IBSQUtrition: Microbiota and Metabolite Profiles Linked to Severity in Irritable Bowel Syndrome

RESEARCH PROTOCOL

Version 2

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IBSQUtrition

Nutrition to improve QOL of IBS Patients

PROTOCOL TITLE

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee (In Dutch, ABR = Algemene Beoordeling en Registratie)
AE	Adverse Event
ANOVA	Analysis of Variance
AR	Adverse Reaction
CA	Competent Authority
CCMO	Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek
CV	Curriculum Vitae
DSMB	Data Safety Monitoring Board
EU	European Union
EudraCT	European drug regulatory affairs Clinical Trials
FFQ	Food Frequency Questionnaire
FODMAP	Fermentable, Oligo-, Di-, Monosaccharides And Polyols: short chain carbohydrates that are poorly absorbed in the small intestine
GCP	Good Clinical Practice
HADS	Hospital Anxiety and Depression Score
IB	Investigator's Brochure
IBS	Irritable Bowel Syndrome
IBS-C	Irritable Bowel Syndrome type constipation
IBS-D	Irritable Bowel Syndrome type diarrhoea
IBS-M	Irritable Bowel Syndrome type mixed: alternating between diarrhoea and constipation
IBS-QoL	Irritable Bowel Syndrome Quality of Life Questionnaire
IBS-SSS	Irritable Bowel Syndrome Symptom Severity Score
IBS-U	Irritable Bowel Syndrome: type unspecified: IBS with no specific alterations in stool pattern.
IC	Informed Consent
IMP	Investigational Medicinal Product
IMPD	Investigational Medicinal Product Dossier
METC	Medical research ethics committee (MREC); in Dutch: medisch ethische toetsing commissie (METC)
MLDS	Maag Lever Darm Stichting: Dutch Association for gastro-intestinal diseases
OTU	Operational Taxonomic Units

PDSB	Prikkelbare Darm Syndroom Belangenvereniging: Dutch association voor IBS patients
QoL	Quality of Life
RCT	Randomized Controlled Trial
(S)AE	(Serious) Adverse Event
SCFA	Short-Chain Fatty Acids
SPC	Summary of Product Characteristics (in Dutch: officiële productinfomatie IB1-tekst)
Sponsor	The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.
SUSAR	Suspected Unexpected Serious Adverse Reaction
Wbp	Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)
WMO	Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen

SUMMARY

Rationale: Irritable Bowel Syndrome (IBS) is a gastro-intestinal disorder that strongly affects Quality of Life and impairs daily functioning. However, the aetiology and pathophysiology has been poorly understood. Studies suggest that intestinal microbiota in IBS is altered, however a general consensus remains elusive. This may be due to the large individual variation in microbiota and IBS symptoms, and the cross-sectional designs. Moreover, other factors like diet, wellbeing and metabolite profiles are often not taken into account. New evidence is suggesting that IBS severity may be an important factor in microbiota composition.

Objective: To determine faecal microbiota composition and metabolite production (such as acetate, propionate and butyrate), and investigate differences between healthy controls and mild or severe patients IBS. Moreover, to investigate whether (clinical) parameters such as symptom severity fluctuated, and if these fluctuations are associated with an alteration in faecal microbiota composition and metabolite production, one month after baseline, compared to healthy controls.

Study design: This study is a longitudinal study, with two data collection points (baseline and after one month).

Study population: For this study, 100 IBS patients and 30 healthy controls will be recruited. After the first data collection point (T1), the 30 most mild and 30 most severe IBS patients will be included for data collection at T2 (after one month). All subjects will be aged between 18 and 65 years.

Intervention (if applicable): Not applicable.

Main study parameters/endpoints: primary endpoint of this study is microbiota composition and metabolite profiles, and the difference between groups and possible change after one month. These are assessed two faecal samples, which are analysed by 16S rRNA gene-based microbiota profiling. Secondary parameters are dietary intake, Quality of Life, depression and anxiety scores, and stool consistency and frequency, which are assessed by validated questionnaires.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: This study has a relatively low burden and risk, since it is an observational study. Subjects only have to visit the study site once (at the information event). All data collection will be done from home, and will be done twice, which include faecal samples online questionnaire.

1. INTRODUCTION AND RATIONALE

Irritable Bowel Syndrome (IBS) is a functional gastrointestinal disorder, which affects 10 to 20% of adults, with a female predominance [1-3]. Although IBS it not a life-threatening disorder, or no histological abnormalities are seen in the intestine, it strongly affects quality of life (QoL) and impairs daily functioning [1]. IBS is characterized by altered bowel habits, abdominal pain and discomfort, and is often diagnosed using the Rome IV criteria [4-6]. Based on predominant stool patterns, IBS can be roughly divided into four subtypes: IBS with constipation (IBS-C), IBS with diarrhoea (IBS-D), Mixed IBS (IBS-M) or Unsubtyped IBS (IBS-U) [4].

The aetiology and pathophysiology of IBS is poorly understood. IBS has been associated with increased intestinal permeability, low grade inflammation, abnormal gut motility and increased visceral hypersensitivity [7, 8]. Evidence is indicating an important role for gut microbes in the pathophysiology of IBS. A meta-analysis pooling 18 studies found an increased risk for developing IBS after bacterial gastroenteritis after 1 year and even 36 months [9]. Moreover, double blind randomized controlled trials (RCT) found improvement in symptoms after short-term treatment with antibiotics such as rifaximin and probiotics [8, 10-14].

Several studies showed differences between microbiota of IBS patients, or subtypes, and healthy controls [13, 15, 16]. The Rome Team Working group (Simren et al 2013) concluded that there is good evidence supporting the concept that intestinal microbiota in IBS is altered. However, these studies lack general consensus and the specific microbial signature in IBS, even within IBS subtypes, remains elusive [17]. These conflicting results may be due to the cross-sectional design, the large individual variation in microbiota composition and relatively small sample size in many studies. Moreover, IBS is a chronic disorder with relapses and remissions, and large variation in severity of symptoms and type of defecation, even within the subtypes [5].

Possibly, microbiota clusters may not match the traditional IBS subtypes, as found by Jeffrey et al (2012) [12]. Recently, Tap et al (2017) defined a microbiota signature which identified 90 operational taxonomic units (OTU's). This signature showed associations with stool consistency, transit time, microbial richness, exhaled CH4, presence of *Methanobacteriales*, and enterotype distribution. IBS severity was found to be the a strong factor that was associated with this microbial signature. Other (clinical) parameters, like IBS subtypes, diet, medication, anxiety and depression scores were not strongly associated with the microbial signature [18]. Because this is the first study to define a microbiota signature that is linked to severity, and this microbial signature had a relatively low

sensitivity, results should be interpreted with caution, the more since the set-up of the study was cross-sectional. Moreover, Tap et al (2017) did not find associations between microbiota profile and anxiety and depression scores, while Jeffrey et al (2012) found that IBS patients with normal-like microbiota had an increased score of depression and anxiety, again highlighting the lack of consensus observations [12]. Further research is thus needed to elucidate the microbiota signature in IBS and its link with other factors like severity, QoL, depression, anxiety and diet over time.

Among IBS patients, 91% report symptom onset related to diet. Food groups such as cereals, spicy foods, vegetables, fatty foods, animal protein, dairy, fruits, nuts, seeds and caffeine are reported as most problematic [19]. FODMAPS (Fermentable, Oligo, Di, Monosaccharides and Polyols; a specific group of carbohydrates) are often problematic in IBS patients as they are fermented by intestinal microbes, thus increasing gas production and possibly provoking IBS symptoms. These effects of diet, and especially consumption of dietary fibre, on intestinal microbiota composition have been demonstrated in studies. Fibres are largely resistant against digestion in the human intestine, and are therefore able to reach the colon, where they increase viscosity and faecal mass [20]. The fibres are fermented by microbiota in the colon, which results in groups of metabolites [21]. Short-Chain Fatty Acids (SCFA), including acetate, propionate and butyrate, are the major group [20, 22]. Recent research indicates that faecal SCFA levels may be altered in bowel disorders [23], and that SCFA can play a role in prevention and treatment of bowel disorders. Studies show that SCFA administration positively influenced Ulcerative Colitis, Crohn's disease and antibiotic-associated diarrhoea [24-26]. In IBS patients, several studies suggest that faecal SCFA levels are altered, but these are limited and inconsistent [27-30]. Moreover, SCFAs are a major product of FODMAP fermentation, and since IBS patients often limit FODMAP intake, the reduction of faecal SCFA may just rather reflect diet than be a feature of disease [27].

To date, much remains unclear about the role of microbiota in IBS, and the association with other parameters like diet and wellbeing. Categorizing IBS patients into the traditional IBS subtypes did not reveal subtype-specific microbiota compositions. Moreover, SCFA levels in IBS have been limited investigated, and controlled for factors like diet. In our study, we aim to investigate microbiota composition and SCFA in IBS patients, categorized by severity of symptoms, and investigate associations with diet, depression, anxiety and QoL, as well as defecation type at time of sampling. Moreover, we aim to assess to what extent severity and other parameters change after one month, and if, and to what extent we see changes in microbiota and SCFA profiles.

2. OBJECTIVES

Primary Objective:

To determine faecal microbiota composition and metabolite production (SCFA such as acetate, propionate and butyrate), and investigate differences mild or severe patients IBS.

Moreover, this study aims to investigate whether (clinical) parameters such as symptom severity fluctuated, and if these fluctuations are associated with an alteration in faecal microbiota composition and metabolite production, one month after baseline, and if this is different between mild or severe IBS patients.

Secondary Objective(s):

- To investigate whether microbiota composition and metabolite production are associated with dietary intake, depression & anxiety and QoL, and whether this differs between mild IBS and severe IBS.
- To investigate if one month after baseline measurements, dietary intake, QoL and depression & anxiety changed, and if they are associated with possible alterations in IBS severity, microbiota composition or metabolite profiles.

3. STUDY DESIGN

The acronym IBSQUtrition stands for Irritable Bowel Syndrome, Questionnaire, QoL, and Nutrition. This research project is in collaboration with three departments of Wageningen University & Research: the division of Human Nutrition, Food and Biobased Research (FBR) and the Laboratory of Microbiology. IBSQUtrition consist of several studies, and aims to investigate the effects of nutrition on IBS symptoms, in order to improve QoL. The first study conducted within this research project was a large online questionnaire-study, which assessed associations between nutrition, IBS symptoms and QoL.

This study focusing on composition and metabolite production of the microbiota has an observational longitudinal design. IBS subjects will be recruited from the online questionnaire-study, which has been conducted previously. In order to understand intestinal microbiota in IBS and its association to other parameters, such as severity and nutrition, IBS patients that completed the online questionnaire and gave permission to receive more information on follow-up studies, will be invited for an information event. We aim to recruit 100 IBS patients. After the first data collection (T1), assessment of IBS severity will be done according to the IBS Severity Symptom Score (IBS-SSS). IBS patients who are classified either as most mild (n=30) or most severe (n=30) will be included for follow-up at T2, which is one month later. Figure 1 shows this schematically.

As mentioned before, IBS is a chronic disorder with relapses and remissions, and large variation in severity of symptoms and type of defecation [31, 32]. Moreover, studies suggest that microbiota can differ over time [33]. Therefore, we will assess parameters at two time points, with one month in between, to have a better understanding of this variability. Since IBS severity is also highly variable, but we would like to specify groups based on IBS severity (mild or severe), we will assess a larger group at T1 (n=100). After T1, we will select IBS patients who are the most mild or most severe. This study design will give us the opportunity to do a cross-sectional analysis at T1, where data is collected at the same moment to reduce variability, and to elucidate whether (and to what extent) there is a change in severity and other parameters after one month (T2), and if microbiota and metabolite profiles also changed, and to what extent. Healthy controls will be tested at T1 and T2 for comparison.

There will be two moments of data collection in this study (T1 and T2). At T1 and T2, all subjects (both IBS subjects and healthy controls) will fill out several online questionnaires on dietary intake, symptom severity, stool form and wellbeing. Moreover, they will collect a faecal sample for analysis. More detailed information on specific methods for data

collection can be found at chapter 8 Methods. Figure 2 gives a schematic overview of the data collection.

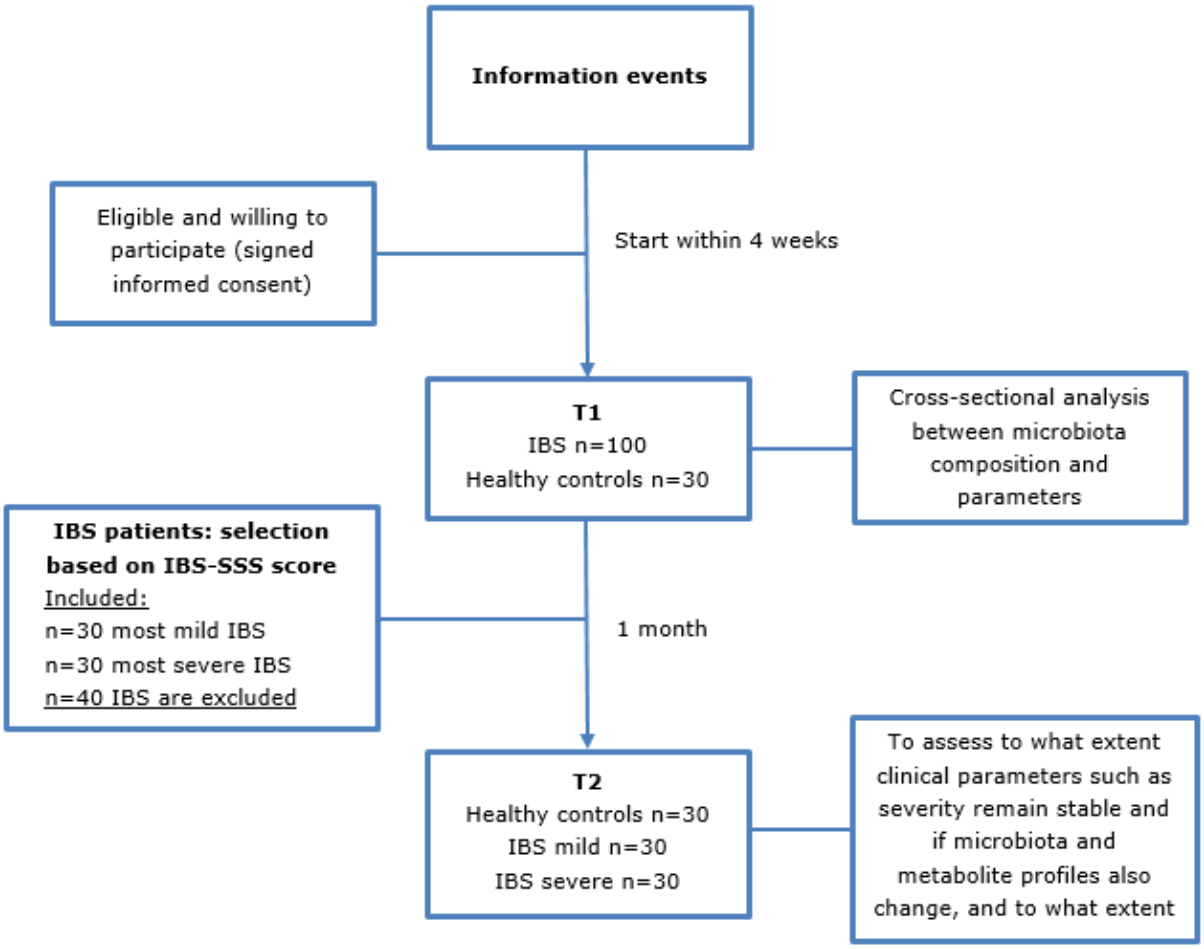

Figure 1. Flowchart of the selection of study population

Figure 2. Overview of the data collection

4. STUDY POPULATION

4.1 IBS patients

This study will be performed in IBS patients who already participated in a previous cross-sectional questionnaire study. For the questionnaire study, patients were recruited online, in close cooperation with the Dutch Association for IBS (Prikkelbare Darm Syndroom Belangenvereniging, PDSB), the Dutch Assocation for gastro-intestinal diseases (Maag Lever Darm Stichting, MLDS), and hospital the Gelderse Vallei Ede, where our PI MD Ben Witteman has an outpatient clinic for IBS patients. After completion of the online questionnaire, IBS patients could choose to receive more information on this study. When IBS patients are willing to participate, they will be invited for an information meeting, where they can receive more information. If recruitment from the subjects of the online questionnaire is not successful, there will be an open call for recruitment of IBS patients. This will be done in collaboration with the PDSB and online using the website http://voedingsonderzoek.wur.nl.

In order to be eligible, IBS patients must fulfil the following criteria:

- Adults, aged 18-65 years. Research shows that microbiota composition and activity can change in elderly [34].
- IBS patients that meet the Rome IV criteria [5], as shown in table 1. This will be evaluated by the principal and/or medical investigator.
- In close proximity of Wageningen (max. 50 km), for practical reasons: collection of the faecal samples.
- Have an Body Mass Index (BMI) between 18.5 and 30 kg/m^2. Microbiota was found to be altered in obese subjects, therefore these subjects will be excluded. [35].
- Signed informed consent.
- After T1, IBS severity will be checked using the IBS-SSS. Subjects will be included for follow-up at T2, when they have the most mild symptoms (n=30) or most severe (n=30) symptoms.

IBS patients will be excluded from participation of this study, when:

- Presence of gastro-intestinal diseases, such as celiac disease, Crohn's disease, or Ulcerative colitis.
- Have a history of intestinal surgery that might interfere with study outcomes. This does not include an appendectomy or cholecystectomy.
- Presence of significant systemic diseases, such as diabetes mellitus, cancer, cardiovascular disease or respiratory disease.
- Female subjects: currently pregnant or breast-feeding.

- Use of antibiotic treatment less than 3 months before start of the study and no use of antibiotics during the study.
- Are a subject in another study during this study.
- Are an employee or student of the Division of Human Nutrition, Food and Biobased Research or Laboratory of Microbiology, of Wageningen University & Research.
- Unwilling or unable to fulfil study criteria.
- If they are not selected in the most mild or most severe group at T2.

Table 1. Rome IV criteria

Rome IV criteria for IBS *Patient has recurrent abdominal pain (≥1 day per week, on average, in the previous 3 months), with an onset of ≥6 months before diagnosis.*
Abdominal pain is associated with: - Pain related to defecation - Change in frequency of stool - Change in form (appearance) of stool
Patient has none of the warning signs: - Age ≥50 years, no previous colon cancer screening and presence of symptoms - Recent change in bowel habit - Evidence of overt GI bleeding (i.e., melena or haematochezia) - Nocturnal pain or passage of stools - Unintentional weight loss - Family history of colorectal cancer or inflammatory bowel disease - Palpable abdominal mass or lymphadenopathy - Evidence of iron-deficiency anaemia on blood testing - Positive test for faecal occult blood

4.2 Healthy controls

Healthy volunteers will be recruited through the database of the division of Human Nutrition, Wageningen University & Research. They will be matched for age (±5 years), gender and Body Mass Index (±1 kg/m^2). Healthy controls will be matched with the mild IBS patients, since the smallest difference is expected between those groups. An e-mail will be sent out explaining the study and a call for recruitment, and will be posted on http://voedingsonderzoek.wur.nl. Healthy controls will be recruited after the IBS subjects finished the study, in order to be sufficiently matched. Subjects will be invited for an information event, where eligibly will be checked. Controls will be eligible for participation in this study when:

- Adults, aged 18-65 years.
- No history of IBS, as assessed by the Rome IV criteria.
- Has a score <75 from the IBS-SSS.
- Are age (±5 years), gender and Body Mass Index (±1 kg/m^2) matched with IBS patients.
- In close proximity of Wageningen (max. 50 km), for practical reasons: collection of the faecal samples.
- Signed informed consent.

Subjects will be excluded from participation, when:

- Presence of gastro-intestinal diseases, such as celiac disease, Crohn's disease, or Ulcerative colitis.
- Have a history of intestinal surgery that might interfere with study outcomes. This does not include an appendectomy or cholecystectomy.
- Presence of significant systemic diseases, such as diabetes mellitus, cancer, cardiovascular disease or respiratory disease.
- Female subjects: currently pregnant or breast-feeding.
- Use of antibiotic treatment less than 3 months before start of the study.
- Are an employee of the Division of Human Nutrition, Food and Biobased Research or Laboratory of Microbiology, of Wageningen University & Research.
- Are subjects in another study during this study.
- Unwilling or unable to fulfil study criteria.

4.3 Screening visit

Possible subjects will be invited for an information event, where additional information on the study will be given and subjects have an opportunity to ask questions. If willing to participate, subjects will sign an informed consent (see Appendix E2). After this, they will receive a questionnaire to assess eligibility. If the informed consent is signed and subjects are eligible, the intended start of date of the study is within 4 weeks. This study will first start with assessment of IBS patients. When all IBS patients have finished the study, recruitment for healthy controls will be open, in order to match controls to the IBS patients.

4.4 Sample size calculation

We used a somewhat comparable study of Mättö et al (2005), which investigated the stability of microbiota composition in faecal samples of IBS patients and controls at baseline, 3 months and 6 months [33]. Based on their study, we expect to find a difference of 3.6 (with a standard deviation of 4.9) in similarity of intestinal microbiota between IBS

patients and controls over time. In our calculation, we used an α=0.05 (probability of Type I error) and a β=0.2 (probability of Type II power).

We used the following formula: $n = 2 \times \frac{(Z\alpha + Z\beta)^2}{(\sigma/\delta)^2}$. When filled in, this results in the following formula: $n = 2 \times \frac{7.9}{(4.9/3.6)^2} = 29.3$

Therefore, our aim is to include 30 research subjects per group: 30 most mild IBS patients, 30 most severe IBS patients, and 30 healthy controls.

5. TREATMENT OF SUBJECTS

Not applicable.

6. INVESTIGATIONAL PRODUCT

Not applicable.

7. NON-INVESTIGATIONAL PRODUCT

Not applicable.

8. METHODS

8.1 Study parameters/endpoints

8.1.1 Main study parameter/endpoint

The main study endpoint is microbiota composition, metabolite production and other faecal markers. This will be assessed with 16S rRNA gene-based microbiota profiling. At T1 and T2, subjects will collect a faecal sample for microbiota profiling. Faecal samples will be used for 16S rRNA gene-based profiling and subsequent analyses of a variety of microbial ecological characteristics, including microbiota composition, α-diversity (diversity within the sample), β-diversity (diversity between the samples) and group differences. Moreover, metabolites such as propionate, butyrate and acetate will be determined, which are an end product of bacterial metabolism in the large intestine [28].

8.1.2 Secondary study parameters/endpoints (if applicable)

Secondary endpoints are assessed at T1 and T2, and include QoL, depression & anxiety scores, symptom severity, stool frequency and form. Dietary intake will only be assessed at T1. All questionnaires will be equal for IBS patients and healthy controls.

8.1.3 Other study parameters (if applicable)

All subjects fill in a general screening questionnaire to assess characteristics such as age, gender, educational level, ethnicity, body weight, height, physical activity and smoking history. In order to check eligibility, the screening questionnaire is different for healthy controls and IBS patients: for the healthy controls the IBS-SSS is added, for the IBS patients the ROME IV criteria is added. At T2, subjects will be asked whether they changed their diet during the last month, and yes, what changes they made.

8.2 Randomisation, blinding and treatment allocation

Since this is an observational study, this is not applicable.

8.3 Study procedures

A general overview is shown in table 2. This study consists of two data collection time points (T1 and T2), which are separated by one month. All questionnaires can be found in Appendix F1. The following parameters will be assessed:

Microbiota and metabolite profiling: Faecal material will be collected at T1 and T2. After collection, faecal material will be immediately frozen at the home freezers of the subjects, transported on dry ice to the laboratory and subsequently frozen at -80 °C until analysis. DNA will be isolated from part of the faecal samples and subsequently be used for 16S rRNA gene-based microbiota profiling using NG-tax as described earlier [36, 37]. Metabolite concentrations (including SCFAs) will be determined by HPLC as described earlier [38, 39]. A variety of multivariate analytical tools available as R scripts will be used to determine differences between microbiota composition and diversity between subjects, as well as determining microbial taxa that are associated with parameters derived from the different questionnaires.

IBS severity: will be assessed using the validated score IBS-SSS [40]. Maximum achievable score is 500. A score <75 indicates no IBS or in remission, 75-175 is mild IBS, 175-300 indicated moderate IBS, and >300 indicates severe IBS. The IBS-SSS score will be assessed at T1 and T2. Since we only have a relatively small group, these cut-off points will not be used, but the most mild IBS patients and most severe IBS patients will be selected as the study population for T2.

Dietary intake: will be assessed using a validated Food Frequency Questionnaire (FFQ). An FFQ is a valid way to assess habitual dietary intake [41]. Subjects will receive an online invitation with login to fill in their FFQ. From this FFQ, energy content (kcal) and carbohydrate, protein, fat, saturated fat, fibre intake (g and en%) will be assessed. Moreover, water content (L) and food groups such as vegetable and fruit intake, will be assessed. If subjects indicate in the FFQ that they follow the FODMAP diet, a dietician will call them to ask what specific groups they are excluding from their diet. This gives more insight when subjects data who follow the FODMAP diet differs significantly from those who are not following the diet.

Quality of Life: quality of life will be at T1 and T2, using the validated 34-item Irritable Bowel Syndrome Quality of Life questionnaire (IBS-QoL). This questionnaire gives an overall score of QoL and holds different subscales, such as dysphoria, interference with activities, body image, health worry, food avoidance, social reaction, sexual and relationships [42].

Depression and anxiety: will be assessed at T1 and T2 using the validated questionnaire Hospital Anxiety and Depression score (HADS) [43]. This is a 14-item questionnaire, which gives a screening score for anxiety and depression.

Stool frequency and form: will be assessed using the validated Bristol Stool Chart. Stool frequency will be assessed using general questions, such as: "Please think of the week month. How many days did you have defecation?" "Please think of an average day on which you have defecation. During this day, how many times did you have defecation?" "Did you feel relief of abdominal pain after defecation?"

Table 2. Schematic overview of the study procedure

	T1	T2
Collection of faecal samples	X	X
IBS Symptom Severity Score (IBS-SSS)	X	X
IBS Quality of Life Questionnaire (IBS-QoL)	X	X
Hospital Anxiety and Depression Scale (HADS)	X	X
Stool frequency and form	X	X
Food Frequency Questionnaire (FFQ)	X	

IBS: Irritable Bowel Syndrome

8.4 Withdrawal of individual subjects

Subjects may discontinue the trial at any moment without the obligation to state the reason for discontinuation. Subjects may be withdrawn from the study by the principal investigator if they do not comply with the rules and regulations of the study. Subjects may be withdrawn from the study by the medical supervisor in case of reported serious adverse events or in case of other medical, social or psychological events as evaluated by the medical investigator and discussed with the principal investigator.

8.4.1 Specific criteria for withdrawal (if applicable)

Not applicable.

8.5 Replacement of individual subjects after withdrawal

If more than five subjects per group withdraw to participate at the beginning of the study, new subjects will be recruited in order to obtain sufficient power.

8.6 Follow-up of subjects withdrawn from treatment

Not applicable.

8.7 Premature termination of the study

A premature termination of the study is not expected, since this is an observational study. If the study is premature terminated, data which was collected before the termination will be analysed.

9. SAFETY REPORTING

9.1 Section 10 WMO event

In accordance to section 10, subsection 4, of the WMO, the sponsor will suspend the study if there is sufficient ground that continuation of the study will jeopardise subject health or safety. The sponsor will notify the accredited METC with undue delay of a temporary halt including the reason for such an action. The study will be suspended pending further review by the accredited METC. The investigator will take care that all subjects are kept informed.

9.2 AEs, SAEs and SUSARs

9.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to the study. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded. Adverse events will be reported to the reviewing METC.

9.2.2 Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that at any dose:

- Results in death;
- Is life threatening (at the time of the event);
- Requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- Results in persistent or significant disability or incapacity;
- Is a congenital anomaly or birth defect;
- Any other important medical event that may not result in death, be life threatening, or require hospitalization, may be considered a serious adverse experience when, based upon appropriate medical judgement, the event may jeopardize the subject or may require an intervention to prevent one of the outcomes listed above.

The investigator will report all SAEs to the sponsor without undue delay after obtaining knowledge of the events.

The sponsor will report the SAEs through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 7 days of first knowledge for SAEs that result in death or are life threatening followed by a period of maximum 8 days to complete the initial preliminary report. All other SAEs will be reported

within a period of maximum 15 days after the sponsor has first knowledge of the SAE.

9.2.3 Suspected unexpected serious adverse reactions (SUSARs)

Not applicable.

9.3 Annual safety report

Not applicable.

9.4 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist. SAEs need to be reported till end of study within the Netherlands, as defined in the protocol. The follow-up of AE's will also be reported in the annual progress report.

9.5 Data Safety Monitoring Board (DSMB) / Safety Committee

Not applicable.

10. STATISTICAL ANALYSIS

Data will be presented as mean ± standard deviation (SD) if normally distributed, or median (interquartile range) when skewed. Normal distribution will be verified by the Kolmogorov-Smirnov test. To test associations between parameters, multiple linear regression will be used. Data will be tested for confounding or effect mediators, and confounding factors will be added to the regression. Microbiota data will be assessed using the statistical program R. Other data will be assessed using SPSS statistics. A p-value of <0.05 will be considered significant.

10.1 Primary study parameter(s)

A variety of in house R-scripts and the Phyloseq package will be used for microbiota composition analyses using R v3.4.0 software (The R Foundation for Statistical Computing, Vienna, Austria). To determine the abundance differences of microbial groups at various phylogenetic levels within individuals a paired Wilcoxon test will be used.

To contrast between the different IBS groups (over time effect), a linear mixed model taking into account the effects of repeated measurements using the lme4 package will be used. Variables that will be added to the model are time of sampling, group, gender, age, and BMI. Diversity of the microbiota will be quantified at different levels (taking into account the impact of abundance) using the Vegan package (https://cran.r-project.org/web/packages/vegan/vegan.pdf) and picante package (http://picante.r-forge.r-project.org/picante-intro.pdf). Analysis of variance with the Tukey Honest Significant post hoc analysis will be applied to compare beta diversity between and within groups. P values will be corrected for multiple comparisons using the Benjamini–Hochberg procedure. To correct for a false discovery rate (FDR), FDR adjusted p-values will be computed, a so called q-value [44]. A corrected q-value of less than 0.2 was considered significant. Student T-tests and ANOVA will be used to evaluate differences between faecal metabolite concentration over time and between groups.

10.2 Secondary study parameter(s)

Secondary parameters are dietary intake, QoL, depression and anxiety scores, symptom severity, stool frequency and form (Bristol Stool score). These will be assessed using various validated questionnaires. For the Bristol stool score, the top 3 type of stools will be determined, ranging from 1 (hard to pass) to 7 (entirely liquid).

Differences between T1 and T2 will be assessed using a repeated measure Analysis of Variance (ANOVA) if normally distributed, otherwise a Kruskall-Wallis test when skewed. Variables such as age, gender, BMI and group will be added to the model.

10.3 Other study parameters

Other study parameters include age, gender, ethnicity, educational level, body weight, height, and lifestyle factors such as physical activity and smoking. These variables will be checked if they differ across the groups using an ANOVA, or when skewed a Kruskall-Wallis test. Moreover, these variables will be assessed if they are a confounder or effect mediator, and if they have to be in the multiple linear regression model.

10.4 Interim analysis (if applicable)

When all IBS patients completed the study, a short interim analysis will be done to check for distribution of age, gender and BMI. These data will be used to recruit age, gender and BMI matched healthy controls. All other data will not be analysed until full completion of the study.

11. ETHICAL CONSIDERATIONS

11.1 Regulation statement

This study will be conducted according to the principles of the Declaration of Helsinki (64th WMA Assembly, October 2013), and in accordance with the Medical Research Involving Human Subjects Act (WMO 1998) and other guidelines, regulations and Acts.

11.2 Recruitment and consent

IBS patients will be recruited through a previous online questionnaire study. Subjects who participated in that study can choose at the end of the questionnaire if they would like to receive more information on follow-up studies like this microbiota profiling study. If they indicated this, they will receive a patient information folder (see Appendix E1). When willing to participate, subjects will be invited for an information meeting. Subjects will receive additional information, and there is time to answer questions. Within 4 weeks after the information meeting, the subject has to sign the informed consent if they wish to enter the study. After this, eligibility will be checked. Healthy subjects will be recruited using the online database the division of Human Nutrition of Wageningen University & Research. If willing and eligible, subjects will be invited for an information meeting (separate meeting than for IBS patients). There, additional information will be given and subjects have the chance to ask questions. Within 4 weeks after the information meeting, subjects have to sign the informed consent if they wish to enter the study.

Subjects will sign the informed consent first, followed directly by the investigator, and the subject will receive a copy of the signed informed consent directly after signing. The informed consent is enclosed in Appendix E2.

11.3 Objection by minors or incapacitated subjects (if applicable)

Not applicable.

11.4 Benefits and risks assessment, group relatedness

This study can help elucidate the association between IBS severity, microbiota composition and diet, QoL, depression and anxiety. There is no additional risk for the subjects, since this is an observational study. Subjects will have to invest time at three points: information meeting (screening), and T1 and T2, where they will fill in online questionnaires and collect a faecal sample. They will only have to visit the study site once, for the information meeting. After screening, all data collection will be done

online. Subjects can collect and store faecal samples at home, and will be picked up by the investigator.

11.5 Compensation for injury

Since this is an observational study with no invasive measurements, there is no additional risk for the subject. Therefore, an exemption for insurance will be asked of the METC. This letter can be found in Appendix G1.

11.6 Incentives (if applicable)

Subjects will receive a financial compensation of €25,- after completion of this study. If IBS patients completed T1, but are not selected for T2, they will receive a financial compensation of €15,-. Subjects are free to withdraw from further participation for any reason and at any time during the study and will receive a proportional repayment for the effort they made.

12. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

12.1 Handling and storage of data and documents

Before the start of the study, subjects will be assigned a study code that will remain the same during the study. The code list with both the codes and the names of the subjects will only be accessible for researchers of this study in a password protected file. For all other purposes, the study code will be used for subjects identification. The informed consent will be stored separately from all other information. No names of the patients will be used in the publication of this study. All researchers have signed a confidentially statement. The handling of personal data will be done according to the General Data Protection Regulation (GDPR), which is enforced at 25th of May 2018, and is registered on the website of the Dutch Data Protection Authority (AP). According to standard data management procedures, all research data are stored for a period of at least 15 years after collection. Thereafter, data and documents will be deleted and destroyed (e.g. by a shedder). Biological samples will be disposed at the end of the study according to biomedical waste procedures.

12.2 Monitoring and Quality Assurance (if applicable)

Monitoring will not be done, this is a relatively short study with minimum risks due to the observational study design.

12.3 Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. Non-substantial changes (such as typing errors, administrative changes like changes in names, telephone numbers and other contact details of involved persons mentioned in the submitted study documentation) will not be notified to the reviewing METC. Substantial amendments will be notified to the METC that gave a favourable opinion. The documentation that will be included in the submission should cover the following information:

1. Covering letter, including the reasons for the amendment in one or two sentences, a brief description of the changes that are included in the amendment and the name of the documents that are modified;
2. An extract of the modified documents, where applicable, showing both the previous and new wording, where applicable.
3. The new version of the modified documents, where applicable, identified with updated number of version and date.

12.4 Annual progress report

Because the study period is less than a year, the investigator will submit a final study report within one year after the end of the study to the accredited METC.

12.5 End of study report

The investigator will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last data collection of the last subject. In case the study is ended prematurely, the investigator will notify the accredited METC within 15 days, including the reasons for the premature termination. Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

12.6 Public disclosure and publication policy

All trial results, both positive and negative, will be disclosed in agreement with the CCMO statement on publication policy. Regardless of the outcome of the study, the results will be submitted for publication to a peer-reviewed scientific journal. The authorship of the article shall be determined in appropriate consultations based on a considerable contribution to set-up and execution of the study and an active participation in publication. The nine industrial partners are entitled to examine the manuscript prior to publication and make comments on it. None of the partied concerned has the right of veto considering publication.

13. STRUCTURED RISK ANALYSIS

13.1 Potential issues of concern

Not applicable.

13.2 Synthesis

Not applicable.

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