Dietary Impact on Intestinal Sulfate Metabolism

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Protocol Number: CTSI# 25182

Trial Registration: pending

Version / Date: Version 10 / 20 January 2021

CONFIDENTIAL

VERSION HISTORY

Version #	Version Date	Significant Changes from Previous Version
Version 1	18 Oct 2016	Original Protocol Version
Version 2	15 May 2018	Raised age and BMI cutoffs for inclusion criteria; removed vegetarianism as exclusion criteria; allowed coffee and tea as beverages in High-S diet; removed physical exam from baseline study data collection; changed titled to align with grant submission process
Version 3	21 June 2018	Added 24-hour urine collection to help characterize dietary sulfur intake
Version 4	19 July 2018	Changed status of vital signs and blood collection procedures to "per PI discretion"
	22 August 2018	Revised section 4.1 according to reviewer comments: "Revise the section 4.1 of the protocol to state the following, to ensure the health and safety of all participants entering and exiting the study: 'Of note, Vital signs and collection of blood samples will be completed based on PI discretion, except for the Screening and Safety Follow-up Visit.'
Version 5	20 December 2018	Decreased diet intervention duration to from 14 days to 7 days and overall duration of the study from ~2 months to ~1 month; Decreased clinic visits from 11 down to 6; Modified High-S and Low-S diets to incorporate the emphasis on animal-based for high sulfur and plant-based for low sulfur described in "Research Design and Methods"; Updated FFQ to most recent version (web-based DHQ-III)
Version 6	19 February 2019	1. Added participant compensation of up to $150. Payments will be received in the form of gift cards. 2. Added additional fecal sample collections. The fecal sample collections are not full stool samples, but are small samples collected in collection tubes. These samples will be stored in the participant's freezer and collected at each study visit. These will be collected on days (±2 days) -4, -2, 3, 5, 9, 11, 16, 18, 23, 25, 30, and 32. The additional samples will enhance analysis of longitudinal changes and help correct for some of the inherent variability associated with stool sampling. 3. We would also like to remove Matthew Hamilton as a study team member.
Version 7	8 May 2019	1. Increased population to 15 volunteers 2. Changed 24-hour urine collection to "per PI discretion"
Version 8	17 September 2019	1. Increased population to 20 volunteers
Version 9	27 February 2020	1. Increased population to 30 volunteers
Version 10	20 January 2021	1. Increased "Inclusion Criteria" age from maximum of 70 years of age to 80 years of age

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LIST OF ABBREVIATIONS

AE	Adverse Event
CH_4	Methane
CH_3SH	Methanethiol
CRF	Case Report Form
CTCAE	Common Terminology Criteria for Adverse Events
DHQ	Dietary History Questionnaire
DNA	Deoxyribonucleic Acid
GC	Gas Chromatography
H_2S	Hydrogen sulfide
H_2	Hydrogen Gas
High-S	High-Sulfur Diet
HIPAA	Health Insurance Portability and Accountability Act
IBD	Inflammatory Bowel Disease
IRB	Institutional Review Board
Low-S	Low-Sulfur Diet
LC-MS	Liquid Chromatography-Mass Spectrometry
NCI	National Cancer Institute
NDSR	Nutrient Data System for Research
SAA	Sulfur-containing Amino Acid
SAE	Serious Adverse Event
SCFA	Short-Chain Fatty Acid
SRB	Sulfur-Reducing Bacteria
TMAO	Trimethylamine N-oxide
UC	Ulcerative Colitis
UPIRTSO	Unanticipated Problems Involving Risks to Subjects or Others

PROTOCOL SUMMARY

Title:	Dietary Impact on Intestinal Sulfate Metabolism
Phase:	1
Population:	30 healthy male and female volunteers. Subjects will be recruited from the Twin Cities metropolitan area and will participate as outpatients in this study.
Number of Sites:	1 (University of Minnesota)
Study Duration:	12 months
Subject Participation Duration:	Approximately 1 month
Agent or Intervention:	Dietary intervention will consist of following diets high in sulfur-containing amino acids (SAAs) and low in SAAs for 7 days each, with a 14-day washout period between interventions
Objectives:	The primary objectives of this proposal are to: 1) Compare low sulfur and high sulfur diets with respect to fecal production of hydrogen sulfide (H_2S) and other end products of microbial fermentation; and 2) Determine whether the diet alters the fecal microbiota and the relative proportion of fecal sulfate reducing bacteria.
Endpoints:	The primary endpoint is the change in H_2S concentration measured by gas chromatography. Comparisons will be made between the baseline measurement (Day 0) and end of intervention (Day 7) for each diet (within-group comparison) and between high- and low-sulfur diet H_2S concentration on Day 7 (between group comparison).

1 BACKGROUND INFORMATION AND SIGNIFICANCE

Ulcerative colitis (UC) is one of the two major inflammatory bowel disease (IBD) subtypes and is characterized by chronic, relapsing inflammation in the colon causing diarrhea and rectal bleeding. UC currently affects 0.25% of the population in North America (~ 1 million people in the United States), having risen dramatically over recent decades, and is now emerging as a global disease.[1] UC is responsible for significant personal, professional, and economic consequences - annual costs associated with medical management approach $5 billion.[2] The causes for flares and remissions in UC activity are largely unknown, although epidemiologic studies suggest a significant role of the environment in the emergence of IBD. Thus, there is rising incidence in Europe and North America,[3,4] higher incidence in developed countries as compared to non-industrialized countries,[5,6] and increasing incidence rates in developing countries as they have Westernized.[7]

Patients with UC instinctively understand that diet is an important variable in diseases involving the intestinal tract, and they have a strong interest in dietary modifications that may aid in maintaining remission of symptoms and improving their disease. When first diagnosed, virtually all patients with IBD ask what they should eat to help control their disease. To a large extent, this reasonable and intuitively logical question remains unanswered, as there is a scarcity of well-designed, prospective interventional studies that can guide dietary recommendations for this patient population. Current allopathic therapeutic strategies rely exclusively on drugs and surgery to control intestinal inflammation and deal with its complications. However, these available therapies are commonly not sufficient to control the symptoms, risk serious side effects, and come at a high financial cost. Therefore, patients and doctors alike seek effective complementary therapies.

The intestinal tract contains complex microbial communities that participate in the final steps of the digestive process. The structure and composition of these communities is highly responsive to dietary input.[8] Some of the end products of microbial metabolism in the gut are highly toxic (e.g., H_2S) and may be linked to the pathogenesis of UC.[9,10] These potentially toxic compounds can damage the epithelial lining of colon, cause breakdown of the gut barrier, and lead to inflammation. Fecal H_2S concentration is increased by increasing either the sulfur amino acid[11] or sulfate content (as additives) of the diet.[12] Sulfur-containing amino acids (SAA) are converted to sulfides by sulfate reducing bacteria (SRB)[13,14], which may expand in response to increased availability of SAA and inhibition of alternative end routes of fermentative pathways, e.g., production of methane or short-chain fatty acids (SCFAs).[15,16]

The scientific foundation for the sulfur-containing toxin hypothesis as a driver of UC rests on provocative and compelling evidence from clinical observations, animal models, and dietary association studies. The characteristic distribution of UC in the colon (always involving the rectum and continuously extending proximally to varying extent of involvement) is suggestive of a toxin being concentrated along the path of intestinal transit. Sulfated dextrans, but not free dextrans, can induce experimental colitis in rodent models.[17] H_2S, a potent toxin in its own right and one of the suspected sulfur-containing toxins in UC, decreases butyrate utilization by colonocytes and causes loss of gut barrier function.[9,18] UC patients have been found to have increased luminal concentrations of H_2S and disease activity correlates with sulfide production

rates.[10,19] Furthermore, some medications that have beneficial effects on UC activity, e.g., mesalamine and aminoglycoside antibiotics, can inhibit SRB.[20,21] The major dietary sources of sulfur are high-protein foods (containing the SAAs methionine and cysteine), cruciferous vegetables from the *Brassicaceae* family, and processed food preservatives.[22] Dietary sources of sulfur exceed endogenous sources in the colon,[23] and dietary intake studies have found a greater risk of UC flares in individuals consuming diets with higher sulfur content.[24,25] When taken together, these mechanisms suggest that minimizing intake of dietary SAAs may improve symptoms and reduce risk for disease exacerbation in individuals with UC.

Diet-derived sulfur is not the only determinant for the availability of sulfur to microbiota in the colon. Saturated fats (mainly animal-derived), as opposed to unsaturated fats (mainly plant-derived) promote taurine conjugation of hepatic bile acids. Taurine provides a source of organic sulfur to SRB such as *Bilophila wadsworthia*, which blooms in mice fed a high-milk fat diet, but not a high-safflower fat diet.[26] Furthermore, the high-milk fat diet induces *B. wadsworthia*-specific Th1 cells in the gut-associated lymph nodes and greater incidence of colitis in genetically susceptible (IL-10-/-) mice. Different bile acids themselves can have potent effects on microbiota, both inhibitory and stimulatory, on different members of microbiota. *B. wadsworthia* is clearly favored by taurocholate, and this is also true of *Clostridium difficile*, which is a common trigger for flares of UC activity.[27,28]

The notion of a "low sulfur diet" for treatment of gastrointestinal conditions has existed for decades. Restricting high-sulfur foods, in combination with milk fat restriction, has historically been recommended to UC patients by some practitioners of integrative medicine. However, and despite these claims, the methodology of this diet was ill-defined and it is not known whether it results in specific changes in gut microbiota, decreases production of toxic sulfur-containing end products, or is even feasible in terms of adherence. The medical literature contains only a single small clinical case series of 4 patients with UC from approximately 20 years ago that demonstrated symptomatic improvement on a low sulfur diet that restricted meat, milk fat, cruciferous vegetables, and inorganic sulfates used as food preservatives.[29] This work did not include objective measurements of disease activity, such as laboratory or endoscopic criteria, which would be standard today. One prospective cohort study[24] published in 2004 measured dietary intake via food frequency questionnaire and found that a high intake of meat and meat products (particularly red and processed meats), eggs, protein, alcohol, energy, fat, sulfur, and sulfate (a common food and beverage additive) predicted an increased likelihood of relapse. However, data on sulfur content of foods was only listed for 29% of foods consumed by participants in the study, indicating incompleteness of the full total of sulfur intake. Notably, sulfur content was not available for red and processed meats, which are known to be high in dietary sulfur. Therefore, more complete food tables for sulfur would be expected to increase the differences in sulfur consumption between participants who did or did not relapse.

The scarcity of rigorous, systematic investigations of dietary interventions in UC patients is a consequence of several factors: (1) controlling diet in human subjects is difficult, (2) dietary input is complex, and (3) clinical end points alone provide few opportunities for mechanistic dissections. However, the rise of high throughput DNA sequencing and metabolomics, combined with novel computational techniques over the past decade, is opening an entirely new era in

nutrition research. The gut microbiota, unlike human individuals, can serve as very accurate reporters of environmental changes.

The long-term goal of our research group is to develop evidence-based nutritional strategies for UC that compliment medical therapies. Our strategy is to link specific diets to their effects on microbiota functionality. Nevertheless, we understand that even when armed with the new powerful tools, identifying an effective diet that is also acceptable to patients will be a challenging and resource intensive journey. Therefore, in this proposal, which is our first step toward that ultimate goal, we will test dietary extremes in highly motivated, healthy volunteers. This will allow us to streamline our experimental toolkit and measure the magnitude of the potential effects on gut microbiota composition and function that can be achieved by controlling the diet, which will help to estimate the size of a formal clinical study in patients with UC.

2 STUDY HYPOTHESES AND OBJECTIVES

2.1 Hypotheses

Our hypotheses are that excessive production of toxic sulfur-containing end products of microbial fermentation in the colon are major drivers of UC, and that it is possible to decrease their production by dietary means.

2.2 Primary Objectives

The primary objectives of this study are:

1. Compare low sulfur (Low-S) and high sulfur (High-S) diets with respect to fecal production of H_2S and other end products of microbial fermentation, such as thiols, hydrogen gas (H_2), and methane (CH_4) (prediction: higher H_2S with High-S diet).
2. Determine whether the diet alters the fecal microbiota and the relative proportion of fecal SRB using 16S rRNA profiling and whole metagenome shotgun sequencing for species-level resolution (prediction: higher SRB with High-S diet).

2.3 Secondary Objectives:

1. Evaluate the tolerability and feasibility of high- and low-sulfur diet regimens.
2. Measure changes in bile acid profiles associated with changes in dietary SAA intake.

2.4 Exploratory Objectives

1. Assess changes in serum TMAO, carnitine, and phosphatidylcholine concentrations associated with changes in dietary SAA intake.

2.5 Study Outcome Measures

2.5.1 Primary Outcome Measures

Primary outcome measures of this study include:

1. Concentration of H_2S, CH_4, H_2, and methanethiol (CH_3SH) in fecal headspace samples measured by gas chromatography spectrometry (GC).
2. Changes in fecal bacterial composition (pre- vs. post-diet) associated with High-S vs. Low-S diets.

2.5.2 Secondary Outcome Measures

1. Compliance with diet interventions and adverse event rates overall and between High-S vs. Low-S diets.
2. Changes in gastrointestinal symptoms overall and within participants in High-S vs. Low-S diets, by clinical assessment and adverse event monitoring.
3. Changes in fecal bile acid profiles overall and within participants in High-S vs. Low-S diets, by liquid chromatography-mass spectrometry (LC-MS).

2.5.3 Exploratory Outcome Measures

1. Changes in serum concentrations of TMAO, carnitine, and phosphatidylcholine within participants and between High-S vs. Low-S diets.

3 RESEARCH DESIGN AND METHODS

This is a randomized, 4- to 5-week crossover pilot study of 15 highly motivated healthy volunteers who will consume two 7-day intervention diets: 1) a diet low in sulfur-containing amino acids (Low-S diet), emphasizing plant-based foods and fat sources; and 2) a diet high in sulfur-containing amino acids (High-S diet), emphasizing animal protein and fat sources. A 14-day washout period will follow each intervention, in which participants will eat their typical diets. Intervention order will be randomly assigned through a computer-generated number sequence. At the conclusion of the second intervention, participants will be instructed to resume normal dietary habits and will be tracked for an additional 7 days to monitor study endpoints and related variables. Frequent communication between participants, the registered dietitian, and other study investigators will be sustained throughout the trial to ensure compliance with dietary and data collection and to reduce attrition.

Participants will be assessed prior to any dietary intervention to determine baseline demographics and characteristics, dietary habits, and measurements of endpoints of interest. High-S and Low-S meal plans will be designed by a registered dietitian trained in nutrient analysis, and detailed descriptions of each intervention will be discussed with participants. Participants meeting inclusion criteria will be consented at the screening clinic visit. Routine clinical examinations, assessments, laboratory evaluations, and sample collections will be completed at 5 clinic visits over a time period spanning approximately 35 days.

3.1 Study Participants and Recruitment

Highly motivated participants will be recruited from the Minneapolis-St. Paul metropolitan area. All participants will comply with the following predetermined inclusion and exclusion criteria:

3.1.1 Inclusion Criteria

Participants will be eligible to participate in the study if all of the following conditions exist:

1. Provide informed consent
2. Ambulatory and community dwelling
3. Able and willing to comply with the study schedule and procedures
4. 18 - 80 years of age

5. BMI between 18.5 – 40.0 kg/m^2
6. Omnivorous or willing to consume animal products through duration of study

3.1.2 Exclusion Criteria

Subjects will be excluded from participation in the study if any of the following conditions exist:

1. Antibiotic use within 3 months
2. Planned antibiotic therapy within the period of the study, e.g., perioperative antibiotics.
3. Use of sulfonamides or sulfasalazine, as these sulfur-containing medications may confound results
4. Use of 5-aminosalicylates (5-ASA), as they are known to inhibit sulfidogenesis
5. History of anaphylactic food allergies, e.g., peanuts, seafood.
6. Food intolerances and allergies, including gluten sensitivity, lactose intolerance, and intolerance of high fiber dietary content.
7. Strict vegan eating practices, with refusal to consume animal products.
8. Planned use of oral probiotics while on study.
9. Serious, concomitant illness that, in the opinion of the Investigator, would interfere with evaluation of safety or efficacy, or put the participant at risk of harm from study participation.
10. Significant alcohol use, defined as > 20 g/day in females and > 30 g/day in males for a period of 3 months within one year prior to screening.
11. Underlying chronic gastrointestinal disease that can cause diarrhea, including short bowel syndrome, irritable bowel syndrome, malabsorption, and celiac disease.
12. History of partial or complete colectomy.
13. History of malabsorptive bariatric surgery.
14. Currently participating in another clinical study.
15. Legally incompetent and unable to understand the study's purpose, significance and consequences, and to make decisions accordingly.

3.2 Diet Intervention Design

Two intervention diets will be designed by a registered dietitian trained in nutrient analysis. A registered dietitian will provide detailed instructions for each intervention diet, and educational materials will be developed to indicate foods with high and low contents of SAAs as well as example meal plans for participants to reference. Dietary instructions will be based on the following table:

Table 1. Guidelines for intervention diets

High-S Diet (Emphasize animal-based foods)	Low-S Diet (Emphasize plant-based foods)
Meats: beef, pork, lamb, veal, venison, goat, processed meats (ham, bacon, bologna, salami, sausages, hot dogs, deli or lunch meats), poultry, seafood, shellfish	Cooked lentils and beans, excluding soy beans or soy bean-derived products
Dairy Products: cheese, milk, yogurt, ice cream, sour cream	Dairy alternatives: almond milk, rice milk, hemp milk, coconut milk, cream substitutes
Eggs	Egg substitutes
Animal-derived fats: butter, lard	Plant-derived fats: oils (e.g., vegetable, olive, safflower, cottonseed, cod liver, coconut, peanut, sesame)
Grains: white breads, pastas, rice	Grains: whole wheat, brown rice, bulgur, buckwheat, barley, oats, fiber-containing breakfast cereals
Limited intake of vegetables	Fresh/frozen vegetables
Limited intake of fruit	All fruit
Beverages: fruit juices, mineral water, sulfited wines, coffee, tea	Beverages: fruit and vegetable juices, coffee, tea, distilled spirits, beer
Soy Products: soy milk, tofu, tempeh, miso, soy crisps, dried soy beans	Snack foods: granola bars, popcorn, pretzels, potato chips, rice cakes
	Nuts and seeds
Condiments: mayonnaise, dairy-based salad dressings	Condiments: oil-based salad dressings; ketchup, mustard, canned sauces and gravies

During the Low-S diet intervention, participants will be advised to completely avoid foods high in SAAs, strictly eating foods included on the "Low-Sulfur Foods" list. During the High-S intervention, participants will be instructed to eat foods strictly from the "High-Sulfur Foods" list. Participants will record time and location of food intake and any deviations from the prescribed diet in the provided study log. Participants will have access to a registered dietitian throughout their participation in the study to assist with any issues regarding the trial or the diet.

3.3 Dietary Intake Assessment

Dietary intake data will be collected before, during, and after the intervention period to estimate usual total energy intake and macro- and micronutrient composition. Two methods of dietary assessment will be used: food frequency questionnaires (FFQs) and 3-day diet records. The National Cancer Institute's Diet History Questionnaire III (DHQ III) is a freely available web-based FFQ. A web-based version of the DHQ III will be completed by participants at their baseline clinic visit (Day 0), in which they will be asked about their usual dietary intake over the past year.

Participants will be instructed to record their daily intake of foods and beverages during each 7-day intervention period. Throughout the intervention, participants will be instructed to maintain normal physical activity, sleep habits, and caffeine consumption (coffee and tea). A registered dietitian will be in contact with participants throughout the interventions to monitor compliance with the High-S and Low-S diets. Three-day diet records will be completed by participants prior to the intervention start date, once during each intervention, and twice during the washout period,

and one week after the conclusion of the second intervention diet to determine independent, ad libitum dietary habits. A registered dietitian trained and certified in the University of Minnesota's Nutrient Data System for Research (NDSR) dietary interviewing and assessment protocols will instruct participants on methods for accurate quantification and recording of dietary intake. DHQ III data will be analyzed with Diet*Calc software developed by the National Cancer Institute. Analysis of 3-day diet records will be performed using NDSR data output.

3.3.1 Tolerability and Feasibility

There are many difficulties associated with examining the effects of dietary components on health, and subject compliance is of major importance to the outcome of any dietary trial. It is extremely difficult to control for subject compliance completely; however, daily diet records during each intervention and 3-day diet records in each standard diet phase, coupled with frequent contact by study staff, will improve participant adherence.

Participants will be provided with a study log at the baseline clinic visit (Day 0). Data recorded in the study log will be reviewed by study investigators at each contact with participants and will be recorded for data analysis. Participants will be instructed to record the following during each day of the study period:

- Adverse events, with specific attention paid to gastrointestinal symptoms including bloating, gas, abdominal pain, constipation, and loose stools
- New medications and/or any medication use – dose and frequency of administration
- Time of bowel movements and stool consistency
- Physical activity

Since the intention of this project is to generate pilot data for a longer randomized trial, approximating compliance for a prolonged period of time (≥ 3 months) is necessary for the design of future interventions. A questionnaire will be developed to assess participants' input on tolerability and feasibility for each dietary intervention.

3.4 Sample Collection

3.4.1 Fecal Samples

Fecal samples will be collected to assess gut microbial composition (via 16S sequencing) and to evaluate concentrations of fecal gases related to sulfur metabolism (via GC). Participants will collect samples in disposable commode specimen containers placed under the toilet seat prior to a bowel movement. Samples collected from the center of the specimen will be used for DNA sequence analysis. Larger collection tubes will be provided for intact stool samples for fecal headspace analysis and dry weight determination. The remainder of fecal specimens collected on-site will be transferred into sterile containers. Specimens will be put in the freezer until transfer to the investigator's lab. Full samples will be collected at designated study visits. Participants will also be asked to collect intermittent samples in collection tubes throughout the study. These will be collected on days (±2 days) -4, -2, 3, 5, 9, 11, 16, 18, 23, 25, 30, and 32. These samples will be stored in the participant's freezer and collected at each study visit. Participants will also record all bowel movements in their study log.

3.4.2 Urine Samples

24-hour urine collections will take place per PI discretion at baseline (Day 0; Day 0±2 days), at the end of the first diet intervention (Day 7; Day 5±2 days), at the end of the washout period (Day 21; Day 19±2 days), and at the end of the second diet intervention (Day 28; Day 26±2 days). Participants will collect all urine for a 24-hour period in a collection bottle(s), which will be stored in the refrigerator. Participants will be asked to note the exact time they begin and complete their urine collection. The entire 24 hour sample (all urine containers) will be returned after completion of the 24-hour period. Samples collected will be used for urine sulfate analysis.

3.4.3 Blood Samples

Blood (10 mL) will be collected per PI discretion in the clinic at visits 1, 2, 3, 4, 5, and 6 in Vacutainer Blood Collection Tubes. Blood for clinical laboratory assessment will be collected into tubes containing lithium heparin as anticoagulant. The tubes will be inverted carefully 3-5 times to mix blood and anticoagulant, and kept at room temperature until centrifugation, which will be done within 15 minutes of collection. Blood for measurement of TMAO and metabolites will be collected into a gel-barrier tube and allowed to clot for 30 minutes. Centrifugation of all samples will be carried out for 15 minutes at 1400 RCF (relative centrifugal force) at refrigerated temperature. The top layer (serum or plasma) will be collected and frozen at -80^{o}C.

3.5 Gas Concentration Measurement

Gases produced by fecal samples will be analyzed by GC. Approximately 10g of fecal material will be transferred to a gas-tight septum jar and incubated at 37^{o}C for 2 hours. After incubation, 5mL gas samples will be removed with a syringe and injected into 10ml headspace vials, pre-purged with nitrogen gas. H_2S and CH_3SH concentration will be measured using a gas chromatograph outfitted with a sulfur chemiluminescence detector (GC-SCD). CH_4 concentration will be measured using a gas chromatograph with a flame ionization detector (GC-FID) and CO_2/H_2 is quantified using a gas chromatograph equipped with a thermo-conductivity detector (GC-TCD). Calibration gases will be used as standards for all quantifications. All measurements will be normalized to mL per gram (dry weight) fecal material. Dry weight is calculated by taking a known mass of wet fecal material, baking it at 95^{o}C under vacuum for ≥ 16 hours, weighing the dried fecal material and calculating the ratio of dry weight / wet weight.

3.6 16S Sequencing

Fecal bacteria composition will be characterized using high throughput 16S rRNA gene sequence analysis.

3.6.1 DNA Extraction from Fecal Samples

DNA will be extracted from ~0.25g aliquots of fecal samples using a PowerSoil DNA Extraction Kit (Mo Bio Laboratories, Carlsbad, CA) performed according the manufacturer's instructions. Approximately 100 ng of DNA will be used for PCR amplification of the 16S rRNA gene in samples.

3.6.2 Amplification of 16S rRNA Gene and Sequencing

The V5+V6 hypervariable regions of the 16s rRNA gene will be amplified using primers specific for that region. Amplicons obtained from individual samples will be pooled in equimolar ratios

prior to sequencing. Paired end sequencing will be performed using the Illumina MiSeq (Illumina Inc, San Diego, CA) platform at the University of Minnesota Genomics Center (UMGC). Sequence data will be analyzed using the MOTHUR program[30] for taxonomic assignment and alpha/beta diversity. Sequence datasets will be compared by principle component analysis.

3.7 Fecal Bile Acid Analysis

Bile acids will be detected and quantified using liquid chromatography – mass spectrometry (LC-MS). Bile acids will be extracted from fecal material by sonication in acetonitrile, with the resulting extract injected directly into the LC-MS. All quantifications will be based on a C13 labeled internal standard and standard curves generated from known concentrations of each bile acid.

The investigator's lab will store all of the stool samples as well as the urine and blood samples collected for metabolomics. This laboratory is located at:

Wallin Medical Biosciences Building
University of Minnesota
2101 6th Street SE
Minneapolis, MN 55414

3.8 Treatment Assignment

This is a randomized, open-label crossover study. Each participant will receive both dietary interventions. The sequence in which participants receive the interventions will be determined through computer-generated random number assignment prepared by the study statistician.

3.9 Withdrawal

3.9.1 Reasons for Withdrawal

A study participant will be discontinued from the study for:

- Completion of the study;
- Request by participant to terminate participation;
- Requirement for prohibited concomitant medication or treatment;
- Unable to comply with requirements of the protocol;
- Lost to follow-up;
- At the request of the institutional review board (IRB);
- The participant's well-being, based on the opinion of the investigator.

Participants who are discontinued from further study agent/interventions will be followed for safety until completion of the normal visit schedule. Participants will be contacted by phone within one week after withdrawal, and again after one month.

3.9.2 Handling of Withdrawal

Participants will be encouraged to complete the study; however, they may voluntarily withdraw at any time. The sponsor-investigator will provide a written explanation of the reason for

withdrawal in a source document and the reason will be recorded on a case report form. Participants will be asked for permission to continue scheduled evaluations, and complete an end-of-study evaluation. Medical care that may be required for management of adverse events in the course of the study will be charged to the medical insurance of the participant.

3.10 Study Termination

If the sponsor-investigator or appropriate regulatory officials discover conditions arising during the study that indicate that the study should be halted, this action may be taken after appropriate consultation between the sponsor-investigator, study statistician, and independent medical monitor. Conditions that may warrant termination of the study include, but are not limited to, the following:

- The discovery of an unexpected, serious, or unacceptable risk to the participants enrolled in the study,
- A decision on the part of the sponsor-investigator to suspend or discontinue testing or evaluation of the intervention.

4 STUDY PROCEDURES AND EVALUATION SCHEDULE

4.1 Clinical/Laboratory Evaluations and Study Schedule

Consent will be obtained before any clinical evaluations are performed. There is one screening visit and 6 visits planned over the approximately 1 month study duration for each subject. The study will last until every evaluable subject has completed the Safety Follow-Up Visit. (See Schedule of Events in the Appendices). Of note, Vital signs and collection of blood samples will be completed based on PI discretion, except for the Screening and Safety Follow-up Visit.

4.2 Initial Screening Visit

The study will be explained to potential participants qualified from initial recruitment. Consent will be obtained at this time that will go over all the procedures and studies involved. During this visit the medical history will be collected and inclusion/exclusion criteria will be thoroughly reviewed. The following procedures will occur after the consent form is signed:

- Thorough review of the study, including involved procedures
- Medical history, including medications over the previous 12 months, and demographics
- Vital signs (heart rate, blood pressure, respiration rate)
- Height and weight measurement
- Review of methods used to record and measure food consumption during the study, to be conducted by dietitian.
- Three-day diet record is initiated following this visit, to be completed prior to visit 1 (baseline).
- Urine collection kit provided, to be completed prior to visit 1
- Link provided for web-based NCI Diet History Questionnaire III (DHQ III) to assess the baseline diet of subjects prior to entry into the study

4.3 Visit 1 - Baseline (Day 0)

The following procedures are conducted during this visit:

- Measurement of vital signs
- Weight measurement

- Collection of blood samples for clinical labs and research measurements
- Fecal sample collection
- 24-hour urine sample content collection
- Dietitian consult and review of 3-day diet record
- Assignment of first intervention diet, to begin immediately after conclusion of visit
- Receive instructions on completing adverse events diary card
- Review of adverse events and concomitant medications

4.4 Visit 2 (Day 7 ± 2 days; Final day of first diet intervention)

- Vital signs
- Weight
- Collection of blood samples for clinical labs
- Collection of stool sample for research measurements
- Review of 3-day diet record with dietitian
- Review of adverse events and concomitant medications
- 24-hour urine sample content collection
- Urine collection kit provided, to be completed prior to visit 3
- Review of adverse events and concomitant medications

4.5 Visit 3 (Day 14 ± 2 days)

- Vital signs
- Weight
- Collection of blood samples for clinical labs and research measurements
- Collection of stool sample for research measurements
- 24-hour urine sample content collection
- Review of 3-day diet record with dietitian
- Review of adverse events and concomitant medications

4.6 Visit 4 (Day 21 ± 2 days; First day of second diet intervention)

- Vital signs
- Weight
- Collection of blood samples for clinical labs
- Collection of stool sample for research measurements
- Assignment of second intervention diet, to begin immediately after conclusion of visit
- Review of 3-day diet record with dietitian
- Review of adverse events and concomitant medications
- Urine collection kit provided, to be completed prior to visit 5

4.7 Visit 5 (Day 28 ± 2 days; Final day of second diet intervention)

- Vital signs
- Weight
- Collection of blood samples for clinical labs and research measurements
- Collection of stool sample for research measurements
- 24-hour urine sample content collection

- Review of 3-day diet record with dietitian
- Review of adverse events and concomitant medications

4.8 Visit 6 (Day 35 ± 2 days; Follow-up visit)

- Vital signs
- Weight
- Collection of blood samples for clinical labs and research measurements
- Collection of stool sample for research measurements
- 24-hour urine sample content collection
- Review of 3-day diet record with dietitian
- Review of adverse events and concomitant medications

4.9 Unscheduled Visits

If at any time an unscheduled visit, including phone calls, should occur throughout the duration of the study, the following procedures will be completed:

- Adverse Events
- Concomitant medications
- Ask for any concerns about study procedures
- Physical examination and review of medical history as needed
- Order any lab tests as clinically indicated

4.10 Participant Compensation

Participants can receive up to a total of $150 for participation in the study; $25 paid for the completion of each study visit (a total of 6 study visits). Payment will be received in the form of gift cards.

4.11 Potential Risks

4.11.1 Risk of gastrointestinal symptoms

Changes in dietary intake can trigger gastrointestinal symptoms. However, these effects are typically minor in nature and occur in a small percentage of healthy volunteers.

4.12 Known Potential Benefits

IBD is a very complex condition and dietary intervention is only one component of a treatment and prevention plan. This study will generate information necessary to carry out a follow-up study specifically designed for patients with UC. We hope it will constitute an important step toward understanding the mechanisms of this disorder and contributions of diet and the gut microbiota to its pathophysiology. The potential benefits, therefore, are primarily societal in terms of enhancing understanding and ultimately finding better ways to treat this condition.

4.13 Risk/Benefit Ratio

Overall the risk of participating in this study is small. The benefit is primarily societal.

5 DATA SAFETY AND MONITORING PLAN

5.1 Definitions

Adverse Event (AE)

An adverse event (AE) is any symptom, sign, illness or experience that develops or worsens in severity during the course of the study. Intercurrent illnesses or injuries will be regarded as adverse events. Abnormal results of study procedures are considered to be AEs if the abnormality:

- Results in study withdrawal
- Is associated with a serious adverse event (SAE)
- Is associated with clinical signs or symptoms
- Leads to additional treatment or to further diagnostic tests
- Is considered by the Investigator to be of clinical significance.

Adverse Reaction
An adverse reaction is any adverse event caused by the investigational agent. Adverse reactions are a subset of suspected adverse reactions.

Suspected Adverse Reaction
A suspected adverse reaction is an adverse event for which there is a reasonable possibility that the investigational agent caused the adverse event.

Serious Adverse Event (SAE)
A serious adverse event (SAE) is any adverse event that is:

- Fatal
- Life-threatening
- Requires or prolongs a hospital stay
- Results in persistent or significant disability or incapacity
- A congenital anomaly or birth defect

Important medical events are events that may not be immediately life-threatening, but are clearly of major clinical significance and may be SAEs. They may jeopardize the subject, and may require intervention to prevent one or the other serious outcomes noted above.

Hospitalization
Hospitalization shall include any initial admission (even if less than 24 hours) to a healthcare facility as a result of a precipitating clinical adverse event; to include transfer within the hospital to an intensive care unit. Hospitalization or prolongation of hospitalization in the absence of a precipitating, clinical adverse event (e.g., for a preexisting condition not associated with a new adverse event or with a worsening of the preexisting condition; admission for a protocol-specified procedure) is not, in itself, a serious adverse effect.

Expected Adverse Event
Expected adverse events are those that are known to be associated with or have the potential to arise as a consequence of participation in the study.

Unexpected Adverse Event

An adverse event or suspected adverse reaction is considered unexpected if it is not listed in the Protocol at the specificity or severity that has been observed.

Unanticipated Problems Involving Risk to Subjects or Others (UPIRTSO)
An adverse event that in the opinion of the Principal Investigator is unexpected and related to the investigational agent.

Assessment of Severity
The sponsor-investigator will make an assessment of severity for each AE and SAE reported during the study. The assessment will be based on the sponsor-investigator's clinical judgment. The severity of each AE recorded will be assigned a severity based on Common Terminology Criteria for Adverse Events (CTCAE) grading.

Assessment of Causality
The sponsor-investigator will estimate the relationship between the investigational agent and the occurrence of each AE or SAE by using his best clinical judgment. Other elements, such as the history of the underlying disease, concomitant therapy, other risk factors, and the temporal relationship of the event to administration of the investigational agent, will be considered and investigated.

An SAE may be recorded when the sponsor-investigator has minimal information to include in the initial report. The sponsor-investigator may change his opinion of the causality in light of follow-up information, with subsequent amendment of the SAE report.

Categories	*Definition*
Definitely related	This relationship suggests that a definite causal relationship exists between the administration of the investigational agent and the AE, and other conditions (concurrent illness, progression/expression of disease state, or concurrent medication reaction) do not appear to explain the event.
Probably related	This relationship suggests that a reasonable temporal sequence of the event with investigational agent administration exists and, based upon the known or previously reported adverse reactions, or judgment based on the investigator's clinical experience, the association of the event with the investigational agent seems likely.
Possibly related	This relationship suggests that treatment with the investigational agent may have caused or contributed to the AE (i.e., the event follows a reasonable temporal sequence from the time of investigational agent administration and/or follows a known response pattern to the investigational agent but could also have been produced by other factors.)
Not related	This relationship suggests that there is no association between the investigational agent and the reported event.

5.2 Recording of Adverse Events

At each contact with the subject, the investigator will seek information on adverse events by specific questioning, reviewing the adverse events diary card, and, as appropriate, by examination. Information on all AEs will be recorded immediately in the source document and the appropriate AE module of the case report form (CRF). All clearly related signs, symptoms, and abnormal diagnostic procedures results will be recorded in the source document.

All AEs occurring during the study period will be recorded. The clinical course of each event will be followed until resolution, stabilization, or until it has been determined that the study treatment or participation is not the cause. AEs still unresolved at the conclusion of the study will be followed up on for one month. SAEs still ongoing at the end of the study period will be followed up to determine the final outcome. Any SAE that occurs after the study period and is

considered to be possibly related to the study treatment or study participation will be recorded and reported immediately.

Expected adverse events include:

- Events associated with venipunctures.
  - Discomfort and slight bruising.
- Events associated with dietary modification.
  - Changes in bowel habits, including diarrhea and/or constipation

Possible, although unlikely, adverse events that may be encountered include:

- Events associated with venipunctures.
  - Bruising
  - Bleeding
  - Lightheadedness, fainting
  - Infection at the venipuncture site
  - Nausea
  - Anxiety
  - Swelling at the venipuncture site

5.3 Adverse Event Management

If an adverse event occurs that requires clinical management, the subject will be evaluated at an unscheduled study visit with a physical exam. Tests and treatments that may be clinically indicated will be ordered.

5.4 Reporting of Serious Adverse Events

5.4.1 IRB Notification by Sponsor Investigator

Reports of all serious adverse events (including follow-up information) must be submitted to the IRB within the reporting timeline requirements, if the SAE falls under the UPIRTSO guidelines. Copies of each report and documentation of IRB notification and receipt will be kept in the Clinical Investigator's binder.

5.4.2 UPIRTSO Events

Upon first learning of a UPIRTSO event, investigators are required to submit a report of the applicable event(s) to the IRB within the required reporting timeline.

5.5 Temporary Interruption of Intervention in an Individual Subject

If a participant does not or cannot comply with the assigned diet intervention, further eligibility will be assessed by the Principal Investigator. A participant may be able to continue in the study if the interruption is temporary (1-2 days).

6 STATISTICAL CONSIDERATIONS

6.1 Sample Size Considerations

This feasibility study plans to enroll 17 participants who will be assigned to receive two dietary interventions in a random order. We anticipate that 2 subjects may drop out from the study for various reasons, including inability to comply with the study visit schedule or new medical

problems unrelated to the study, e.g., use of antibiotics for an infection. The intention of this study is to generate data which will inform the sample size necessary for a larger trial, so power calculations have not been performed.

We will compare primary, secondary, and exploratory outcome measures within each intervention (Day 0 vs. Day 7) and between the two interventions (High-S vs. Low-S) using paired *t*-tests.

6.2 Randomization

This is a randomized, open-label crossover study. Each participant will receive both dietary interventions. The sequence in which participants receive the interventions will be determined through computer-generated random number assignment prepared by the study statistician.

6.3 Blinding

The clinical co-investigators will remain blinded to the intervention. Due to the nature of the intervention, the PI and other personnel giving dietary instructions, and participants will not be blinded. All investigators will be blinded to the randomization schedule and measured endpoints.

6.4 Planned Interim Analyses

Independent monitoring of the clinical study for compliance will be conducted periodically (at a minimum of every 6 months) by qualified staff of the University of Minnesota in accordance with the established monitoring plan. No interim analyses are planned for this study.

6.5 Safety Review

To minimize risk, cumulative safety data will be reviewed by the sponsor-investigator and an independent medical monitor. Medical monitoring will be initiated by the occurrence of adverse events as they arise. This safety monitoring will include careful assessment and appropriate reporting of AEs as noted above. Medical monitoring will include a regular assessment of the number and type of SAEs. Study enrollment and dosing will be stopped and an ad hoc review will be performed if any of the following occurs:

1. Occurrence of a life-threatening allergic/hypersensitivity reaction (anaphylaxis), manifested by bronchospasm with or without urticaria or angioedema;
2. An overall pattern of symptomatic, clinical, or laboratory events that the sponsor-investigator, scientific liaisons, regulatory affairs manager and statistician consider associated with the intervention and that may appear minor in terms of individual events but that, collectively, may represent a serious potential concern for safety;
3. Any SAE that is possibly, probably, or definitely related to the intervention;
4. Two or more subjects experience the same CTCAE Grade 3 or higher adverse event that is possibly, probably, or definitely related to study product administration.

7 DATA AND RECORD KEEPING

7.1 Data Capture Methods

Data for this study will include safety, laboratory and outcome measures. Clinical data (including AEs and concomitant medications) and clinical laboratory data will be managed by the principal

investigator, co-investigators, study coordinator, and biostatistician. Data will be entered into REDCap, an internet data entry system provided by the Clinical and Translational Science Institute. The data system includes password protection and internal quality checks to identify data that appear inconsistent, incomplete, or inaccurate. Clinical data will be entered directly from the source documents.

7.2 Source Documents

A source document is defined as the location where study-related data are initially recorded. Source documents for this study will include hard copy paper and/or electronic forms, laboratory printouts, and medical records onto or into which data will first be recorded.

7.3 Study Records Retention

Per University of Minnesota policy all documents concerning the use of human subjects in research will be maintained for at least 3 years from completion of IRB-related work and at least 6 years for HIPAA. No record will be destroyed without the written consent of the sponsor-investigator.

The sponsor-investigator will permit authorized representatives of the University of Minnesota and regulatory agencies to examine (and when required by applicable law, copy) clinical records for the purposes of clinical site monitoring, quality assurance reviews, audits, and evaluation of the study safety and progress.

7.4 Protocol Deviations

A protocol deviation is any noncompliance with the clinical trial protocol. The noncompliance may be either on the part of the subject, the investigator, or the study site staff. As a result of deviations, corrective actions are to be developed by the site and implemented promptly.

When a deviation from the protocol is necessary for an individual subject, the Investigator must complete a description of the deviation from the protocol and justification on the Protocol Deviation Form. It will not be considered a protocol deviation if a subject is unable to provide a stool sample at any visit that requires a stool sample.

8 QUALITY CONTROL AND QUALITY ASSURANCER

8.1 Dietary Intake

Agreement of participants' short- and long-term dietary intake will be assessed through comparison between NDSR and DHQ databases at baseline and Visit 6.

9 ETHICS/PROTECTION OF HUMAN SUBJECTS

9.1 Ethical Standard

The investigator(s) will ensure that this study is conducted in conformity with the principles of The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Participants of Research of The National Commission for the Protection of Human Participants of Biomedical and Behavioral Research (April 18, 1979) and codified in 45 CFR 46, 21 CFR 312,

and/or ICH E6; 62 Federal Regulations 25691 (1997). The University of Minnesota holds a current federal-wide assurance (FWA) issued by OHRP for federally funded research.

9.2 Institutional Review Board

The Human Participants Protection Program at the University of Minnesota will be asked to review and approve this protocol, associated consent documents, and recruitment materials. Approval of any amendments to the protocol or consent materials will also be requested before they are implemented.

10 INFORMED CONSENT PROCESS

The informed consent process will be initiated before a volunteer agrees to participate in the study and will continue throughout the individual's study participation. The participant will sign the informed consent document before any procedures are undertaken for the study. A copy of the signed informed consent document will be given to the participant for his/her records. The consent will explain that participants may withdraw consent at any time throughout the course of the trial. Extensive explanation and discussion of risks and possible benefits of this investigation will be provided to the participants in understandable language. Adequate time will be provided to ensure that the participant has time to consider and discuss participation in the protocol.

The consent form will describe, in detail, the study interventions / procedures and risks / benefits associated with participation in the study. The rights and welfare of the participants will be protected by emphasizing that their access to and the quality of medical care will not be adversely affected if they decline to participate in this study.

10.1 Participant Confidentiality

Participant confidentiality is strictly held in trust by the participating investigator, his staff, and their agents. This confidentiality includes documentation, investigation data, participant's clinical information, and all other information generated during participation in the study.

No information concerning the study or the data generated from the study will be released to any unauthorized third party without prior written approval of the sponsor and the participant.

The study monitor or other authorized representatives of governmental regulatory agencies may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records (office, clinic, or hospital) and pharmacy records for the participants in this study. The clinical study site will permit access to such records.

10.2 Principal Investigator Responsibility when Participant Withdraws or is Discontinued

If a participant terminates the study early, and is willing, the tests and procedures that would occur at all forthcoming study visits will be completed.

10.3 Future Use of Stored Specimens

Blood samples collected during the study will be sent to and processed by hospital laboratory personnel at University of Minnesota Medical Center, Fairview. Blood will be discarded.

11 PUBLICATION POLICY

The International Committee of Medical Journal Editors (ICMJE) member journals have adopted a trials-registration policy as a condition for publication. This policy requires that all clinical trials be registered in a public trials registry such as ClinicalTrials.gov, which is sponsored by the National Library of Medicine. This protocol will be registered on ClinicalTrials.gov.

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13 Appendix 1. Schedule of Events

Procedures	**Screening**	**Baseline**	**Visit 2**	**Visit 3**	**Visit 4**	**Visit 5**	**Visit 6**
		0	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 35**
Obtain consent	X						
Medical history	X						
Vitals*	X	X	X	X	X	X	X
Height	X						
Weight	X	X	X	X	X	X	X
Clinical labs[1*]		X	X	X	X	X	X
Fasting blood for TMAO and metabolites*		X		X		X	X
Provide stool sample**		X	X	X	X	X	X
24-hour urine collection*		X	X	X		X	X
Diet assignment		X			X		
Dietary History Questionnaire		X					
Provide food diary	X	X	X	X	X	X	X
Review food diary		X	X	X	X	X	X
Adverse events/Concomitant medications		X	X	X	X	X	X

[1]Clinical labs include: fasting lipid panel and C-reactive protein (CRP)

*Optional based on PI discretion

**Intermittent stool samples (in frozen collection tubes) will be dropped off at each respective visit following collection. Samples will be collected on days (±2 days) -4, -2 3, 5, 9, 11, 16, 18, 23, 25, 30, and 32.