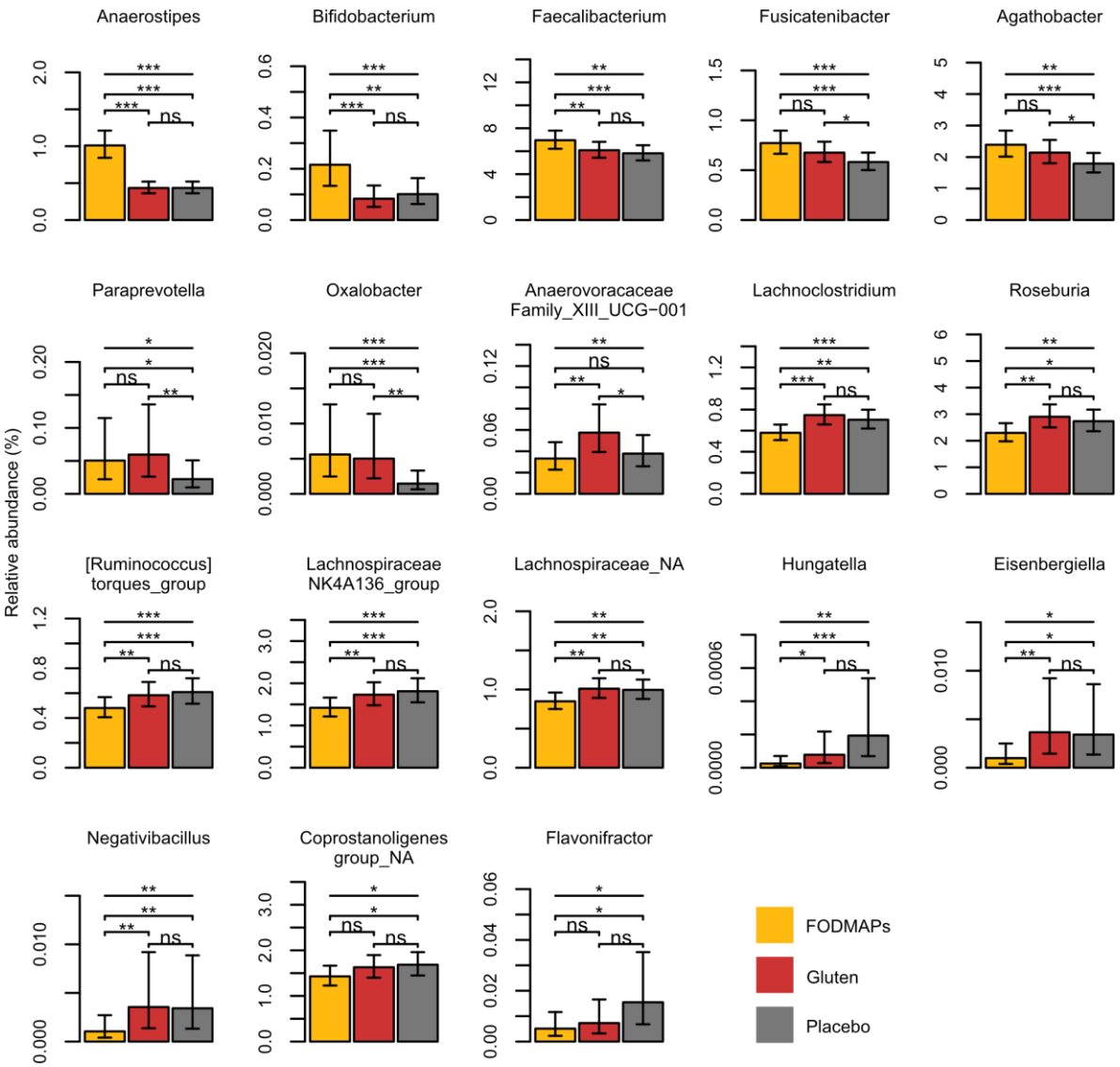


Figure 13. Genera selected from Random Forest modelling for FODMAP related models (FODMAPs vs placebo, FODMAPs vs washout and FODMAPs vs Gluten). Genera showing significant differences (relative abundance, % of total bacteria) between the interventions FODMAPs, gluten, and placebo are reported. Data are presented as estimated marginal means and confidence interval (%)
P-values <0.05 are presented using the star system. * = p<0.05, ** = p<0.01, *** = p<0.001

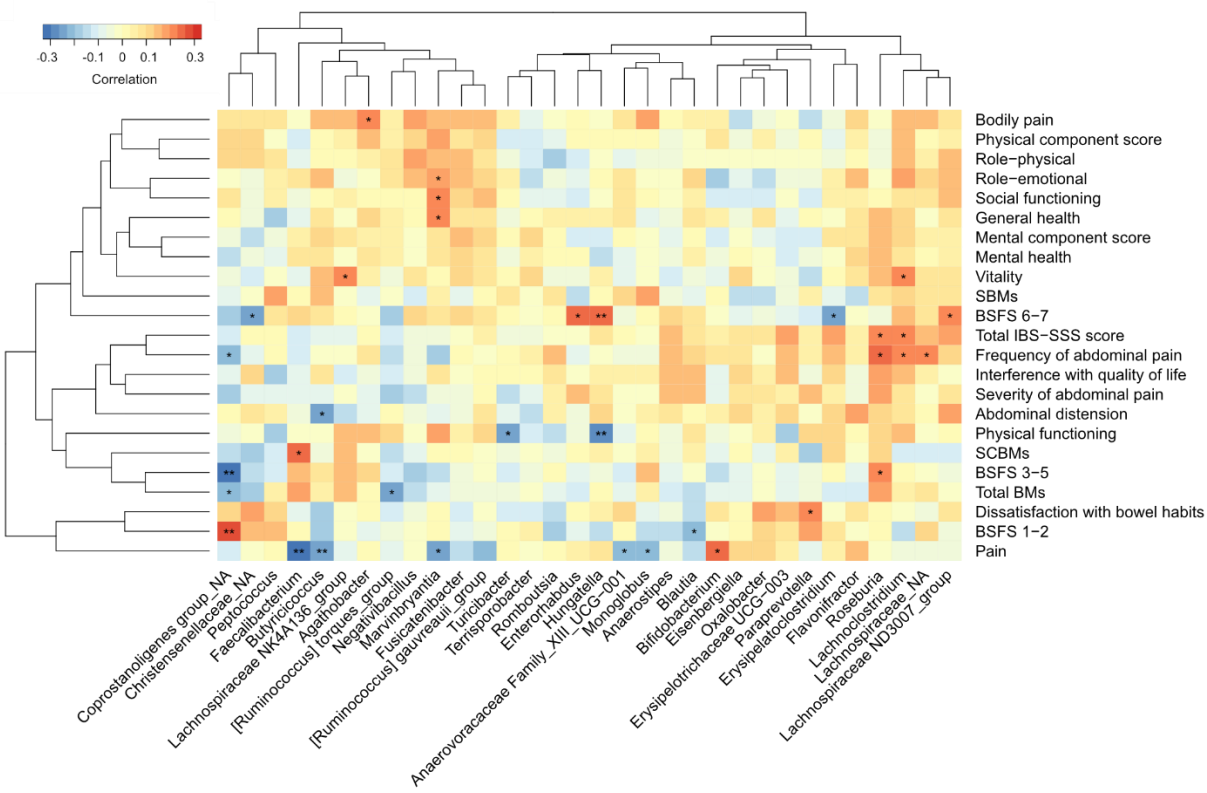


Figure 14. Partial Spearman correlation between bacterial genera selected from Random Forest modelling for FODMAP related models (FODMAPs vs placebo, FODMAPs vs washout, and FODMAPs vs gluten) and the questionnaires IBS-SSS, SF-36v2 (health and quality of life), and the bowel diary, adjusted for age and sex.
* = p<0.05, ** = p<0.01, *** = p<0.001
Abbreviations: BM, bowel movements; BSFS, Bristol stool form scale; FODMAPs, fermentable oligosaccharides-, disaccharides-, monosaccharides, and polyols; SBMs, spontaneous bowel movement; SCBMs, spontaneous complete (a sensation of complete evacuation) bowel movement; Pain, abdominal pain linked with bowel emptying

5.2. Effect of interventions on short-chain fatty acids

Only weak effects on SCFAs were observed in the present study, with only isobutyrate showing a decreased plasma level following FODMAPs (**V**). Such a result is in accordance with FODMAPs being the preferred substrates for microbiota, thereby reducing protein fermentation and production of branched-chain amino acids.[221] The absence of a more general effect of FODMAP intake on SCFAs could relate to different locations of fermentation of SCFA (in the colon) and sampling (feces),[80,113,175] even though this does not explain their unperturbed plasma levels.

Absence of a general effect of gluten on SCFAs could relate to carbohydrates being consumed simultaneously with gluten.[206] Interestingly, isovalerate showed a reduced abundance in feces after gluten intake (**V**), which is contrary to expectations as it is a branched-chain fatty acid favored by protein breakdown.[222] Notably, all fecal SCFAs were generally lower after gluten intake, although only isovalerate reached significance. A previous study of a low (2 g/day) and high (18 g/day) gluten diet for 2 weeks did not find any effect on SCFAs in feces or plasma.[114] Another study provoking with 30 g gluten for 4 days found an increase in most SCFAs, even though the gluten contained minor amounts of non-absorbable starch.[130] Taken together, it is

possible that the observed effect of gluten on isovalerate was spurious, but it could also be that gluten systematically lowers SCFAs, although no mechanistic explanation exists so far.

5.3. Effect of interventions on the metabolome

FODMAP intake affected the metabolome CR = 0.88, $p_{permutation}<0.0001$, whereas gluten exposure had more modest effect (CR = 0.72, $p_{permutation} = 0.01$) (**IV)**. The main metabolites affected by FODMAPs were bile acids (decreased), phenolic- derived metabolites (increased), 3-indolepropionate (increased) and unknown phenyl sulphates (some increased and some decreased), **Figure 15**. These groups of metabolites clustered together, suggesting robust findings. The intake of gluten affected the lipid metabolites carnitine derivates, an acyl-CoA derivate, a medium-chain fatty acid, and an unknown lipid.

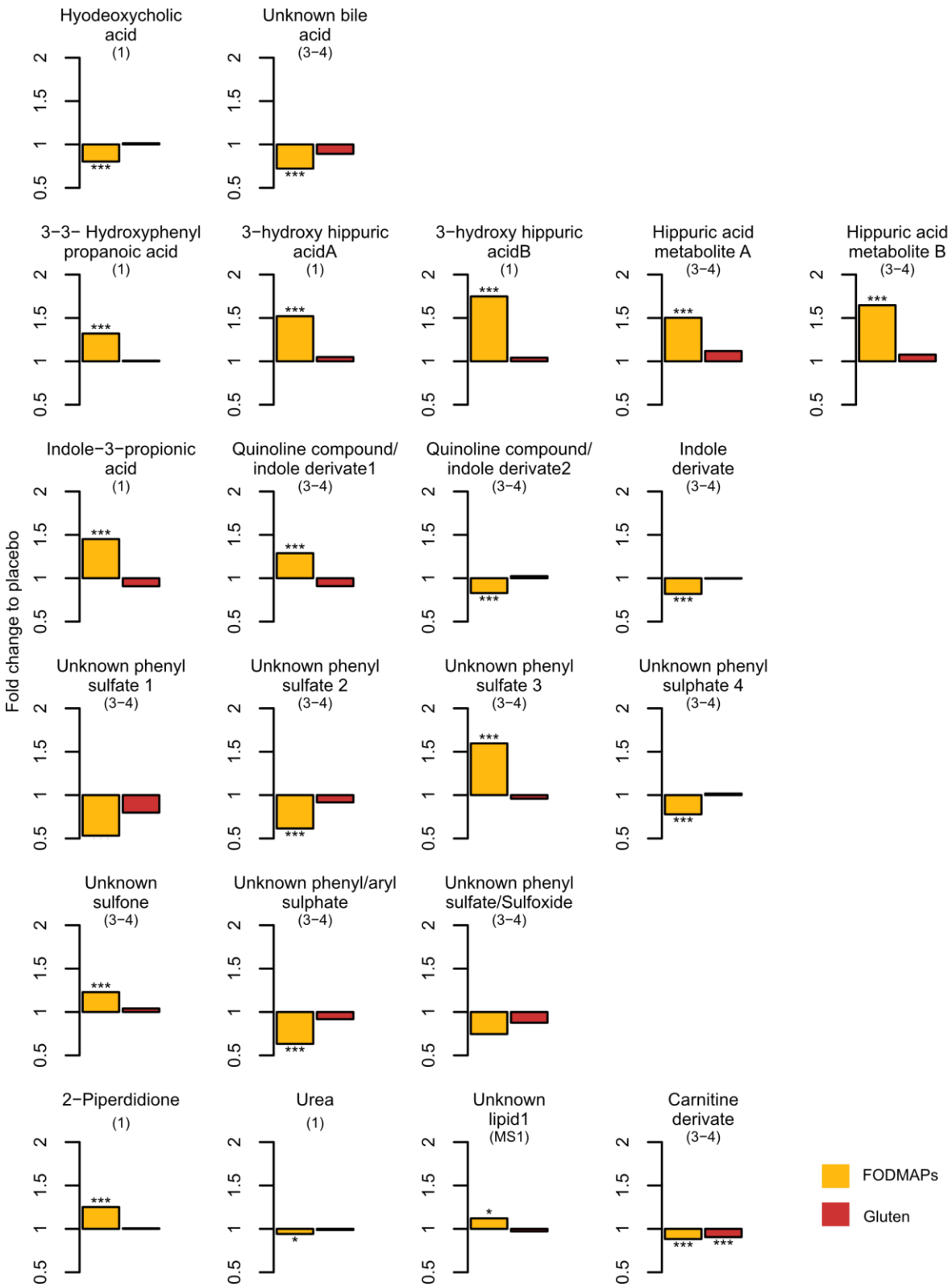


Figure 15. Annotated features from FODMAP-related models selected from RF modelling. Data are presented as fold change for FODMAP and gluten vs. placebo based on marginal means from mixed model analysis. The level of metabolite identification is presented in parentheses (From MS1 to Schymanski's scale 1-5).[138] Further details are reported in **IV**.
* = p<0.05, ** = p<0.01, *** = p<0.001

Several of the metabolites identified following the FODMAP intervention relate to health outcomes. Secondary bile acids have been associated with colon cancer.[223] The phenolic-derived metabolites 3-hydroxyhippurate and 3-3-hydroxyphenylpropionate are microbial breakdown products of polyphenols,[242] and have anti-inflammatory[224] as well as vasodilatory properties.[225] The assumed downstream end-product of these molecules, hippurate,[226,227] has also been inversely associated to BMI, hypertension, type 2 diabetes, and Crohn's disease.[228–232] Thus, the observed increase in polyphenol metabolites suggests potential positive health effects following FODMAP exposure. 3-indolepropionate and other indole derivates are end-products of tryptophan metabolism.[233] These metabolites act as aryl hydrocarbon receptor and pregnane receptor ligands,[234,235] thus playing an important role in the regulation of epithelial renewal, intestinal barrier integrity, and immune response.[236,237] In animal studies, 3-indolepropionate has been linked to reduced fasting glucose and insulin;[238] whereas in human trials, it has been associated with insulin sensitivity and reduced risk of type 2 diabetes.[239] Consequently, the observed increase in 3-indolepropionate implies positive metabolic effects from FODMAP consumption. Lastly, the unknown phenyl sulphates specific for the FODMAP intervention constitute a group of unidentified metabolites that warrant further investigations. Histamine,[152] glucose, and polyols[153] were previously reported to decrease with low FODMAP intake; whereas p-hydroxybenzoic acid and azelaic acid[152] showed the opposite trend. However, these effects were not observed in this study.

Most FODMAP-related metabolites exhibited no association to IBS symptoms (**Figure 16**), in line with a previous study.[158] Only 3-indolepropionate correlated weakly to abdominal pain and quality of life. This is in contrast with 3-indolepropionate having a neuroprotective effect[240] and being associated with improved intestinal barrier integrity,[241] while IBS symptoms are linked with degradation of the intestinal barrier.[48] However, associations did not survive adjustment for multiple testing and further investigations are warranted. Interestingly, circulating bile acids did not correlate with IBS symptoms. Bile acids are considered potential neurotransmitters in IBS and believed to be involved in IBS pathogenesis,[242,243] although previous results have been inconsistent.[242,243]

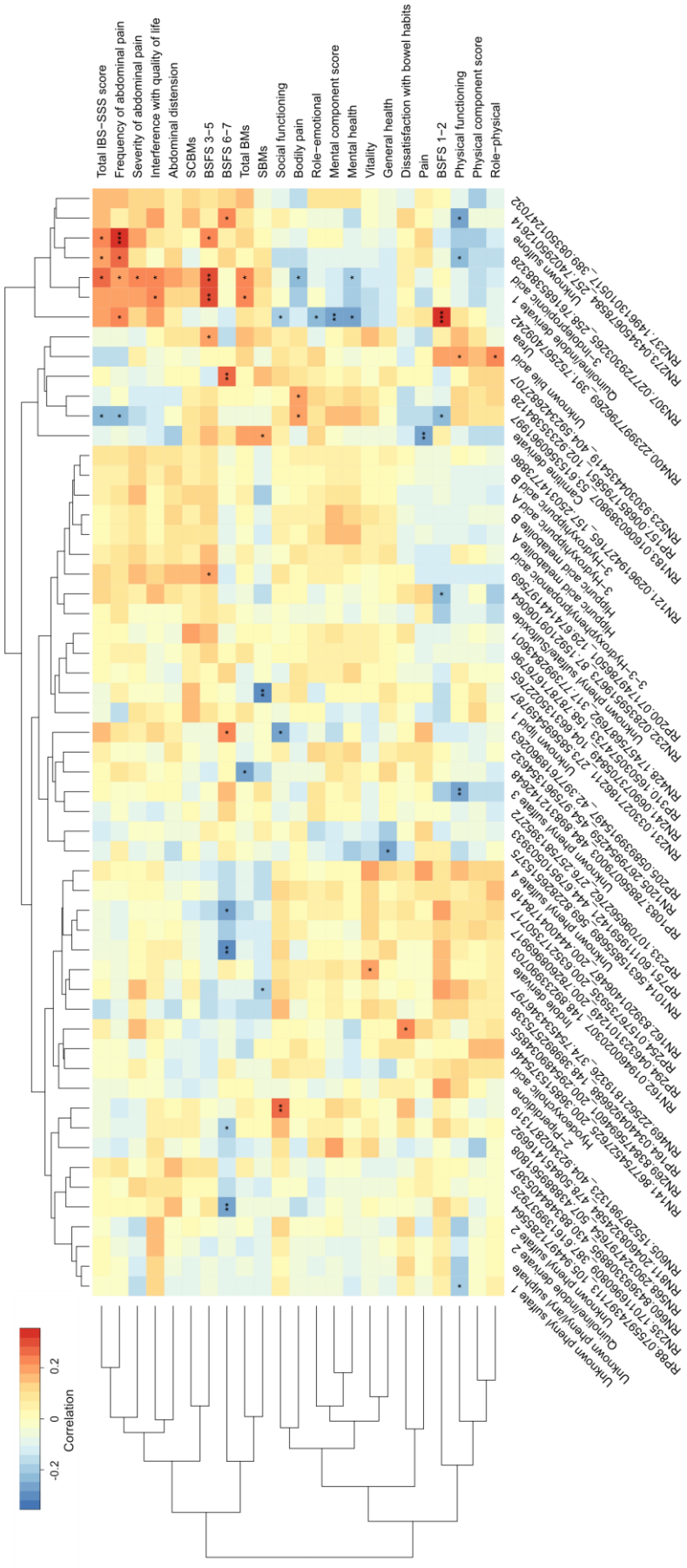


Figure 16. Partial Spearman correlation between metabolites selected from Random Forest modelling for the FODMAP-placebo model and the questionnaires IBS-SSS, the bowel diary and health and wellbeing questionnaire (SF-36v2), adjusted for age and sex. * = p<0.05, ** = p<0.01, *** = p<0.001

BM, bowel movements; BSFS, Bristol stool form scale; FODMAPs, fermentable oligo-, di-, monosaccharides, and polyols; SBMs, spontaneous bowel movement; SCBMs, spontaneous complete (a sensation of complete evacuation) bowel movement; Pain, abdominal pain linked with bowel emptying

Evidence supporting a link between gluten and metabolic health remains inconsistent.[244–247] A minor effect on lipids (**Figure 17**) is in line with a previous study, which reported metabolic changes, including on lipids, in relation to gluten intake.[247] That study investigated the effect of a daily intake of gluten (9.7 g/day) vs. a gluten-free diet and concluded that gluten intake was not associated with harmful health outcomes.[247] However, no such study has been conducted for a high intake of gluten as in the present case. Of note, no meaningful interpretable correlations for gluten-related metabolites and IBS symptoms were found.

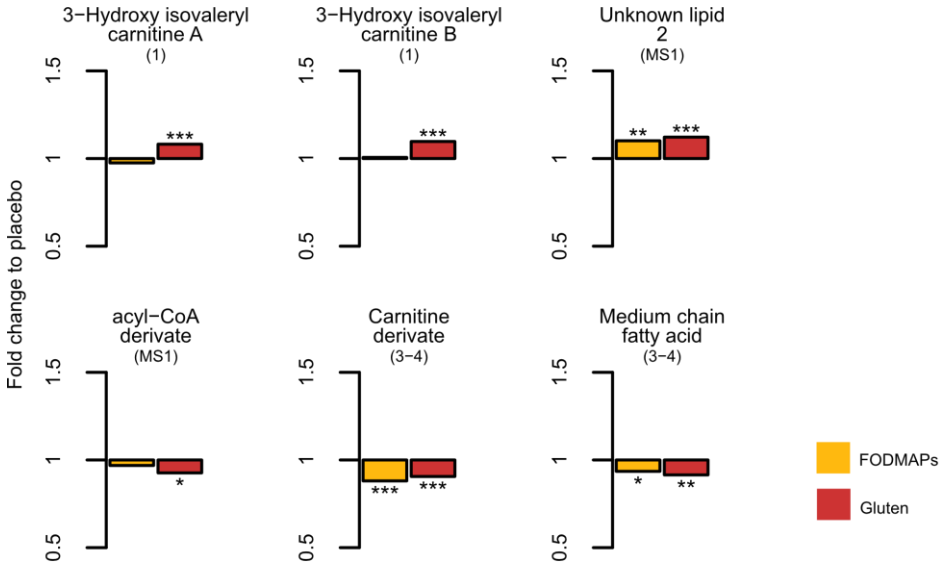


Figure 17. Annotated features from gluten-related models selected from Random Forest modelling. Data are presented as fold change for FODMAPs and gluten against placebo based on estimated marginal means from mixed model analysis. The level of metabolite identification is presented in parentheses (From MS1 to Schymanski's scale 1-5).[138] Further details are presented in **IV**.
* = p<0.05, ** = p<0.01, *** = p<0.001

5.4. Integrated multiomics

The degradation of polyphenols results in intermediates, such as 3-3-hydroxyphenylpropionate and 3-hydroxyhippurate, and depends on microbiota.[226,227] In the present study, the main genus correlating with these features was *Agathobacter* (**V**), whose involvement had not been reported previously. Moreover, FODMAP intake increased the proportion of *Bifidobacterium*, and *Fusicatenibacter*, which in turn associated to 3-3-hydroxyphenylpropionate, previously reported in an online atlas of human plasma microbial-derived metabolites.[248] More human studies are warranted to determine the involvement of microbial conversions of polyphenols.[249,250]

Several genera increased after FODMAP intake; they include *Anareostipes* and *Fusicatenibacter*, which have been previously associated with 3-indolepropionate.[248] while it is known to be produced by microbiota, details about the process remain scarce.[251] FODMAP intake reduced the level of the secondary bile acid hyodeoxycholic acid, although bacteria producing this compound[117] were not influenced by FODMAPs. Hence, the effect on hyodeoxycholic acid and potential positive health consequences (secondary bile acids are

associated with colon cancer)[223] seem to relate to FODMAP intake. Several genera correlated to the phenyl sulphate cluster of metabolites. However, only *Oxalobacter* increased with FODMAP intake (**Figure 18)**. Further efforts are needed to investigate these metabolites and study their interplay with microbiota. Few previous studies have investigated the combined analysis of microbiota, SCFA, and metabolites in IBS after exposure to FODMAPs. One study found that *Porphyromonadaceae spp.* correlated strongly with urinary histamine.[152] This finding was not replicated in the present plasma metabolomics analysis.

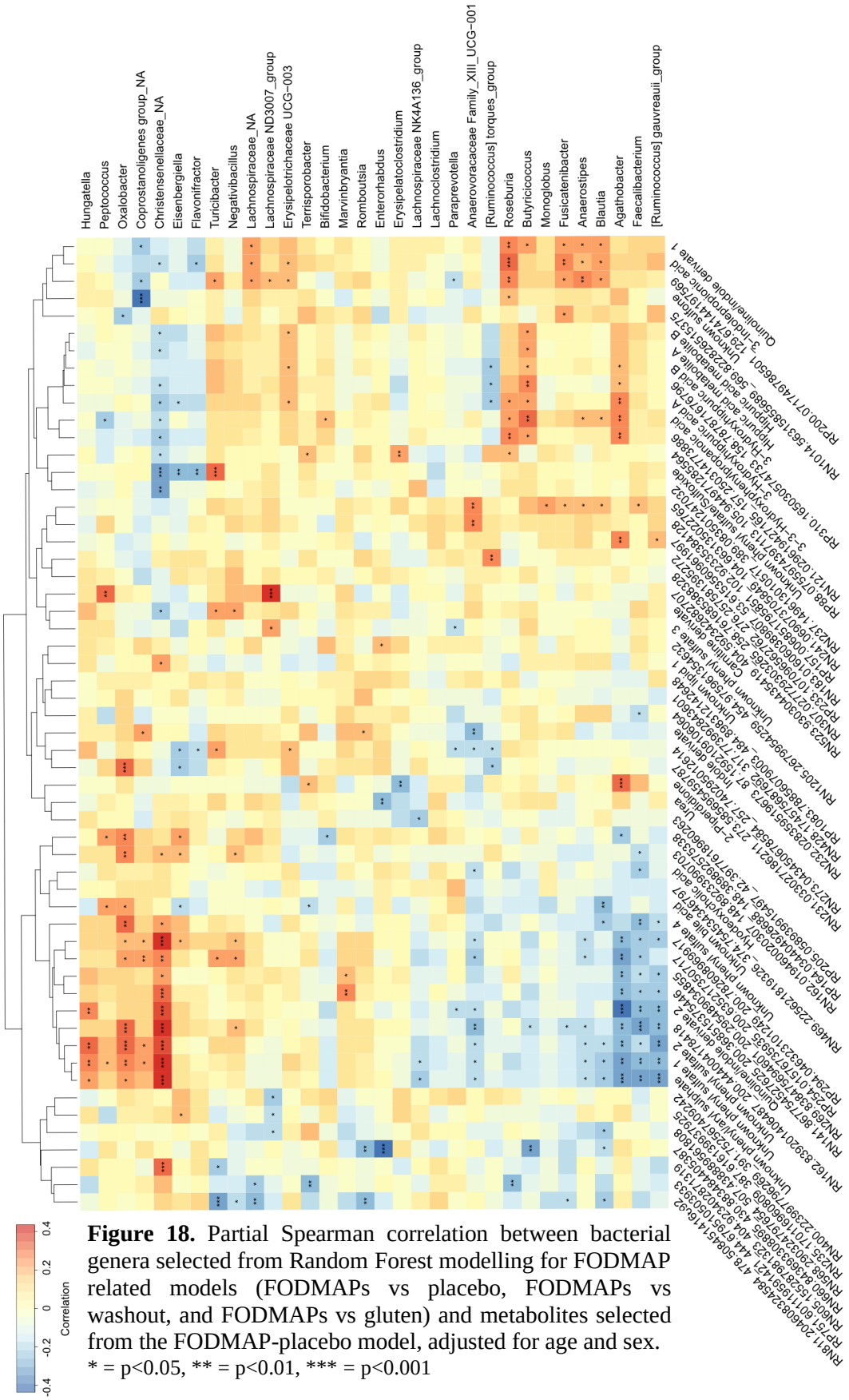


Figure 18. Partial Spearman correlation between bacterial genera selected from Random Forest modelling for FODMAP related models (FODMAPs vs placebo, FODMAPs vs washout, and FODMAPs vs gluten) and metabolites selected from the FODMAP-placebo model, adjusted for age and sex.
* = p<0.05, ** = p<0.01, *** = p<0.001

5.5. Differential response

Regression and classification analysis did not identify any differential responses to dietary interventions (**IV**). PARAFAC was used to account for multidimensional data, thus capturing the behavior of metabolites during the provocation test. As no time-dependent changes were detected to associate to IBS symptoms, postprandial metabolite dynamics following exposure to FODMAP and gluten did not seem to relate to IBS severity.

Despite a wide exploration of methodologies, no differential response to IBS and diet was found. While these findings are consistent with some previous reports,[175,176] other studies could distinguish between responders and non-responders.[153,154,166–174] There are several methodological concerns with these trials such as small sample sizes,[162] and interpretation of effect size as well as data-analytical methodology. For gluten, one study found responses to a gluten-free diet versus a gluten-containing diet relating to microbiota and the metabolome.[155] However, variables defining these differences were not reported and the study was largely underpowered for such sub-analysis.

The present effort aimed for a wide coverage of baseline data as predictors, however, it is possible that other key variables were omitted.[252] Psychological factors are important in IBS, and stratification based on psychological markers may be necessary for better understanding of treatment efficacy.[253] In addition, other omics layers could potentially provide mechanistic clues underpinning differential responses.

Gluten intervention had a weak effect on both IBS symptoms, as well on microbiota and the metabolome. At the same time, the large variability in the data could potentially lead to a differential response, in line with previous studies.[204,211] However, no subgrouping was found. The reasons for the absence of a differential response are likely similar for gluten and FODMAP. They likely stem from the heterogenous character of IBS and possibly a lack of baseline variables. One final possibility is that there is no underlying rationale for differential responses to these diets in IBS.

Regardless of the discouraging results for differential response of IBS in relation to FODMAP and gluten exposure, the methodological approaches used here can be applied to other settings, in which a differential response is expected. RF-based methods can be of use only when the differential response is guided (supervised) by a target variable (e.g., differential response to treatment); whereas PARAFAC can be applied more generally.

6. GENERAL DISCUSSION

The present study included in this thesis is the hitherto largest double-blind exposure trial with FODMAPs, and gluten assessing their effect on IBS. Results indicate that gluten had no effect, and FODMAPs only a modest effect on IBS symptoms. FODMAP modified the microbiota and metabolome, affecting factors known to improve metabolic health, less risk of type 2 diabetes, and reduced inflammation. Only weak correlations between molecular data (microbiota, SCFAs, the metabolome) and IBS symptoms were observed, and none survived multiple testing. Moreover, no clinical or molecular basis was found for differential response to the interventions. Finally, reported BSFS and water content conformed only modestly.

A low FODMAP diet has been considered an effective treatment to alleviate IBS symptoms.[183] Of note, most of the IBS and FODMAP trials have been small and not blinded or solely single blinded.[13] Also, most studies have been designed as FODMAP eliminations rather than provocations. The high risk of bias in IBS and low FODMAP dietary studies has repeatedly been highlighted.[13,254] Further aspects pertaining to the effect of FODMAPs on IBS are:

- *First*, a low FODMAP diet is remarkably effective in comparison to baseline.[152,158,198] Given that there is valid criticism of comparing interventions against the baseline (lack of power, changes over time or regression towards the mean)[269] and that only few studies have compared treatment effects against an appropriate control,[255] there are reasons to question the effectiveness of excluding FODMAPs in IBS.
- *Second*, a low FODMAP diet has shown only small or no difference in comparison to other dietary regimens such as general dietary advice, traditional dietary advice, and a gluten-free diet.[73,179,199,280] Interestingly, double-blind provocation trials with FODMAPs have only led to a modest increase in IBS symptoms,[158,204,211] suggesting that data from non-blinded trials overestimate the true FODMAP effect.
- *Third*, evaluations of dietary triggers have shown surprising results. Food diaries in an IBS trial demonstrated no association between fructan intake and IBS symptoms, and lower gluten intake was associated with more severe IBS symptoms.[256]
- *Fourth*, there is no evidence of a clear dose-response to FODMAP intake in IBS across studies. Widely different doses were reported to cause similar severity of symptoms.[77,78] Interestingly, also healthy subjects develop gastrointestinal symptoms after FODMAP intake.[89] Only a few double-blind studies have evaluated differences in response between healthy individuals and those with IBS.[193,257] Besides inconsistent results these studies were also small and used elevated doses of FODMAPs. Larger studies evaluating doses commonly consumed by both healthy individuals and those with IBS are thus warranted.
- *Fifth*, a discussion about a clinically meaningful effect needs to be raised in the field. In fact, it is frequently mentioned that IBS-SSS requires a change of 50 points to indicate clinically significant improvement. This cut-off can be traced to Francis et al,[19] and has had an enormous impact on the interpretation of IBS symptoms in interventions. However, such cut-off has only limited support in the literature, and has to my

knowledge only been validated once.[258] Moreover, cutoff levels in clinical trials do not always follow this benchmark, which skews any comparison.

- *Sixth*, IBS is a multifactorial disease and general guidelines recommend that it be treated from a holistic perspective, integrating medical treatment, lifestyle and dietary adaptations and behavioral therapy.[12] Several trials have shown that interventions other than dietary adaptations can be effective in reducing IBS symptoms, they include acupuncture, cognitive behavioral therapy, hypnotherapy, meditation, and yoga.[12,101,102] Even so, strong evidence for their efficacy is missing due to methodological concerns such as lack of blinding.[12] A recent publication raised the paradox that both a low FODMAP diet and exposure based cognitive therapy i.e., the consumption of FODMAPs to target the fear of inducing IBS symptoms, are effective in reducing the latter. A theory for this paradox was that since IBS is heterogenous, patients with different etiologies enroll in different type of IBS related trials.[259] However, this is not in line with the fact that randomized control trials have shown that both hypnotherapy and yoga were equally effective as a low FODMAP diet.[260,261]

Considering these issues, conducting a large double-blind study was relevant to enable an objective evaluation of the FODMAP and gluten interventions.[262] Gut-brain interactions are important in IBS,[35] although the underlying mechanisms are unknown. The different scores at baseline, run in/washout and interventions indicate that FODMAPs cause only a modest increase in IBS symptoms. However, they also suggest a pronounced relief in IBS symptoms solely by participating in the trial. [42]

Divergencies between reported BSFS score and water content, along with reported changes in IBS subtype over time,[263–265] may contribute to the inconsistent effect of interventions on IBS subtypes in clinical trials. In accordance with a previous observation,[42] the lack of association between BSFS and IBS symptoms points to the strong importance of visceral sensory stimuli and central processing of sensory information from the gut.

In the present study, there were only weak correlations between microbiota, the metabolome and IBS symptoms, and none surviving multiple testing. The weak associations are well reflected in the large diversity reported in previous studies relating to the heterogenicity of IBS,[266] large placebo response,[267] differences in study design,[13] and methodological disparities.[54,167] Again, highlighting the need for large double-blind trials not to confound responses in IBS-SSS with placebo/nocebo response.

Even though elevated inter-individual variability in the response to the interventions could disclose molecular subtypes in IBS, no such findings were reported. However, other non-measured sources of data such as psychological factors could also contribute to treatment outcome.[253] Hence, the question about differential responses remains open.

Against the limited evidence of symptom alleviation from FODMAP exclusion, we also need to consider that a low FODMAP diet introduces the risk of nutrient deficiencies, eating disorders, and economic implications for the individual.[74,75] Therefore, a discussion is needed to address whether a low FODMAP diet should be recommended. In agreement with existing guidelines, the present study has shown that gluten does not have an effect on IBS symptoms.

7. LIMITATIONS

The performed study has several strengths: Primarily the large sample size, the double-blind character of the study, provocation rather than elimination, and comparison to placebo rather than baseline. In view of the limitations within the field, namely small sample sizes, lack of blinding, use of inadequate comparator group, the strengths of the study provided unique opportunity to contribute robust evidence of the effect of FODMAPs and gluten on IBS symptoms.

However, it also has several limitations. Since the interventions in the clinical trial were limited to one week, it is possible that the effect on molecular data and their relation to IBS symptoms would have been more pronounced with longer interventions. The consistency of reported BSFS was based on comparison with water content measurements, which revealed stronger concordance for hard stools compared to lose stool. However, the perception of stool consistency has been demonstrated to not only depend on water content, but also water holding capacity of stool solids, total amount of stool solids, as well as the presence of steatorrhea (in diarrhea)[268] and stool solids (in constipation).[269] Markers of fecal consistency need further evaluation.

The most common matrix for analyzing microbiota is fecal samples.[270] This represents a practical collection of human material in large scale studies, but they offer an inaccurate measure, as they do not reflect the microbial composition in the intestinal mucosa. Hence, it is possible that crucial effects induced by the interventions and triggered earlier in the intestinal tract, are not reflected in feces. However, collecting material in the intestine is invasive and not practical for large scale studies, but intense research for more accurate sampling is ongoing.[270] Moreover, technologies for microbiota analysis have drastically improved in the recent decades.[271] However, the unclear relationship between amount of microbiota and number of reads is a huge problem.[272,273] Different data treatments have been investigated to address this problem indirectly, [272–274] e.g., via relative abundance, which was the method used in the present work. There are still several challenges in microbiota analysis and new methods are in the pipeline such as quantitative PCR or flow cytometry for evaluation of the number of bacteria in each sample, but they are yet not commonly adopted in microbiota research,[275,276] and were not applied in the present study.

Both microbiota and metabolome data were used in this work, but other measurements could have contributed with complementary insights into IBS, FODMAPs and gluten. Instead of 16S rRNA, full metagenomic sequencing could have been used to capture not only functional aspects of bacteria, but also information about the virome and mycobiome, possibly uncovering could uncover effects or interactions with bacteria.[46,50,107] Furthermore, additional omics layers, such as gene expression, transcriptomics and proteomics, could contribute to a more extensive mechanistic understanding.[156] Moreover, in this study metabolites were analyzed in plasma, but future trials could broaden metabolome investigations to include also urine, and feces. Such coverage could disclose the mechanisms and health effects of dietary interventions. Finally, identification of metabolites was limited in the present study and is considered a major bottleneck in metabolomics, with intense research under way to overcome this problem.[277]

8. CONCLUSIONS

- FODMAPs caused only a modest effect, whereas gluten had no effect on IBS symptoms. The evidence supporting a low FODMAP diet to mitigate IBS symptoms is weak and several methodological improvements are needed in future clinical trials. Based on this and previous studies, gluten does not have an overall systematic effect on IBS symptoms.
- In IBS, subjective reporting of the BSFS conforms modestly to objective stool water content. Significant proportions of reported BSFS 1-2 (hard) and BSFS 6-7 (loose) values did not correspond to water content for constipation and diarrhea, respectively. Correct IBS subtyping is vitally important, as it is assumed that different subtypes will benefit from different treatment regimens. Also, incorrect subtyping could mislead the interpretation of results in clinical trials. The present findings show that unsupervised training for reporting the BSFS score was not efficient when compared the actual water content. Supervised training for the correct usage of BSFS has been shown to improve accuracy, and should be applied for IBS. High intake of FODMAP and gluten does not affect stool water content.
- FODMAPs were associated with changes to microbiota with beneficial health outcomes. An increasing abundance of the genera *Anaerostipes, Bifidobacterium, Faecalibacterium,* and *Fusicatenibacter* is associated with reported positive health effects including anti-inflammatory properties and inhibition of pathogens colonizing the gut mucosa. Conversely, a decreased abundance of *[Ruminoccocus]torques* is associated with reduced gut permeability and inflammation. Gluten had no effect on microbiota.
- FODMAP-affected genera, specifically *Agathobacter, Anaerostipes, Fusicatenibacter,* and *Bifidobacterium,* correlated with increased plasma concentrations of phenolic-derived metabolites and 3-indolepropionate. These metabolites have consistently been related to improved metabolic health, lowered risk of type 2 diabetes and reduced inflammation. Gluten modestly affected carnitine derivates, an acyl-CoA derivate, a medium-chain fatty acid, an unknown lipid, with no interpretable relation to health factors. Elevated doses of FODMAPs and gluten had only minor effect on SCFAs.
- For most FODMAP-related microbiota, SCFAs, and metabolites, no associations were observed with IBS severity. A weak correlation was observed for 3-indolepropionate with abdominal pain, and quality of life, which warrants further investigation. There were no meaningful, interpretable correlations between gluten related metabolites and IBS symptoms.
- Results highlight how a reduced induction of symptoms by FODMAP intake must be weighed against the health aspects of FODMAP consumption. Hence, the appropriateness of recommending a low FODMAP diet should be discussed.
- There was considerable inter-individual variability in the intervention responses. However, no clinical or molecular basis was found for differential responses in IBS, neither after rapid- or prolonged provocations with FODMAPs and gluten. This is likely

related to the heterogenous character of IBS. Mechanistic understanding of differential responses in IBS, could relate to unmeasured variables at baseline, such as other omics layers or psychological variables.

9. FUTURE PERSPECTIVES

- There is a need for additional large double-blind trials to evaluate the effects of FODMAP on IBS, where FODMAP is compared to adequate control treatment, and not merely with baseline.
- The dose-response of FODMAPs in IBS is unclear. IBS and FODMAP trials should include both healthy individuals and those with IBS, as both groups develop gastrointestinal symptoms. This would determine if there is a true difference in the response to FODMAPs and, if so, to what extent and at which doses.
- There is a discrepancy in perceived fecal consistency (BSFS) and water content in IBS. Suitability of scales for subjective evaluation of fecal consistency must be further evaluated. Moreover, training for the usage of such scales should be implemented.
- Regulation of the gut-brain axis could explain the etiology of IBS symptoms. To gain understanding of these interactions, omics studies integrating biological information at several levels, such as gene expression, proteins, metabolites, and microbiota are needed.
- FODMAPs seem to affect several health-related metabolite groups, such as bile acids, as well as phenolic- and tryptophan-derived metabolites. Future trials should benefit from targeted assays to quantify such metabolites.
- To improve the treatment regimen for individuals with IBS, personalized options are preferred. Therefore, further investigation of differential responses to dietary intervention are warranted. The present results suggest that research into IBS response types should further explore microbiota and metabolites, but without omitting also consider other variables of potential interest, such as gene expression, proteins, and psychological parameters.

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