STUDY PROTOCOL

Fecal bile acids, fecal short chain fatty acids and the intestinal microbiota in patients with irritable bowel syndrome (IBS) and control volunteers: Diet Challenge

Principal Investigator:
Huiping Xu, PhD
Indiana University School of Medicine
410 W. Tenth Street, Suite 3000
Indianapolis, IN 46202

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Patients with irritable bowel syndrome (IBS) may exhibit atypical profiles of intraluminal bile acids and short chain fatty acids (SCFA). Intraluminal bile acids may influence intestinal secretion, permeability,[1-4] transit,[5,6] motility[7] and visceral hypersensitivity.[8] Bile acid sequestration,[6,9,10] administration,[11] and modulation of bile acid synthesis[12-15] have shown promising effects on stool characteristics, colonic transit and symptoms during treatment of chronic diarrhea and constipation. Intraluminal SCFA may also exert important effects on intestinal fluid and electrolyte absorption,[16-17] motility[18-19] and symptoms in IBS.[20] Altered SCFA profiles have been identified in IBS, although the pattern of these alterations is incompletely understood.[21-23] Identifying the underlying mechanisms that drive differences in intraluminal bile acids and SCFA could be critical to understanding the pathogenesis of IBS.

There is emerging evidence that alterations in bile acids and SCFA associated with IBS could be associated with changes in the gut microbiota. In addition to modulating levels of intraluminal organic acids, it has been hypothesized that gut microbiota may alter local immune responses, modulate visceral pain responses,[24] and impair gut barrier function.[25] Our **overall goal** is to investigate the relationship between fecal bile acids, SCFA and the gut microbiota in IBS. Results of this pilot study could reveal insights into the interplay of the gut microbiota and small molecule mediators of IBS to suggest targeted clinical strategies for improved diagnosis and management of this important syndrome.

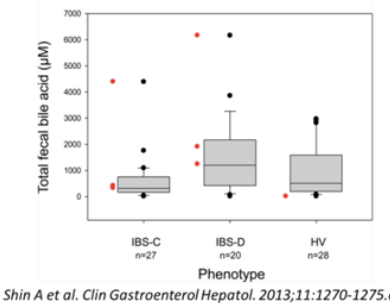
SPECIFIC AIMS & HYPOTHESIS: The central hypothesis of this proposal is that specific shifts in the GI microbiome composition correlate with altered colonic SCFAs and BAs and contribute to IBS symptoms

Specific Aim 1: Identify GI microbiome signatures in IBS subtypes (IBS-C and IBS-D) and matched controls, and test if microbiome signatures in these groups correlate with fecal SCFAs and bacterial fermentation of an indigestible carbohydrate (inulin) after a dietary challenge (fecal inulin).
Aim 1a Hypothesis: Abundance of specific SCFA-producing bacterial taxa (e.g. clostridial clusters IV and XIVa) correlates with fecal SCFAs and will distinguish IBS-C from IBS-D and IBS subtypes from controls.
Aim 1b Hypothesis: Abundance of specific SCFA-producing bacterial taxa is inversely correlated with fecal inulin and will distinguish IBS-C from IBS-D and IBS subtypes from controls.

Specific Aim 2: Determine if GI microbiome signatures in IBS subtypes and controls correlate with fecal BAs or markers of SCFA production (fecal SCFAs or inulin) and test if BAs correlate with fecal SCFAs or inulin.
Aim 1b Hypothesis: Abundance of BA-dehydroxylating taxa (e.g. genus *Clostridium*) correlates with decreased total fecal BAs in IBS-C and increased total fecal BAs in IBS-D compared to controls.
Aim 2b Hypothesis: Abundance of BA-dehydroxylating taxa is associated with fecal SCFAs and fecal inulin.
Aim 2c Hypothesis: Fecal inulin and SCFAs differ in IBS-D patients with and without BA malabsorption.

SIGNIFICANCE

Primary fecal BA significantly higher in IBS-D and non-secretory BA (LCA) higher in IBS-C



Shin A et al. Clin Gastroenterol Hepatol. 2013;11:1270-1275.e1.

Mechanistic effects of bile acids in pathogenesis of Irritable Bowel Syndrome Despite its global prevalence[26] and impact on quality of life,[27] current therapeutic options and actionable biomarkers for the treatment of IBS are limited. Bile acid malabsorption is identified in up to 50% of patients with functional diarrhea or diarrhea-predominant IBS (IBS-D).[28-29] Clinical studies have shown increased primary fecal bile acids, cholate and chenodeoxycholate, in IBS-D and increased non-secretory secondary bile acids, lithocholate, in constipation-predominant IBS (IBS-C).[30] The effect of bile acids on colonic mucosa is in part, determined by bile acid chemistry. Chenodeoxycholate and deoxycholate have been associated with increased fluid secretion, intestinal permeability and mucosal damage.[1-4] Stimulation of propagated pressure waves has been shown in the proximal human colon with intraluminal chenodeoxycholate.[7] In mice, deoxycholate induces peristalsis through TGR5-mediated effects.[31] Administration of chenodeoxycholate[11] or elobixibat, an inhibitor of the ileal bile acid transporter, in patients

with IBS-C and chronic constipation increases colonic transit compared to placebo.[12-14] And newer farsenoid X-receptor agonists such as obeticholic acid[15] and GW4064[32] are being considered for the treatment of bile acid or secretory diarrhea through attenuation of Cl^- secretory responses and stimulation of FGF19, an inhibitory regulator of bile acid synthesis. Meanwhile, treatment with bile acid sequestrants in IBS-D patients reduces colonic transit,[10] improves stool consistency[9] and improves symptoms.[6] The rationale for targeting bile acids as a treatment for IBS arises from our growing understanding of bile acid mediated mechanisms. Altered bile acid excretion can now serve as a validated biomarker in a largely heterogeneous patient population for which targeted therapy is often challenging. However, better characterization of the mechanisms of bile acid biotransformation and the interaction between bile acids and other metabolites (e.g. SCFA) is needed to understand causes and downstream effects of altered bile acid excretion in IBS.

Investigating the Role of the Microbiome in IBS The strongest evidence for a direct role of the intestinal microbiota in IBS pathogenesis is the link between infective gastroenteritis and subsequent development of IBS.[33-34] Numerous studies have evaluated the fecal microbial communities in IBS (reviewed in 35,36). Despite the lack of a definitive microbial signature, changes in the relative abundance of specific microbial groups among IBS patients have been recognized.[37,38] Reported observations include lower biodiversity,[39-41] decreased *Bifidobacterium,*[37,42,43] increased *Veillonella,*[37,43] an increased prevalence of the phylum Proteobacteria[44-45] and reduced *Faecalibacterium.*[42,46] Regrettably, experimental findings are also often contradictory or inconsistent, which may in part be attributable to differences in the employed experimental techniques, heterogeneous patient populations and external factors such as diet and medications.[35,36] Recent emphasis has been placed on studying the functional consequences of dysbiosis in IBS and identifying microbial biomarkers associated with phenotype or symptoms[31, 47] such as colonic transit and clinically significant depression.[48] Lower biodiversity in IBS has been associated with a lower abundance of butyrate-producing bacteria and methanobacteria.[41,49] IBS patients have also been found to harbor higher counts of acetate and proprionate-producing *Veillonella* and *Lactobacillus.*[22] Furthermore, significant decreases in *Bifidobacterium* and *C. leptum*, organisms involved in bile salt metabolism, have been observed in IBS-D[50] suggesting a role for microbial metabolic activity. Clarifying the functional consequences of dysbiosis through investigation of microbial communities and metabolites is needed to provide mechanistic insight into the role of the microbiome in IBS.

Microbial Production of Short Chain Fatty Acids and their Significance in IBS Normally, ~5-10% of all dietary complex carbohydrates is not absorbed in the small intestine and passes into the colon.[51] Ninety percent of the starches entering the colon are fermented by colonic bacteria to SCFA of which acetate, butyrate and propionate comprise the major proportion. SCFA may stimulate intestinal transit,[18] induce contractile responses,[19] and promote intestinal fluid and electrolyte absorption[16,17]. Intracolonic infusion of 0.5% acetic acid enhances sensitivity to colorectal distension in rats.[52] Prior studies have shown lower levels of total SCFA, acetate, propionate and higher levels of n-butyrate in IBS-D compared to controls[23]. In one study, significant increases of acetate and propionate were found in Japanese IBS patients, correlating with increased GI symptoms and worse quality of life. Findings suggest chemical stimuli may serve as an origin or exacerbating factor in IBS through effects on visceral hypersensitivity.[22] In contrast, proteomic analyses of stool from IBS patients showed increased bile acids and decreased branched chain fatty acids relative to controls; however, changes in SCFA were not significant.[53] Given conflicting results and various mechanisms by which these metabolites may affect gut physiology and symptoms in IBS, the relationship between SCFA production and intestinal microbes in IBS warrants further study.

Relationship between intestinal microbiota and intraluminal organic acids Bile salt hydrolases from several intestinal bacterial species including *Bacteroides, Clostridium perfrigens, Bifidobacterium, Lactobacillus and Listeria monocytogenes* deconjugate bile acids.[54] Both conjugated and deconjugated bile acids have been shown to induce intestinal secretion.[55] Although the physiologic advantages of deconjugation are incompletely understood, there is evidence that bile salt hydrolases aid in bacterial colonization of the lower GI tract,[56] promote detoxification of bile salts and increase resistance to bile salt toxicity.[57] Free bile acids rapidly undergo dehydroxylation by intestinal bacteria, converting primary to secondary bile acids. This metabolic pathway is found in ~0.0001% of total colonic flora[58,59] and the bacteria capable of dehydroxylation have previously been classified as *Clostridium.*[60,61] Changes in fecal metabolite profiles leading bowel dysfunction and symptoms in

IBS may reflect underlying shifts in the fecal microbial community. Alternatively, alterations in underlying gut physiology such as transit may contribute to the development of dysbiosis, suggesting a bi-directional interaction. It has been hypothesized that an observed decline in anaerobes following acute gastroenteritis may be a consequence of accelerated transit resulting in a loss of the anaerobic niche, and loss of the bacteria required for carbohydrate fermentation to SCFA.[62-64] Theoretically, accelerated transit may also reduce the time available for bacterial transformation of bile acids. Further investigation of the relationship between fecal microbiota and changes in the metabolome (e.g. bile acids and SCFA) is required for continued development of diagnostic and therapeutic strategies in IBS.

INNOVATION

The utility of SCFAs as a biomarker in IBS remains uncertain. Measuring *in vivo* SCFA production in humans[65] () is challenging. Identifying microbiome biomarkers of IBS has also been challenging due to clinical heterogeneity in patient phenotypes, technical variation in microbiome profiling methods, and inherent microbiome variability. Aims 1 and 2 of our proposal will identify factors associated with altered SCFAs in IBS by (1) **comprehensively assessing the composition of the fecal microbiota in IBS patients using deep 16S sequencing and (2) measuring SCFAs and bile acids in parallel.** In Aim 1, we will use a **novel approach to measure SCFA production** and test if SCFAs are a clinically useful IBS biomarker. Our approach is highly innovative because we will study carefully phenotyped participants and measurable pathways (SCFAs, bile acids) that could lie at the interface of IBS symptoms and the GI microbiome. We will utilize a new approach to monitor SCFA production by measuring fecal inulin in the stool. Studying the associations between bile acids and markers of SCFA production in Aim 2 is also distinctly innovative as effects of fecal bile acids on SCFA production in IBS are unknown. Our findings could generate critical preliminary data demonstrating a mechanistic pathway by which the GI microbiome is linked to IBS symptoms.

PREVIOUS WORK

Role of Fecal Bile Acids in IBS The current proposal will expand on prior work that the PI has completed during her research fellowship at Mayo Clinic during which the she conducted original clinical research investigating total and individual fecal bile acids and association with colonic transit in IBS to demonstrate significant increases in primary fecal bile acids in patients with IBS-D.[30] The PI also participated as a member of the team demonstrating quantitative differences in bile acid synthesis, fecal bile content, colonic transit, and intestinal permeability in IBS to identify actionable biomarkers for the treatment of IBS.[5,66] The current study will also expand on an ongoing companion protocol IRB # 1606244063 to further evaluate fecal bile acids, fecal SCFA, the fecal microbiota, and fecal inulin in the setting of a controlled dietary intervention with inulin supplementation.

RESEARCH PLAN

***Study Design*:** This trial will include a prospective investigation of fecal bile acid excretion, fecal bile acid profile, SCFA excretion, SCFA profile, fecal inulin and fecal microbiota in IBS patients and healthy controls.

***Study population/Participants*:** The *target study population* will be patients with IBS (both diarrhea- and constipation-predominant) and healthy controls. Our target population will focus on IBS-D and IBS-C in order to study the extremes of the disease phenotype. The *accessible population* includes patients attending Indiana University Gastroenterology Clinics, Primary Care Clinics, individuals from the local community (Marion County, IN within a 150 mile radius), and individuals from the Indiana CTSI research registry and from the national ResearchMatch registry. Participants may be identified by review of ICD10 codes, the electronic medical record, the Indiana University Motility Diagnosis Registry, and by public advertisement. Participants may also be identified and invited 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. The *intended sample* will include the aforementioned subjects meeting the following eligibility criteria:

***Inclusion Criteria*:** Patients with IBS, ages 18-75 fulfilling Rome IV criteria[67] and asymptomatic controls (healthy volunteers) 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.

Exclusion Criteria:
a. Participants with microscopic/lymphocytic/collagenous 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
b. Prior radiation therapy of the abdomen or abdominal surgeries except for C-section, tubal ligation, vaginal hysterectomy and appendectomy or cholecystectomy, > 6 months prior to study initiation
c. Ingestion of any prescription, over the counter, or herbal medications which can affect GI transit or study interpretation (e.g. opioids, narcotics, anticholinergics, norepinephrine reuptake inhibitors, nonsteroidal anti-inflammatory drugs, COX-2 inhibitors, bile acid sequestrants) within 6 months of study initiation for asymptomatic volunteers or within 2 days before study initiation for IBS patients. Stable low doses of tricyclic antidepressants or serotonin reuptake inhibitors will be permitted for individuals who have been taking them for a period of greater than one month. Rescue therapy to facilitate stool collection will be permitted where needed.
d. Any females who are pregnant or breast-feeding
e. Antibiotic usage within 3 months prior to study participation
f. Prebiotic or probiotic usage within the 2 weeks prior to study initiation
g. Regular use of tobacco products within the past 6 months.

Recruitment and retention: Subjects will be recruited by public advertisement (e.g. newspaper advertisement, flyers, and through notification of previously identified CTSI and ResearchMatch research participants). Subjects may also be identified and invited during clinical visits to any Indiana University site through the Indiana University diagnosis motility registry, or through review of electronic medical records including ICD10 diagnosis codes. Flyers will be made available at our clinical Indiana University sites and mailed to potentially eligible subjects. 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 $100 for completing study participation and will be reimbursed $25 for Visit 1 and $25 for Visit 2. They will receive an additional $50 after completing and returning the daily dairy and completing Visit 3. 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. Volunteers who opt to participate in the optional phone call occurring between 30 and 90 days of study initiation will be offered an additional $10 for their time.

Main variables**:** Main variables will include fecal bile acids, fecal SCFA, fecal microbiota, fecal inulin, and stool characteristics (frequency and consistency).

Sample collection**:** Stool samples will be collected from all participants after study initiation. Samples will be collected during the last 2 days of a 4-day low fiber, 100 g fat diet. Stool will be refrigerated by participants and brought to the laboratory on ice within 4 days of collection. Once received, day 4 (pre-inulin) and day 5 (post-inulin) stool specimens will be split into separate samples for fecal microbial, fecal SCFA, fecal inulin, and fecal bile acid analysis and immediately frozen at -80^{o} Celsius. One aliquot from each daily specimen (day 4 and day 5) will be stored for (1) fecal microbial (brown screw-top tube), fecal SCFA (microcentrifuge tube), fecal inulin (microcentrifuge tube with ethanol) and back-up (microcentrifuge tube) testing. The remaining bulk of the 48-hour stool specimens will be reserved for total fecal bile acids. All specimens will be 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 from stool genomic DNA using degenerate primers[68] with index sequences[69] and sequenced in pools on an Ilumina MiSeq as described previously[70,71] in the Nelson lab.
d) qPCR validation experiments: We will design primers for specific microbial taxa of interest, for example specific pathogens or taxa identified as enriched in IBS patient sub-groups, 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. Primers will also be analyzed using the program Spyder[72] to assess specificity in and outside of taxonomic groupings. Target sequences will be then be synthesized by a commercial vendor (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.
***Data analysis of molecular methods*:**
16S sequencing data will be processed by the DADA2 package[73] to generate separate lists of microbial taxa and their relative abundances in stool.[74] 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, *phyloseq*[75] and *vegan*, bacterial taxa richness and α/β diversity indices.

***SCFA and bile acid measurement*:** The stool sample submitted by study participants will be split at the lab for DNA isolation and frozen aliquots will be shipped to the Metabolite Profiling Facility at Purdue University and the Mayo Clinic Department of Laboratory Medicine and Pathology for SCFA and bile acid measurement respectively. Total and individual fecal bile acid excretion will be measured by high-performance liquid chromatography/tandem mass spectrometry (per 48 hours on a 100 g fat diet).[76,77] Analysis and analytical performance have previously been documented.[30] The lower limit of quantitation of each of the individual measured BAs is 0.06 µmol (in methanol extract). Measurement of SCFA will be performed by liquid chromatography-mass spectrometry using previously published methods.[78]

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.[79]

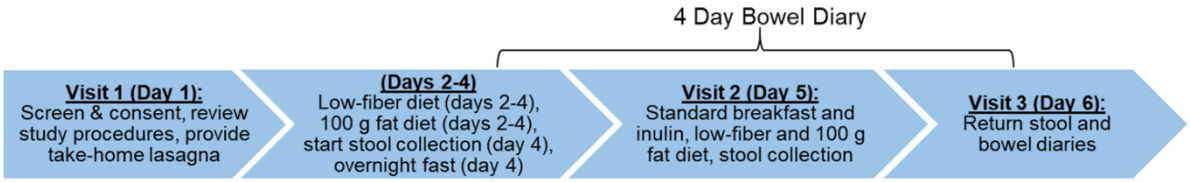
Inulin (Orafti®) is officially recognized by The United States Food and Drug Administration as an approved dietary fiber. This fiber occurs naturally in a great number of plants and vegetables. Orafti® inulin is extracted through hot water processing and is of 100 % vegetable origin. In order to minimize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

***Stool Frequency and Consistency*:** Participants will complete a validated daily diary to record bowel habits (frequency, consistency and form).[80] 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: Throughout the 4 day low fiber, high-fat diet, participants will be instructed to complete a food record. 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. The first take-home meal (Visit 1) will consist of a meat or vegetarian lasagna, a dessert and beverage. The second and third take-home meals are to be consumed at 4 and 8 hours after the breakfast with inulin ingestion on Day 5 and will consist of vegetarian or non-vegetarian sandwiches and meat or vegetarian lasagna meals with a dessert and beverage.

***Experimental Procedures*:**

Figure: Schedule of Events



VISIT 1 (Screening Visit, Informed Consent) - Eligible volunteers will undergo a screening visit by a physician, be asked to sign informed consent after explanation of the study. All questions will be answered. Inclusion/exclusion criteria will be reviewed. A physical exam will be performed. Medical history and medication intake will be reviewed. The screening visit may occur virtually or in-person and signing of the informed consent will be done either online via Redcap or in-person. Upon signing the consent and confirming eligibility after the screening visit, participants will be scheduled for study-days and participants will fill out the irritable bowel syndrome severity scoring system questionnaire (IBS-SSS) if they have IBS.[81] The IBS-SSS may be completed in-person or online. All participants will also be asked to complete the web-based Automated Self-Administered Dietary Assessment Tool developed by the National Cancer Institute (https://epi.grants.cancer.gov/asa24/). If needed, guidance on this tool may be offered by a trained interviewer. Study materials will be dispensed: (1) stool kits and instructions for stool collection (day 4 and day 5 collection containers), (2) a take-home non-fermentable meal (lasagna meal as described above)) (3) a validated bowel pattern diary including the Bristol stool form scale for participants to record stool symptoms over a two week course overlapping with stool collections and diet intervention. Bowel pattern diary will be provided in several forms to 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 diary using the method of their choice

Day 1 and Visit 1 may not be the same. This will depend whether the patient may need washout for medications or the day that the patient can make for the Day 5 visit. It will also depend upon the discretion of the PI or Sub I
Day 1: This will start the day prior to the initiation of the low fiber, high fat diet.
Day 2: Day when the participant starts the low fiber, high fat diet.
Days 2-4: The participant will continue a low fiber, high fat diet and start the stool collection on Day 4 (to be combined in the day 4 container).

VISIT 2 (Day 5): Participants will return for a standard breakfast with inulin ingestion following an overnight fast. Participants will continue the stool collection through Day 5 following inulin ingestion (to be combined in the day 5 collection container). Starting 2 days prior to Day 4 (Visit 2), participants will consume a low fiber diet (maximum one piece of fruit and 100 g vegetables a day, and white flour instead of wholemeal products) avoid alcohol, and start a 4-day 100 g fat diet. On the evening before Visit 4, a standardized, completely digestible, and non-fermentable meal (lasagna meal) will be consumed. Participants will present for Visit 4 after an overnight fast and receive a standard pancake breakfast with a vegetarian or non-vegetarian side (approximately 550 to 600 kcal) with 10 g inulin (Orafti®) and 200 mL of water. Take-home meals will be provided (vegetarian or non-vegetarian sandwich meal and a meat or vegetarian lasagna meal with desserts and beverages) to be consumed at 4 and 8 hours following inulin ingestion. Participants will be instructed to complete the IBS-SSS again within one hour of consuming their final take-home meal.

VISIT 3 (Day 6): Participants will return bowel diaries, IBS-SSS forms (via mail, fax, electronic submission or completion of the phone surveys) and return stool samples for analysis. Study exit will be confirmed at this time.

Optional dietary follow-up (Day 30-90): To further assess the utility of the 24 hour dietary recall, willing participants will be invited to participate in a an optional follow-up activity during which they will complete the Automated Self-Administered Dietary Assessment Tool with or without assistance of a trained interviewer. Interested volunteers will be contacted by phone and provided instructions on how to complete the tool online by the study team.

***Confounding variables*:** Potential covariates include age, BMI, sex and concomitant use of acid suppression. These variables will be collected upon screening to ensure accuracy.

***Statistical considerations*:**
The primary endpoints for analysis will be:

1) Total bile acid excretion
2) Total and individual fecal excretion of SCFA
3) Fecal microbial population
4) Fecal inulin

The secondary endpoints for analysis will be:

1) Percent primary fecal bile acid excretion
2) Stool characteristics (frequency, consistency, ease of passage) based on bowel diary

The associations of fecal SCFAs (continuous variable) with phenotype (using HV as the reference group) and specific microbial taxa will be assessed using a general linear model (GLM) adjusting for relevant covariates. The relative abundance of specific bacterial taxa will be measured and compositional data analysis will be performed to assess the associations of relative microbial taxa abundances with fecal SCFAs or other endpoints (fecal inulin) and to assess whether phenotype alters observed associations. GLM will include specified endpoints, phenotype, the interaction between the endpoint of interest and phenotype, and relevant covariates as prediction variables for SCFAs or other endpoints of interest. False discovery rate (FDR) control will be performed using the Benjamini-Hochberg procedure to correct for multiple testing.[82] We will explore (a) multivariable associations between overall community composition (e.g. Bray-Curtis dissimilarity) with phenotype, fecal SCFAs, or fecal inulin using the PERMANOVA approach adjusting for covariates and (b) the influence of bowel functions on the association between microbial endpoints with fecal SCFAs or inulin in IBS.

Associations of total fecal bile acids with relative abundance of microbial taxa and other specified endpoints (fecal SCFAs or inulin) will be assessed using GLM adjusting for covariates. FDR control will be performed using the Benjamini-Hochberg procedure. Among IBS-D participants, we will test associations between fecal SCFAs or inulin and bile acid phenotype (with or without bile acid malabsorption) using ANCOVA adjusting for covariates. We will explore (a) multivariable associations between overall community composition (Bray-Curtis dissimilarity) with phenotype or total fecal bile acids using the PERMANOVA approach adjusting for covariates and (b) associations between primary fecal bile acids with relative microbial abundances in IBS patients.

***Sample size and Power*:** We will enroll 24 patients with IBS-D, 24 patients with IBS-C, and 24 healthy controls. In order to compensate for withdrawals and loss to follow-up, up to 50 patients could be enrolled to meet the 24 studied in each group.

Sample size assessment is based on results of primary endpoints for Aim 1 from a preliminary dataset acquired by our lab and calculated using ANOVA among three cohort groups. We will have 80% power at the 5% significance level to detect the effect size shown in the table for fecal SCFAs between 19 IBS-C, 19 IBS-D, and 19 HV. Estimated effect sizes for other endpoints of interest are shown in the Table.
Table: Estimated effect sizes

Response (Data show mean+SD)	HV	IBS-C	IBS-D	Effect size (cohen's f)	N per group
Relative abundance of clostridial cluster IV (%)	0.181+0.221	0.392+0.354	2.01+4.06	0.39	22
Relative abundance of clostridial cluster XIVa (%)	0.0814+0.148	0.04+0.0316	0.137+0.31	0.181	99
Relative abundance of genus *Clostridium* (%)	0.486+0.368	0.84+0.647	2.57+3.85	0.461	16
Overall colonic transit (days)	1.55+0.977	1.52+1.39	1.08+0.649	0.461	65
Total SCFA excretion (µg/mg)	9.94+5.87	16.5+10.1	11.5+2.9	0.235	19

***Inclusion of Women, Minorities, and Children*:** Individuals will not be excluded based on gender, race, or ethnicity. Patients with irritable bowel syndrome are comprised of both men and women and have diverse racial and ethnic backgrounds. The prevalence of irritable bowel syndrome among white, blacks, and Hispanics in the U.S. is very similar. Gender and racial/ethnic differences in fecal bile acid and SCFA excretion have not been reported. The communities of Marion County are anticipated to be 65% white, 29% black or African American and 6% other minorities. Children, 18-21 years of age, and adults 21 years or older will be eligible for participation in the study as active participants. However, children below 18 years of age will be excluded.

***Hazardous Procedures, Situations, or Materials and Protection of Human Subjects*:** Potential exposure to radiation will be recognized. A Data Safety Monitoring Plan will be proposed. Participant records will have a unique identifier and other identifier information, including name, address, email, home address will be deleted. Only de-identified or anonymized clinical data will be transferred to processing laboratories.

***Quality assurance/Quality Controls*:** Forms will be completed by subjects and reviewed by study staff to ensure completeness. Study coordinator will maintain study records. Study personnel performing stool analysis will be blinded to subject identity.

***Feasibility*:** Resources available through our clinical research motility program at Indiana University and through the Nelson lab for fecal microbial assessment make completion of this study highly feasible. The PI and her mentors have demonstrated a well-established track record of the techniques to be employed for this proposal in previous studies.

***Internal Validity*:** Threats to internal validity include measurement bias, confounding, and chance. We will protect against measurement bias with validated assays and blinding of all study staff when applicable. We will protect against confounding by inclusion of covariates in multivariate analysis. We will control for chance by having sufficient sample size and power as previously calculated.

***External Validity*:** Results will reflect the population of Indianapolis, IN and will be generalizable to patients with IBS and similar demographic distribution.

***Strengths and Limitations*:** Major strengths of the study include prospective study design, the ability to noninvasively measure transit, assess fecal microbiome, and fecal organic acids. Other strengths include the microbiome expertise of the Nelson lab, bile acid expertise of the Mayo Clinic Lab Medicine and Pathology, expertise of the Metabolite Profiling Facility at Purdue University, and expertise of the Complex Carbohydrate Research Center at the University of Georgia. Limitations include cost, potential for participant withdrawal or loss to follow-up and multiple study dates which will be addressed by coordinating schedules to subject's availability and limiting recruitment to the local community.

***Benchmarks*:** Primary endpoints will serve as benchmarks for success anticipated to achieve aims. It is anticipated that completion of Aims 1 and 2 will result in 2-3 separate publications, with at least one publication in a high-impact journal. All data will be analyzed and results submitted submitted in abstract form to scientific meetings. Findings from this study will be used to support future grant applications stemming from subsequent research studies investigating novel approaches to restoring the microbiome in IBS and relieving symptoms

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