STUDY PROTOCOL

A novel approach to measuring intestinal production of short chain fatty acids: A proof-of-concept healthy volunteer study

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Background & Rationale

Intestinal short-chain fatty acids (SCFA) are produced by microbial fermentation of undigested carbohydrates.[1] SCFAs are an important in regulating numerous physiological processes of the human intestine through their effects fluid and electrolyte absorptio[2,3] motility[4,5] and symptoms in IBS.[6] Altered SCFA profiles have been identified in common gastrointestinal conditions such as irritable bowel syndrome (IBS); however, the pattern of these alternations is incompletely understood. Part of the challenge in accumulating consistent data regarding the significance fecal SCFAs in conditions such as IBS, lies in the technical challenges that accompany attempts to measure intestinal SCFAs. SCFAs are volatile, and luminal SCFAs serve as a primary metabolite for human colonocytes. Thus, direct measurement of fecal SCFAs may not accurately represent the quality or quantity of SCFAs that are produced in the colon. There remains a need to develop novel approaches for accurate assessment of colonic SCFA production that will be applicable for clinical and research purposes. Inulin is a non-digestible carbohydrate that is approved by the United States Food and Drug Administration as a dietary fiber and is fermented by colonic bacteria to produce SCFAs. There are no studies that have investigated inulin fermentation as a possible strategy for measuring fecal SCFA production. Furthermore, it is unclear if undigested fecal inulin can be recovered and measured in humans following oral ingestion. The aim of this proposal is to test a novel approach to the assessment of fecal SCFA production in humans through the measurement of fecal inulin after a one-time inulin ingestion among healthy adult volunteers.

1.0 Objective(s)

1.1 Primary Objective: To conduct a proof-of-concept study demonstrating the ability to measure fecal inulin and short chain fatty acid content in stool after a one-time ingestion of a standardized 10-gram dose of dietary inulin.

1.2 Secondary Objective(s):

1.2.1 To assess associations of bowel symptoms with fecal inulin and SCFA content after a one-time ingestion of dietary inulin

1.2.2 To assess associations of individual factors (baseline dietary intake, demographics, body-mass index) with fecal inulin and SCFA content after a one-time ingestion of dietary inulin.

1.2.3 To examine characterize fecal microbiota composition following a one-time ingestion of dietary inulin and associations of microbiota composition with fecal inulin and SCFA content

2.0 Outcome Measures/Endpoints

2.1 **Primary Outcome Measures:** Fecal inulin, total and individual fecal SCFAs

2.2 Secondary Outcome Measures: Bowel symptoms (stool frequency, stool form, ease of passage) based on a validated bowel diary, baseline dietary intake (Automated Self-Administered Dietary Assessment)

2.3 Covariates for inclusion: Demographics and body mass index

3.0 Eligibility Criteria

3.1 Inclusion Criteria

- Healthy adults ages 18-75 years with no prior history of GI disease or symptoms.

- Participants should be on a stable and consistent diet regimen and should not be following an extreme diet intervention such as gluten-free or a low fermentable oligo-, di-, monosaccharides, and polyols diet (FODMAP) diet at the time of study participation

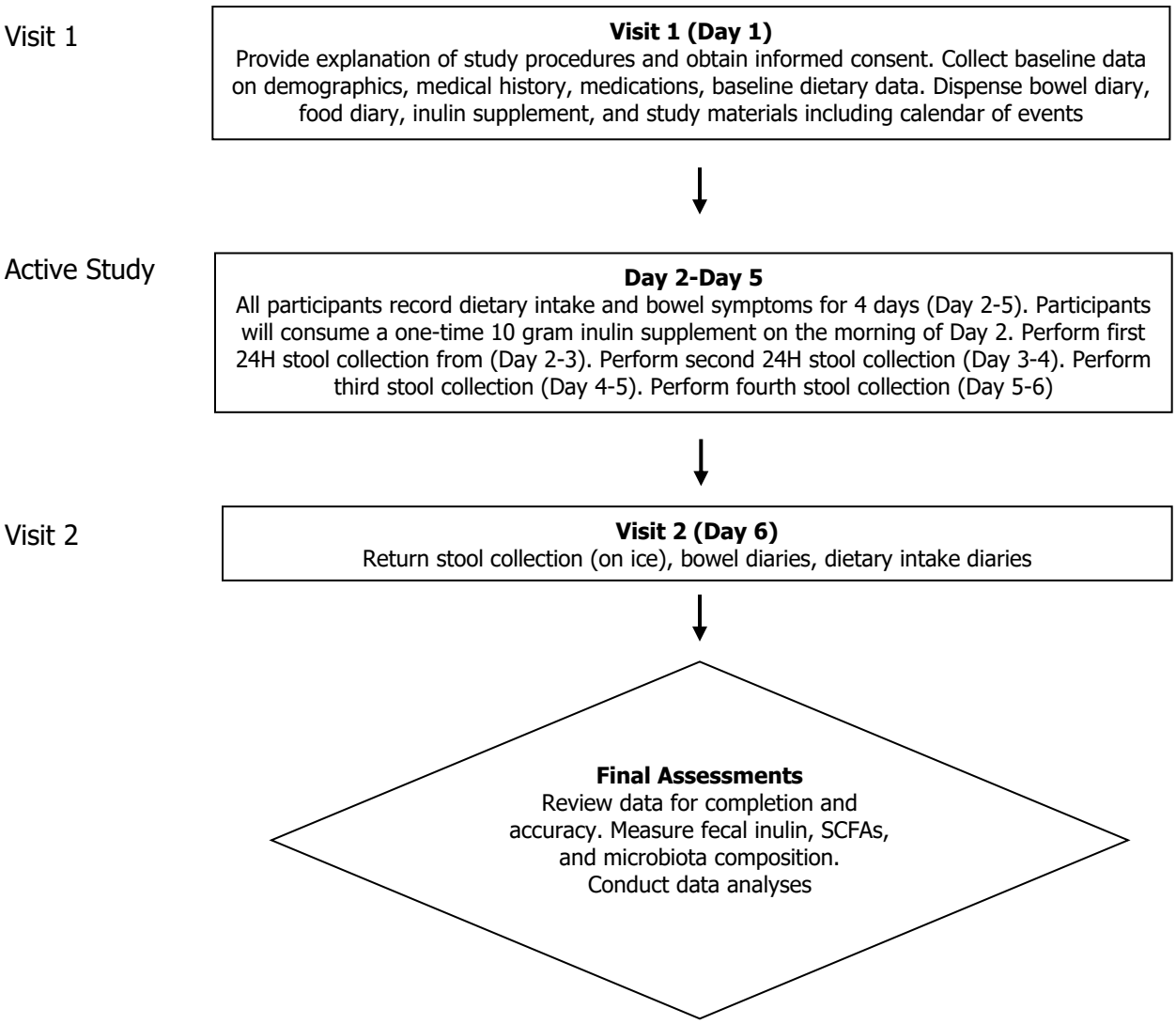
3.2 Exclusion Criteria

- History of microscopic colitis, inflammatory bowel disease, celiac disease, visceral cancer, chronic infectious disease, immunodeficiency, uncontrolled thyroid disease, history of liver disease or history of elevated AST/ALT > 2.0x the upper limit of normal
- Prior radiation therapy of the abdomen or major abdominal surgeries except for C-section, tubal ligation, vaginal hysterectomy and appendectomy or cholecystectomy, or other minimally invasive procedures (as deemed by the PI) > 6 months prior to study initiation.
- Ingestion of any prescription, over the counter, or herbal medications which can affect study interpretation within 6 months of study initiation for asymptomatic volunteers. Rescue therapy to facilitate stool collection will be permitted where needed.
- Any females who are pregnant or breast-feeding
- Antibiotic usage within 3 months prior to study participation
- Prebiotic or probiotic usage within the 2 weeks prior to study initiation
- Inulin usage within the 2 weeks prior to study initiation
- Regular use of tobacco products within the past 6 months

4.0 Study Design

We will conduct a prospective study of fecal inulin, fecal SCFAs, bowel symptoms, fecal microbiota in healthy adults. Baseline data on dietary intake, demographics, body mass index, medical history and medication-use will be collected in adults who meet the eligibility criteria defined above. The accessible population includes individuals from the local community (Marion County, IN within a 150 mile radius). Participants may be identified by public advertisement (e.g. newspaper advertisement, flyers, social media platforms) or during clinical visits to any Indiana University site. Participants may also be recruited from the local community, the Indiana CTSI Research Network, Regenstrief Data Core, and the national ResearchMatch research registry. All volunteers will sign written informed consent prior to enrollment and initiation of study procedures after study explanation.

Flow diagram for cross-sectional survey-based study



5.0 Enrollment

Up to 50 healthy volunteers will be recruited by public advertisement (e.g. newspaper advertisement, flyers, social media advertisements) and through notification of CTSI and ResearchMatch research participants. Subjects may also be identified and invited during clinical visits to any Indiana University site. Flyers will be made available at our clinical Indiana University sites. Flyers may also be posted in public common areas throughout the Indiana University campus and local community (within a 150 mile radius) or posted on other public or forums where such free advertisements are allowed including social media websites (e.g. Facebook, Craigslist).

All study tests and visits will be paid for by the study without cost to the participant. Participants will receive a total reimbursement of $50 for completing study participation and will

be reimbursed $10 for Visit 1 and $40 for Visit 2. Volunteers who present for a screening visit, but are ultimately determine ineligible will be provided a parking voucher to cover parking costs for that visit.

6.0 Study Procedures

6.1 **VISIT 1 (Screening Visit and Informed Consent**) - Eligible volunteers will undergo a screening visit by a physician, be asked to sign informed consent after explanation of the study. This visit may be completed in-person or as a virtual video visit. All questions will be answered. Inclusion/exclusion criteria will be reviewed. Medical history and medication intake will be reviewed. Upon signing the consent (paper version or via REDCap), participants will be scheduled for study-days and participants will fill out a validated bowel disease questionnaire (BDQ). All participants will complete the web-based Automated Self-Administered Dietary Assessment Tool developed by the National Cancer Institute (https://epi.grants.cancer.gov/asa24/) and a food frequency questionnaire (https://www.epic-norfolk.org.uk/for-researchers/ffq/). Study materials will be dispensed or mailed after completion of the visit: (1) stool kits and instructions for stool collection, (2) inulin supplement, (3) a bowel pattern diary including the Bristol stool form scale for participants to record stool symptoms over four days overlapping with stool collections, (4) a dietary intake diary. The bowel pattern and dietary intake diaries will be made available in several forms for the participant in order to allow flexibility for diary completion (i.e. link to secure web-based diary, paper diary or instructions for phone surveys). The participant may opt to complete the diaries using the method of their choice.

6.2 **Day 2** – Day 2 reflects the first day of active study. Day 2 may occur anytime up to 30 days after Day 1. This will depend whether the patient may need washout for medications or on the day that the patient can return for the final visit. It will also depend upon the discretion of the PI or Sub I. The participant will consume the inulin supplement before 9 am on Day 2. The first 24 hour stool collection will begin starting at 7 am on Day 2 and will continue until 7 am on Day 3. All stool collections will be refrigerated. The participant will begin recording their bowel symptoms and dietary intake data using 4-day diaries (recorded using either paper diaries or the web-based Automated Self-Administered Dietary Assessment Tool (https://epi.grants.cancer.gov/asa24/).

6.3 **Day 3** – The participant will start their second 24 hour stool at 7 am on Day 3 and continue until 7 am Day 4.

6.4 **Day 4** - The participant will start their third 24 hour stool at 7 am on Day 4 and continue until 7 am Day 5.

6.5 **Day 5** – The participant will start their fourth 24 hour stool collection at 7 am on Day 5 and continue until 7 am Day 6.

6.6 **VISIT 2 (Day 6):** Participants will return will return bowel diaries, dietary intake diaries, and stool samples. Diaries may be returned by mail, fax, or by electronic

submission. will complete the Automated Self-Administered Dietary Assessment. Study exit will be confirmed at this time.

Sample collection: Stool samples will be collected from all participants after study initiation. Stool will be refrigerated by participants and brought to the laboratory on ice within 4 days of collection. Once received, stool specimens each daily specimen into 4 samples for fecal microbial (brown screw-top tube), fecal SCFA (microcentrifuge tube), a fecal inulin (microcentrifuge tube with ethanol), and one back-up residual sample (microcentrifuge tube) and immediately frozen at -80^{o} Celsius.

Assessment of fecal microbiota:

a) Nucleic acid extraction: When received at the lab, frozen aliquots of stools samples will undergo processing for isolation of nucleic acids using the appropriate DNA isolation kit. Controls will be included at all steps to monitor for potential reagent contamination. DNA quality and quantity will be monitored by gel electrophoresis and fluorescent dsDNA assay. Genomic DNA will be stored at -80ºC for further use in construction of sequencing libraries and qPCR. Back up stool specimens will also be stored at -80oC.

b) Multiplex 16S allele PCR and sequencing: The V4 region of 16S alleles will be PCR amplified using the appropriate library preparation kit from stool genomic DNA to be sequenced in pools on an Ilumina MiSeq as previously described.[7]

c) qPCR validation experiments: We will design primers for specific microbial taxa of interest, for example specific taxa that contribute to the biosynthesis of SCFAs, so that their absolute abundance can be determined by quantitative PCR (q-PCR). Q-PCR targets will be chosen from the study 16S sequences using Primrose and PrimerQuest (IDT Inc.). Specificity of the primers will be assessed by Blast against the study 16S sequences and the NCBI database. Target sequences will be then be synthesized by a commercial vendor (e.g. Genescript) and cloned into a carrier vector. Q-PCR assays will be benchmarked against characterized fecal specimens that do or do not contain the target microorganism to assess assay specificity, and against fecal specimens that were determined not to contain the target taxa by 16S sequencing and which were spiked with different concentrations of the target vector to assess assay sensitivity. These assays will be used to assess presence and loads of specific organisms in the study fecal specimens.

d) Shotgun Metagenomic Sequencing (SGS): In order to overcome limitations of 16S allele PCR and sequencing which may include: (1) biases in the range of taxa amplified by universal primers, (2) limited resolution of the 16S rRNA gene between closely related species, and (3) dependence on the presence of a single marker gene, we propose further analysis of the microbial communities using coupled shotgun metagenomics sequencing. Specimens will be paired-end (2 x 150 bp) sequenced using the Illumina HiSeq and one lane of the sequencer allocated to the pooled sample. Sequencing data will be transmitted to the Bioinformatics Team for further analysis.

Data analysis of molecular methods:

a) 16S sequencing data will be processed by the DADA2 package[8] to generate separate lists of microbial taxa and their relative abundances in stool.[9] For downstream biostatistical analysis, to minimize unequal sampling effects, 16S sequences from each individual will be sub-sampled to equal sequencing depth. Using the statistical R packages, phyloseq75 and vegan, bacterial taxa richness and α/β diversity indices will be computed to quantify community-level variability among samples. Principal coordinate analysis, non-metric dimensional scaling and heatmap methods will be applied to cluster samples based on β diversity.

b) For SGS data, taxonomy will be assigned using MG-RAST to generate lists of bacterial, viral, fungal, protozoal and phage tax. Calculations for relative abundance will be similar to those used for 16S sequences. Total abundance will be estimated from numbers of taxa-specific reads and reads from internal standards.

SCFA measurement: Stool samples will be processed for extraction of fecal SCFAs to be quantitatively analyzed by high performance liquid chromatography-mass spectrometry or NMR and mass spectrometry as previously described.[10]

Inulin measurement: Fecal inulin content will be measured using short acid hydrolysis and high-performance liquid chromatography to measure inulin as hydroxymethylfuraldehyde by the Complex Carbohydrate Research Center at the University of Georgia. This approach is adapted from a method that has previously been published to measure inulin in plasma and urine.[11]

Inulin (Orafti®) is officially recognized by The United States Food and Drug Administration as an approved dietary fiber. It is naturally sourced from chicory root fiber; 92% inulin content, granulated powder. This fiber occurs naturally in a great number of plants and vegetables. Orafti® inulin is extracted through hot water processing and is of 100 % vegetable origin. In order to minimize any potential symptom exacerbation that may occur with consumption of such a dietary fiber, inulin powder supplementation will be limited to a one-time ingestion of a 10-gram dose with water.

Stool Frequency and Consistency: Participants will complete a daily diary to record bowel habits (frequency, consistency and form).[12] Participants will be given the option of completing the diary on paper, which will be dispensed at Visit 1 and collected at study conclusion.

Dietary Intake: Participants will be instructed to complete a food record during active study participation (using either a paper record or the electronic ASA24 website). Participants will further be instructed to maintain a stable diet throughout the duration of the study; and to avoid other extreme diet interventions such as a gluten-free diet or low FODMAP diet.

7.0 Study Calendar

We will conduct a prospective study that will require participants to complete a screening/baseline visit followed by 4-day active study period and a final follow-up visit. The active study may start anywhere up to 30 days after the initial study visit. Thus, participants may be active in the study for up to 35 days. Up to 50 healthy volunteers may be enrolled. We plan to conduct the study over a 24 month time period.

8.0 Reportable Events

All potential research concerns will be monitored by the study research team from the time of initiation until completion. Potential risks associated with this study include loss of confidentiality, discomfort associated with stool collections, or discomfort associated with dietary intervention. The study team will take care to recognized and minimize any discomfort that collecting stool or completing questionnaires may cause for participants. Inulin ingestion will be kept to a one-time ingestion of 10 grams and participants will be counseled appropriately prior to its use. Any potential adverse events will be followed by the study physician until they normalize. All serious events will be reported immediately to regulatory authorities and the Indiana University IRB. Any incidental findings that may require medical or profession intervention will be discussed with the participant by the study physician and the participant will be referred to the appropriate care team.

9.0 Data Safety Monitoring

The study PI (Huiping Xu, PhD) and all members of the study team will perform safety monitoring of all research subjects. Data quality, subject recruitment, subject accrual, subject retention, outcomes, procedures designed to protect privacy of subjects, outcomes, and adverse events data will be monitored by the PI and designated members of the study team on a regular basis.

10.0 Study Withdrawal/Discontinuation

Drop-outs may occur due to withdrawal of consent or because of discomfort related to study procedures. In order to compensate for withdrawals and loss to follow-up, up to 50 patients could be enrolled to meet the target of 30 completed volunteers.

11.0 Statistical Considerations

We plan to enroll 30 healthy volunteers. In order to compensate for withdrawals and loss to follow up, up to 50 patients could be enrolled. As the primary objective of this study is to describe the technical feasibility of measuring, sample size calculations are not based on the primary objective. Instead, the sample size estimation is based on secondary endpoints of the association between fecal SCFAs after inulin supplementation and fecal microbiota composition. Our sample size of 30 subjects provides an 80% power to detect a Pearson correlation of 0.5 based on the Fisher's z test at the 5% significance level. We consider the correlation of 0.5 an important correlation. Such a correlation is large but achievable based on data from a preliminary study of 14 healthy volunteers conducted by our lab, where the Pearson correlation between microbiota composition (e.g. individual taxa) and individual fecal SCFA levels were found to be above 0.5 for 5% of the taxa found in the stool sample.

12.0 Statistical Data Management

All study data will be recorded onto an electronic database (REDCap) which will be accessible only by certified research personnel. Only de-identified anonymized data will be analyzed. Quality assurance steps will include: built in range checks, testing of the database prior to

moving to production mode. The following quality control methods will be used: extraction and cleaning of data every 6 months.

13.0 Privacy/Confidentiality Issues

Potential risks include breach of confidentiality or invasion of privacy. To minimize risk of loss of confidentiality, participant records will be assigned a unique identifier in a coded manner accessible only by study personnel. All data collected on paper will be kept in a locked drawer within the GI Research Office until data collection is complete and transferred to an electronic storage system, after which papers will be discarded and destroyed. Data will also be captured and stored electronically in REDCap and in a protected Microsoft Excel spreadsheet on a Department Server. The storage location will be backed up manually every month. Electronic data will be automatically exported to common statistical packages such as SPSS, SAS, Stata, R/S-Plus at the time of conducting analyses. Only de-identified or anonymized clinical data will be transferred processing laboratories.

14.0 Follow-up and Record Retention

The study is expected to last 24 months in duration. All electronic records will be retained indefinitely. All data collected on paper will be kept in a locked drawer within the GI Research Office until data collection is complete and transferred to an electronic storage system, after which papers will be discarded and destroyed.

15.0 References

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