Template Version 02.02.17

PROTOCOL

Administrative Information

1 Title

Xanthohumol Microbiome and Signature in Crohn's Disease (XMaS in Crohn's) Trial

2a Trial registration

ClinicalTrials.gov Identifier: We have developed a preliminary record for the proposed clinical trial on ClinicalTrials.gov, which we will finalize upon IRB and OCRA approval.

2b See attached table

3 Protocol version

August 27, 2021 Version 1.13

4 Funding

National Center for Complementary and Integrative Health (NCCIH)
Grant Number: 1R01AT010271-01
Grant Number: R01 AT010271-02S1
Hopsteiner, Inc.
XanthoFlav Pure product
Metagenics, Inc.
Xanthohumol and placebo capsules

5a Roles and responsibilities

Principal Investigator: Ryan Bradley ND, MPH, National University of Natural Medicine
Clinical Investigator: Ryan Bradley ND, MPH, National University of Natural Medicine
Clinical Investigator: Jennifer Ryan, ND, MS, National University of Natural Medicine
Post-doctoral Investigator and Clinical Investigator: Blake Langley, ND, MS, LAc National University of Natural Medicine
Co-Investigator: John Phipps PhD, National University of Natural Medicine
Co-Investigator: Joseph Aslan, PhD, Oregon Health and Science University
Post-doctoral Investigator: Brenna Bray PhD, National University of Natural Medicine
Study Coordinator: Emily Stack, National University of Natural Medicine
Study Coordinator: Lita Buttolph, National University of Natural Medicine
Study Coordinator: Anders Gundersen, National University of Natural Medicine
Biostatistician: Doug Hanes PhD, National University of Natural Medicine

5b Trial Sponsor: National Center for Complementary and Integrative Health (NCCIH)
Program Officer: Kim, Hye-Sook, PhD, 301-827-6910, hye-sook.kim@nih.gov

5c Role of Study Sponsors

Office of Clinical and Regulatory Affairs (OCRA), serves as a resource for the planning, implementation, and oversight of clinical research funded by NCCIH. Prior to initiating clinical projects, OCRA staff facilitates finalizing study designs, analytic plans, and clinical study documents. After studies are initiated, OCRA staff facilitates ongoing communication between study teams and NCCIH staff, and regularly obtains reports from active clinical studies regarding participant accrual, as well as data and safety monitoring. Materials and data will remain widely available to the research community in accordance to NIH Principles and Guidelines.

5d Clinical trial site (NUNM) Principal Investigator is Dr. Ryan Bradley. Dr. Bradley's team will consist of three co-Investigators, Dr. Jennifer Ryan, ND, MS a clinical research-trained, licensed physician who has previously recruited participants with CD at NUNM; Dr. John Phipps, PhD a clinical research-trained toxicologist; Dr. Blake Langley, ND, MS, LAc a licensed physician and acupuncturist, who participated as a Clinical Investigator in the phase I portion of this trial; and Dr. Joseph Aslan, PhD, a biochemist and molecular biologist at OHSU. In addition, Dr. Brenna Bray, PhD, a post-doctoral fellow trained as a biomedical and neuro-scientist, will also assist with the study. The study team also includes Ms. Emily Stack, a certified phlebotomist and seasoned clinical trials coordinator, Dr. Lita Buttolph, PhD, DSOM, an experienced clinical trials coordinator and licensed acupuncturist, and Mr. Anders Gundersen, MS, an experienced clinical trials coordinator.

Dr. Bradley will be the responsible party for all clinical trial operations and regulatory requirements. He will oversee the day-to-day operations of the clinical trial. Dr. Phipps will oversee Ms. Stack, Dr. Buttolph, and Mr. Gundersen in the day-to-day operations of the trial, including collection, processing and storage of all biospecimens. Dr. Phipps will also assist with all reporting requirements to the sponsor, IRB, and FDA.

Dr. Bradley will coordinate efforts with the OSU and PNNL co-PIs to finalize all protocol details, sample collection requirements, storage requirements and shipping details, prior to initiation of participant recruitment.

Introduction

6a Background and rationale

The Centers for Disease Control and Prevention estimates 1.3% of U.S. adults (3 million) are diagnosed with Inflammatory Bowel Disease (IBD), and the prevalence of the disease is increasing.[1] Crohn's Disease (CD) is the most prevalent form of IBD, characterized by predominantly small bowel inflammation (although up to 50% may also have colitis), including inflammation in the ileum. CD causes significant symptoms including diarrhea, abdominal pain, bleeding, micronutrient malabsorption, iron deficiency anemia, increased risk of colon cancer, and unintended weight loss. Medical therapy for CD is problematic and no treatment has consistently delivered success in the treatment of CD due to the episodic nature and variability in abnormality.

Current treatments are costly, and associated with adverse outcomes including increased risk of lymphoma and infections.[2]

The pathophysiology of CD is multi-factorial including potential genetic, environmental and microbial factors that may serve as potential triggers, and perpetuators, of the disease process. One consequence of inflammation in the ileum so common in CD is reductions in the absorption of bile acids (BA).[3] BAs are important agonists to the nuclear farnesoid X receptor (FXR) in the intestines, as well as in the liver. The FXR receptor is known to regulate intestinal permeability, as well as innate immunity, including production of pro-inflammatory cytokines IL-1**β**, IL-2, IL-6, tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-**γ**).[4,5] FXR agonists have been tested in animal models and early clinical trials and show promise for altering inflammatory signaling pathways.[4,6] Definitive clinical trials in humans with CD have not been performed.

Xanthohumol (XN) is a flavonoid constituent of hops (*Humulus lupulus*), with antioxidant and anti-inflammatory activities. XN acts as a prebiotic for intestinal microflora, and along with its bacterial metabolites, alters the gut microbiome.[7] *In silico* models suggest XN and its metabolites are receptor agonists for the FXR receptor[8], and our preliminary *in vitro* data suggest XN activates FXR-regulated genes (SREBP1, G6Pase, and PEPCK) at low concentrations, suggesting XN acts as a pharmacologic agonist of FXR (unpublished data). Additional preliminary data from our collaborators demonstrates the bioavailability of XN, including increased bioavailability of XN when combined in a rice protein matrix.[9] Thus XN has the potential to be a novel therapeutic in CD, by acting as a FXR agonist, reducing inflammation, increasing BA re-absorption, and reducing gut permeability.

XN is well represented in the diet as it is commonly found in beer. XN is not currently FDA approved for the treatment, cure, prevention or mitigation of disease. XN is likely safe when used orally in amounts commonly found in foods. Hops and hops oil have Generally Recognized as Safe (GRAS, Flavoring Extract Manufacturers' Association records 2578 and 2580 respectively) status in the US.[10] The Stevens lab at Oregon State University has completed the first human pharmacokinetics study of XN in which 48 study participants ingested a single dose of XN of 20, 60, or 180 mg (16 men and women per dose group).[11] No treatment-related adverse effects were reported in this study (ClinicalTrials.gov Identifier: NCT01367431). Risks associated with any dietary supplement include gastrointestinal symptoms such as gas, bloating, digestive upset and change in bowel movement frequency or consistency.

Xanthohumol has been extensively studied in animals. Gerhauser and colleagues studied the safety of XN administered orally to Sprague-Dawley rats for four weeks.[12] At a dose of 1000 mg XN/kg body weight, authors reported a reduction of liver weight, but found no macroscopic or histopathologic changes of the liver, kidney, lung, heart, stomach and spleen. The mammary glands of treated rats appeared less developed compared to the control animals. In a two-generation study of the teratogenic effects of XN, Gerhauser's group found no differences in development of SD rats treated lifelong with a daily dose of 100 mg/kg body weight.

In another safety study of XN, mice exposed to XN for four weeks via the drinking water at a concentration of 0.5 mM (=177 mg XN per liter) showed no changes in blood counts and in the integrity of the bone marrow, liver, exocrine pancreas, kidneys, muscles, thyroid, ovaries or the surrenal cortex.[13] We estimate that the XN exposure in this study is equivalent to a human dose of 265 mg per day, which is much higher than the highest dose we propose in our clinical trial, i.e., 24 mg/day. In Zucker fa/fa rats study, there was no difference in uterus weight (n=6, females) or testes weight (n=6, males) between dose groups and the zero dose control group (n=6, females/males).[14] Animals were given daily oral doses of 1.86 mg/kg, 5.64 mg/kg, or 16.9 mg/kg body weight for six weeks. These doses correspond to 20 mg, 60 mg and 180 mg XN in a 64 kg human by allometric interspecies scaling.

The recently completed Phase 1 of XN at NUNM resulted no statistically significant differences in any mean laboratory values between XN vs. placebo groups at Closeout/Week 8 (all p values>0.10). At Closeout/Week 8, 2 laboratory parameters met halting criteria in Group B, including 1 mild anemia and 1 AST elevation. Notably, all lab abnormalities present at Closeout/Week 8 were resolved upon re-testing. There were no statistically significant differences in the frequency of any laboratory abnormalities between groups, at any timepoint in the trial (p-values all>0.2). No participants discontinued their study intervention due to AEs. There were no "Grade 3/Severe" or FDA Serious AEs reported in either group. There were no apparent trends in incident AEs in either group over the time course of the trial. Additionally, investigators previously evaluated the effect of 12.5 mg per day of xanthohumol within a formula in adults with IBD, including Crohn's disease. Complete blood count and comprehensive metabolic panel data indicated no adverse changes; mean values were within normal laboratory reference ranges at all time points. All adverse events were non-serious and most were related to preexisting symptoms.[15]

There are no known toxicities for human subjects taking oral supplements containing XN. The general population is primarily exposed to XN through consumption of beer. A major brand beer contains about 0.5-1 mg prenylflavonoids (mainly XN and iso-XN) per liter and a bitter microbrew ale may contain as much as 4 mg of total prenylflavonoids.[16] In Europe, a beer/beverage, containing 4 mg of XN per liter, has been on the market for several years without adverse effects having been reported by the consumers. Based on the available human data and the results from the above cited animal studies, we expect that the study participants will not develop adverse effects at the proposed dosage regimens during the 8-week exposure period.

We aim to extend our preliminary findings related to potential application of XN as a therapeutic by testing its effects in a sample of human adults with CD. We will recruit 32 participants with CD, and randomize them to either 24 mg of XN plus rice protein, or a rice protein placebo for 8 weeks. The study drug will be identical to the preparation used in the Phase 1 trial that preceded this Phase 2 protocol, i.e., XN will be administered as 24 mg of a 99+% pure raw material provided by Hopsteiner USA (NY) combined with 288 mg of rice protein (California Natural Products, Lathrop, CA), 109.3 mg microcrystalline cellulose (Stauber USA, Fullerton, CA), 4.3 mg Aerosil®

200 fumed silica (Vivion Inc., San Carlos, CA), and 4.3 mg magnesium stearate (Nitika Pharmaceutical Specialties, Maharashtra, India), and formulated into a capsule by Metagenics, Inc. (Gig Harbor, WA). XN has been combined with a rice protein carrier due to improved bioavailability.[9]

Our *primary working hypothesis* is that XN at the given dose will be safe and tolerable in adults with active CD. We will test this hypothesis by collecting blood samples at screening, baseline, and every two weeks to monitor changes in laboratory values including liver enzymes, renal function markers, and hematological markers. We anticipate very low frequency of treatment discontinuation due to adverse effects.

Our *working hypotheses* of Aims 2, 3, and the Exploratory Aim are that XN and its microbiota-derived metabolites may reduce inflammation and reduce biomarkers of barrier permeability in the inflamed gut. In order to test this hypothesis, we will collect blood, urine, and stool samples at baseline and every two weeks to monitor gut microbiome profiles and measure XN metabolite profiles. We will also measure fecal metabolome (including bile acid profiles) and lipidome profiles, as well as established fecal and plasma indicators of IBD severity, including fecal calprotectin and C-reactive protein. Because the actions of XN and its metabolites target specific abnormalities in CD, we hypothesize that the proposed studies will deliver a mechanism-based biological signature of XN exposure and its effect on CD mitigation.

6b Choice of Comparator: Control participants will receive placebo capsules containing 288 mg of rice protein (California Natural Products, Lathrop, CA), 109.3 mg microcrystalline cellulose (Stauber USA, Fullerton, CA), 4.3 mg Aerosil® 200 fumed silica (Vivion Inc., San Carlos, CA), and 4.3 mg magnesium stearate (Nitika Pharmaceutical Specialties, Maharashtra, India), and formulated into a capsule by Metagenics, Inc. (Gig Harbor, WA).

7 Objectives

A methodological limitation of assessing the efficacy of CD treatments is that the gut microbiome of CD subjects is highly personalized in terms of state and dynamics. By correlating time-resolved fluctuations in the gut microbiome of CD subjects with their concomitant changes in the profile of XN metabolites, BA profiles, and other markers of gut microbiome-associated biochemical activity as well as with established surrogate end-points of the disease, we *hypothesize:*

1) XN at the given dose will be safe and tolerable in adults with active CD;
2) XN will improve biomarkers and improve the Crohn's Disease Activity Index (CDAI) an activity index of CD activity;
3) We can identify a biological signature(s) of XN through its metabolic byproducts in blood, urine, and stool as well as its effect on bile acid reabsorption; and
4) Specific biological signature(s) of XN exposure will uniquely contribute to changes in CD-associated disease activity indices.

To test these hypotheses and generate a conceptual model, we define four *Specific Aims*:

Aim 1. Determine the clinical safety and tolerability of xanthohumol in adults diagnosed with CD.
We will determine the safety and tolerability of xanthohumol in adults diagnosed with CD over 8 weeks in a Phase 2 trial. The primary hypothesis is that in the XN arm, the number of participants with an adverse event or abnormal lab value will not be more than 50% higher than the number in the placebo group.

Aim 2. Assess changes in levels of inflammation and symptom severity related to CD.
Our primary outcome measure for this secondary objective will include fecal calprotectin and circulating levels of inflammatory cytokines as biomarkers of systemic inflammation. The sample size provides >80% power, at a threshold of alpha=0.05, for the detection of a 25% difference in marker changes in response to XN between the healthy adults in the Phase 1 trial and participants in this trial. Additionally, we will measure changes in Crohn's Disease Activity Index (CDAI) score for participants at each clinical visit to first determine eligibility and, second, compare end results to baseline for any change in score. Finally, results will be compared to the previous Phase 1 trial in healthy adults.

Aim 3. Collect samples and measure XN metabolism, gut permeability and impact on the microbiota.
We will assess XN metabolism, gut permeability and impact on the microbiota. Thus, our outcome measures for this objective include microbiota profiles, XN metabolites and bile acid metabolites.

Aim 4. (Exploratory) Generate a computational, theoretical model for assessing and understanding of the interactions between XN and the gut microbiome, and how the identified interactions may mitigate CD.
We will measure biomarkers of gut inflammation and gut integrity, and composition of intestinal microbiome species through various methods including measurement of circulating plasma endotoxin, CD14, intestinal fatty acid-binding protein, lipopolysaccharide-binding protein; lipidomic profiling; and TLR2/TLR4 activation.

8 Trial design

The design will be a triple-masked, randomized, placebo-controlled trial. Upon confirmation of eligibility and documentation of informed consent, those participants still interested and eligible to participate will be randomized to either: XN (24 mg/day plus 288 mg of rice protein plus 117.9 mg of excipients) or placebo (rice protein 288 mg/day plus117.9 mg of excipients) taken by mouth once daily for 8 weeks.

Methods

Participants, interventions and outcomes

9 Study Setting

The clinical study will take place at an academic medical center, the National University of Natural Medicine's Helfgott Research Institute in Portland, OR, USA.

10 Eligibility criteria

Crohn's Disease (n=32):

Inclusion criteria

- Adults 21-70 years of age
- Active Crohn's disease not in remission based on a CDAI score >150
- Willing to take isolated Xanthohumol as a dietary supplement for 8 weeks
- Willing to have blood drawn bi-weekly and fast for 10-12 hours before blood draws
- Willing and able to collect bi-weekly stool samples at home
- Willing and able to collect a 24-hour urine sample before each study visit
- Willing to receive a brief abdominal exam including palpation at each study visit
- Able to speak, read and understand English
- Must be able to provide written informed consent
- Non-smokers (including tobacco and Cannabis products, combusted or vaporized)
- For individuals of child-bearing potential, willingness to use an IUD, oral contraceptives or two other concurrent forms of birth control (e.g., 2 of the following categories: condoms, spermicide-containing gels, films or sponges, and/or vaginal rings) to prevent pregnancy while enrolled

Exclusion criteria

- Highly variable dosing of anti-inflammatory medications (dose changes more than 1x per week)
- Currently or recent (within last 14 days) taking any dietary supplements containing xanthohumol, flavonoids, or other known "anti-inflammatories" including: curcumin, turmeric, fenugreek, hops, rosemary, ginger, white willow, devil's claw, fish oil (doses>1 g/day), or quercetin. Candidates will be given the option to "wash out" for 14 days and re-contact the study team.
- Consumption of more than 1 beer per day.
- Currently receiving intravenous nutrition support therapy (or within the last 14 days)
- Currently taking anti-coagulant or anti-platelet prescription medications (or they were taken within the last 14 days)
- Currently taking antibiotic, antiparasitic, or antifungal medications orally or intravenously (or they were taken within the last 14 days)
- Initiation of or changes to supplements or medications within 14 days prior to screening.
- Initiation of or changes to an exercise regimen within 14 days prior to screening.
- Initiation of or changes to a food plan within 14 days prior to screening.

- Current involvement or within 14 days prior to screening of a significant diet or weight loss program (such as NutriSystem, Jenny Craig, Atkin's or other low-carb diet programs) or very low-calorie liquid diet programs (such as Optifast, Medifast, and/or HMR)
- Hospitalization (for any reason other than a scheduled medical procedure) within 3 months prior to screening
- Gastrointestinal surgery within 3 months prior to screening
- Malignancy within the last 5 years (with the exception of basal cell carcinoma, squamous cell carcinoma, and/or carcinoma in situ of the cervix)
- Women who are lactating, pregnant or planning pregnancy within the next four months
- Typical intake of more than 2 alcohol-containing beverages per day, more than 14 per week, or more than 4 in any single day within the past 14 days.
- Smoking tobacco or use of nicotine products Use of illicit drugs/substances (such as but not limited to cocaine, phencyclidine (PCP), and methamphetamine) within 14 days of screening
- Use of inhaled or ingested *Cannabis* products, including CBD
- Currently participating in another interventional research study, or participated in another interventional research study within 14 days of screening
- Do not have a medical provider available to consult with and/or manage medications for their CD as needed
- Received a vaccination within 14 days prior to clinical screening.

Candidates will be given the option to stabilize or eliminate alcohol intake, allowable dietary supplement and medication intake, exercise, and diet to the requirement stated above for 14 days, and/or eliminate cannabis use and illicit substance and may re-contact the study team after.

11a Interventions

The intervention is XN (24 mg/day plus 288 mg of rice protein plus 117.9 mg of excipients) or placebo (rice protein 288 mg/day plus 117.9 mg of excipients) taken by mouth once daily for 8 weeks.

11b Withdraw/Discontinuation

A participant may elect to withdraw from the trial at any time they choose, if they experience AEs or for no stated reason. However, participants will be actively withdrawn if they demonstrate new onset, moderately severe gastrointestinal AEs, attributable to the intervention. Participants will also be withdrawn if they have any *critical* value return on routine clinical laboratory measures of safety, including changes in blood counts, liver function tests and/or renal function tests.

Safety measures include self-reported adverse events, % new abnormal and mean changes in specified clinical laboratory parameters including red blood cell count, ALT, AST, GGT, bilirubin, alkaline phosphatase eGFR, and BUN: creatinine ratio.

11c Adherence

Data on adherence to the treatment protocol will be assessed qualitatively and quantitatively by asking participants if they have missed any doses, and why doses were missed if relevant, during each bi-weekly visit, plus by pill count at the week 4 and week 8 clinical visits by the Study Coordinator. A limited supply of product, adequate for 5 weeks, will be dispensed at the Randomization visit and at the Week 4 clinical visit.

11d Permitted concomitant care or intervention during trial

- Anti-inflammatory medications including oral 5-aminosalicylates (e.g., sulfasalazine, mesalamine)
- Immunomodulators (e.g. azathioprine, 6-mercaptopurine, methotrexate)
- Use of over-the-counter medications as indicated on the label for less than 2 days per week
- Multi-vitamin and/mineral
- Dietary supplements except as detailed below

Allowed emergency intervention during trial

- Glucocorticoids (e.g., prednisone, budesonide)

Prohibited concomitant care during trial

- Dietary supplements containing xanthohumol
- Dietary supplements containing flavonoids
- Other known "anti-inflammatories" including curcumin, turmeric, fenugreek, hops, rosemary, ginger, white willow, devil's claw, fish oil (doses>1 g/day), or quercetin.
- Anti-coagulant or anti-platelet prescription medications
- Antibiotic, antiparasitic, or antifungal medications orally or intravenously

12 Outcomes

Table 1

OUTCOME	MEASURE	DESCRIPTION
Primary: Safety & Tolerability		
Clinical Safety/Cumulative Toxicology	Liver (AST, ALT, GGT) and renal function (eGFR, BUN:Cr) tests plus hematology (CBC)	Routine clinical toxicology measures
Secondary: Clinical Status		
Gut inflammation	Fecal calprotectin	Enzyme-linked immunosorbent assay

Inflammation	Plasma pro-inflammatory cytokines (TNF-α, IL-1β, IL-2, IL-6, IL-8, IL-10, and IL-12p70) and C-reactive protein	Flow cytometry-based multiplex assay C-reactive protein from Quest Labs
Composite Symptoms	Crohn's Disease Activity Index (CDAI)	Symptom severity will be determined using the CDAI
Tertiary: Mechanistic Pathways		
Metabolic byproducts of XN	XN and XN metabolites in blood, urine and stool	Ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry
Bile acid re-absorption	Bile acid concentrations in blood and feces	Ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry
Exploratory/Other		
Gut integrity and enterocyte damage	Plasma concentrations of soluble CD14, intestinal fatty acid-binding protein, and lipopolysaccharide-binding protein by ELISA	ELISA will be used to measure soluble markers of microbial translocation from the intestine
	Plasma endotoxin by Limulus amebocyte lysate assay	Plasma endotoxin by Limulus amebocyte lysate assay will measure the microbial translocation
	TLR2, TLR4 activation by transfected HEK293 reporter cells	HEK293 cells pre-transfected with TLR4 and TLR2
Lipid metabolism	Lipidomics profiling	Liquid chromatography UPLC-QTOF

Primary Outcome: Clinical Safety/Cumulative Toxicology
Standard clinical Complete Blood Counts and toxicology panels including liver and renal function tests will be measured at each bi-weekly clinical visit. Values will be interpreted based on: new abnormal value, change since baseline, new emergency or alert values, and/or the emergence of new diagnostic concerns. All participants' labs will be reviewed by a clinical investigator, and any abnormal lab values meeting halting criteria will be recorded in the database.

Should a participant experience a severe or emergent event: 1. The AE and its severity will be documented; 2. The participant's medical provider will be notified and consulted; and, if unavailable, 3. The DSMB Chair (Dr. Robert Martindale, MD, PhD; Division Chief of General Surgery, OHSU) will be consulted if the study team is unable to reach the participant's physicians.

Secondary Outcome: Fecal Calprotectin
Levels of calprotectin, a marker of CD, will be determined in stool samples using a commercial ELISA kit.

Secondary Outcome: Inflammatory markers
To characterize inflammatory status, circulating levels of several pro-inflammatory cytokines (TNF-α, IL-1β, IL-2, IL-6, IL-8, IL-10, and IL-12p70) will be measured in plasma samples. The method uses nanoparticles coated with capture antibodies for the selected targets, and fluorescently labeled detection antibodies to create complexes with specific fluorescent wavelengths. After incubation with the sample, the resultant suspension is analyzed by flow-cytometry, allowing quantitative determination of the concentration of the target molecules. Compared with a conventional ELISA technique, this approach requires less sample material, allowing for smaller blood draws from participants. C-reactive protein will also be measured at Quest Diagnostic Laboratory via routine methods; this biomarker will be measured with the routine safety labs measured at each bi-weekly visit.

Secondary Outcome: Change(s) in patient-reported symptoms
The Crohn's Disease Activity Index (CDAI) is a questionnaire developed to assess symptom severity in CD.[17] It is primarily a research tool and has been validated for that purpose. The index consists of eight factors, summed after adjustment with a weighting factor (described in detail in section 18a). Consensus values have been established for clinically meaningful response to an intervention, and for symptom remission.

Tertiary Outcome: XN and XN metabolites
Identification and quantification of XN and the XN metabolites isoxanthohumol; 8-prenylnaringenin; O-desmethylxanthohumol; dihydro-desmethylxanthohumol; α, β-dihydroxanthohumol will be achieved through ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF). This method separates chemical species using liquid chromatography, followed by identification of the resulting isolated fractions on the basis of mass-to-charge ratio.

Tertiary Outcome: Bile acids
Identification and quantification of bile acids will be achieved through liquid chromatography-mass spectroscopy. This method separates chemical species using liquid chromatography, followed by identification of the resulting isolated fractions on the basis of mass-to-charge ratio.

Exploratory Outcome: Gut integrity and enterocyte damage
The intestinal barrier function will be assessed by measuring plasma concentrations of soluble CD14, intestinal fatty acid-binding protein, and lipopolysaccharide-binding protein with commercial ELISA kits.

Exploratory Outcome: Circulating Endotoxin

As an additional measure of gut integrity, levels of lipopolysaccharide (endotoxin), a component of Gram-negative bacteria, will be determined with the Limulus amoebocyte assay. The version of the assay used in this study will be a microplate-based chromogenic, endpoint assay.

Exploratory Outcome: Bacterial Translocation

Bacterial translocation out of the gut lumen will be measured by TLR2 and TLR4 activation. The proposed reporter-cell assay uses cultured HEK293 cells that have been transfected with a chimeric protein consisting of the extracellular portion of the TLR2 or TLR4 receptor, conjugated with a truncated placental alkaline phosphatase enzyme. Ligand binding to the receptor initiates the production of a colored metabolite which can be quantified in a spectrophotometer.

Exploratory Outcome: Lipidomics

As an additional component of the biological signature of XN in CD, lipidomic analysis will be performed by UPLC-QTOF. These data will be combined with the results of XN metabolite and bile acid profiles in modeling the biological signature.

13 Participant timeline:

Table 2

	Phone Screening	**Clinical Screening**	**Baseline Clinical Visit - Week 0**	**Randomization Visit – within 48hrs after Baseline**	**Bi-weekly Clinical Visits (Weeks 2,6)**	**Midpoint Clinical Visit- Week 4**	**Trial Closeout- Week 8**
Oral waiver of consent	X						
Initial eligibility screening	X						
Written informed consent		X	X				
W9 Form		X					
Written release of medical records				X			
Demographics		X					
Randomization				X			
Assessment of Symptoms/AEs			X		X	X	X
PROMIS-29			X		X	X	X
Disease Activity scale (CDAI)	X	X	X		X	X	X

Adherence assessment (verbal recall)					X	X	X
Adherence assessment (pill count)						X	X
Labs: CBC, & Toxicology Panel (Chem 13), CRP		X	X		X	X	X
Blood collection		X	X		X	X	X
Urine collection				X	X	X	X
Stool collection				X	X	X	X
XN or placebo dispensed				X		X	
Stipend ordered						X	X
Participant Discharged							X

13b Missed visits

In the case of an inability to conduct study visits as intended the study team will move forward with the following procedures according to the circumstantial requirements.

1. Participants refuse or are unable to come in for a Week 2 or Week 6 (Interim) visit:
   Study Procedures:
   a. Request the participant drop off stool and urine samples outside the building in the sample dropbox or ship the samples to the study site using a sample shipment kit sent by the study team
   b. Participants will complete surveys and AE questionnaires electronically via REDCap and over the phone with Coordinators. CDAI will be filled out to the greatest extent possible virtually.
   c. Study team will assess the participants willingness to go to a remote Quest facility for a blood draw
2. Participants refuse or are unable to come in for a Week 4 (Midpoint) visit:
   Study Procedures:
   a. Follow number 1 study procedures with the addition of:
      i. Request the participant keep all unused product and drop it off in the outside sample drop box or ship the samples to the study site using a shipment kit sent by the study team

ii. Participant will be mailed their 2nd and last allotment of study product

3. Participants refuse or are unable to come in for a Week 8 (Closeout) visit:
   Study Procedures:
   a. Follow number 2 study procedures with the exception of:
      i. Study product dispensation
      ii. Incentive to go to a Quest draw site will be increased to $200.00 as necessary
4. Provisional research institute closure:
   Study Procedures:
   a. Participants will be informed to continue taking their assigned study product until they reach their Week 8 time point
   b. Participants will not attend any interim visits
   c. New study product will be mailed to participants if the week 4 visit is attained and institute remains closed
   d. If institute closure persists for scheduled participant closeout visit, participant will be informed to discontinue study product at the end of Week 8, final surveys and AE interviews will be completed remotely. CDAI will be filled out to the greatest extent possible virtually. Incentives to go to a Quest draw site will be increased to $200.00.

14 Sample Size

A sample size of 12 per group provides 80% power to detect a 50% difference in proportions of participants between groups by chi2 test experiencing a laboratory abnormality or adverse event at a significance threshold of α=0.05. In addition, based on our preliminary trial data for change in TNF-α, and applying a 2-sided t-test of means, n=24 allows for n=12 per arm and provides >90% power, at a significance threshold α=0.05, to detect a mean change of -0.5 =/- 0.25 pg/ml in XN-treated participants with CD, based on an estimated mean change of 0.1 +/- 0.25 pg/ml from placebo. Will we randomize up to 32 participants to allow for attrition up to 25% [resulting in a final sample of at least n=24/arm.

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15 Recruitment

Participants will be recruited from the region via posted fliers and the NUNM clinic via posted fliers and IRB-approved mailings to current patients. Members of the study team will present information about study participation to CD support and community groups and set out flyers for attendees to pick up. In addition, we will use Craigslist and social media (i.e. Facebook and Instagram) as well as the NUNM weekly newsletter. Candidates from the community would

simply respond to a dedicated study telephone line or email, and express their interest, and are then contacted by a Study Coordinator.

We will also recruit via targeted clinical recruitment in the EPIC electronic health records system by working with an internal EPIC specialist to create a list of candidate participants based on ICD-9/10 codes from NUNM electronic health records. Similarly, our OHSU collaborator will use the Data Warehouse resource of the Oregon Clinical and Translational Research Institute (OCTRI) to identify potential candidates from OHSU's EPIC database. Upon creation of a candidate list, IRB-approved study materials, i.e., an invitation letter and a copy of the flyer, will be mailed to them. They will not be re-contacted if they do not respond to an initial invitation. We will present at medical conferences and have flyers available for conference participants. Additional retention strategies include the use of stipends dispensed for clinical screening ($50) and each clinical visit completed ($190 each) following enrollment for up to a total of $1,000. Additional compensation ($200) will be provided in the event that closure of campus (e.g. due to COVID-19) requires the participant to go to Quest Diagnostics' phlebotomy clinic for their required blood draw. Participants may be reimbursed for travel and/or lodging expenses if travel cost is a barrier to participation.

Inclusion Enrollment

Planned

Table 3

Racial Categories	Ethnic Categories				
	Not Hispanic or Latino		Hispanic or Latino		Total
	Female	Male	Female	Male	
American Indian/Alaska Native	0	0	0	0	0
Asian	1	1	0	0	2
Native Hawaiian or Other Pacific Islander	0	0	0	0	0
Black or African American	2	2	1	1	6
White	11	10	2	1	24
Total	14	13	3	2	32

This study will enroll biologically male and female participants and therefore will include women. We aim to recruit a sample that is 50% male and 50% female in order to maximize the generalizability of our findings to both men and women. Furthermore, Inflammatory Bowel Disease (IBD) is more common in females (relative risk: 1.53 compared to males) and therefore we are confident women will be well represented.

This study will recruit generally from the Oregon and Washington region. Based on the 2016 census, the ethnic distribution of the Portland, OR area is: Caucasian 70%, Hispanic/Latino 10%, Asian 8%, Black 6%, Pacific Islander 1%, Native American 0%, and Other 0%. Participants who come from beyond the immediate area may be compensated for travel and lodging expenses if it is a limiting factor.

Because Black/African Americans are under-represented in the Portland metro area, we will specifically target recruitment strategies to reach Black/African Americans in the region.

No NIH-defined children will be enrolled.

Assignment of interventions (for controlled trials)
Allocation
16a Sequence generation
Randomization will occur in up to 8 blocks of 4 based on biological sex. Initial randomization series will be generated using readily available random sequence generators designed to do so such as Randomization.com, which allows for block randomization.

16 b Allocation Concealment mechanism
Envelopes will be opaque and signed across the seal. The randomization "code" will be kept in a password protected folder that will only be accessible by NUNM's Information Technology staff.

16c Implementation
Masking will be accomplished by: using trained staff not involved in trial operations to: 1. Produce the "code" (e.g., X2=XN and Y1=Placebo); 2. Label XN and placebo bottles generically as "X2" and "Y1"; 3. Generating the blocked randomization sequence using Randomization.com; 4. Create 32 sequentially-numbered, sealed envelopes that contain the random group assignments. Upon confirmation of eligibility and interest, Study Coordinators involved with trial operations will then open the next sequential envelope in the stack at the time of randomization, the envelope number will be recorded, and allocation disclosed to both participant and coordinator at that time as simply "X2" or "Y1", indicating which bottles will be dispensed.

17a Blinding (masking)
Triple-masked without PI, Study Coordinator, or Participant knowing participants' allocation assignment.

17b Un-blinding/masking
Un-blinding is permissible if the Data Safety Monitoring Board requires it upon review and in the case of a Serious Adverse Event (SAE).

Data collection, management, and analysis

18a Data collection methods

1. *Adverse Events Report Form*

Adverse event (AE) monitoring will occur using a standardized multi-system AE questionnaire based on the National Cancer Institute's Common Terminology Criteria for Adverse Events v4.0 (2009). The Multi-System Adverse Event questionnaire will be administered at each bi-weekly study visit. However, an AE can also be reported to the study staff at any time and collected via a "Standardized Adverse Events Questionnaire: Spontaneous Reporting" form. The "Spontaneous Reporting" form captures the source of the AE report, the severity/grade of the report, any clinical actions taken, and the current state of the AE. Following a spontaneous report, the study team, sometimes in coordination with a participant's physician, will develop a response plan, which is then also documented.

2. Disease Activity Scale: CDAI

Disease activity will be determined using the CDAI. Relevant components of the CDAI will be collected initially at the Screening visit. Abdominal examination will be performed by a clinical investigator at each visit as outlined in Table 2. If an abdominal exam is unable to be performed, the value for this variable may be gleaned from medical records or derived from participant-reported medical history.

The index consists of eight factors, each summed after adjustment with a weighting factor. The components of the CDAI and weighting factors are the following:

Crohn's Disease Activity Index (CDAI) Questionnaire	
Clinical or laboratory variable	**Weighting factor**
Number of liquid or soft stools each day for seven days	x2
Abdominal pain (graded from 0-3 on severity) each day for seven days	x5
General wellbeing, subjectively assessed from 0 (well) to 4 (terrible) each day for seven days	x7
Presence of complications in past seven days	x20
Taking Lomotil or opiates for diarrhea	x30
Presence of an abdominal mass (0 as none, 2 as questionable, 5 as definite)	x10
Percentage deviation from hematocrit of <47% in men and <42% in women	x6
Percentage deviation from standard weight	x1

Specific complications that influence score include any of the following, weighted equally: 1) arthritis or arthralgia; 2) iritis or uveitis; 3) erythema nodosum, pyoderma gangrenosum, or aphthous stomatitis; 4) anal fissure, fistula, or abscess; 5) other fistula; 6) temperature over 100F; all within in the last 7 days.

Remission of Crohn's disease is defined as CDAI below 150 with severe disease defined as a value of greater than 450.[17,18]

3. Health-Related Quality of Life

PROMIS-29 is a short-form questionnaire designed to standardize collection of patient-reported outcomes across studies.[19] It addresses the following domains: physical function, anxiety, depression, fatigue, sleep disturbance, ability to participate in social roles and activities, and pain.

4. Lab Tests

- Clinical toxicology parameters (CBC, AST, ALT, eGFR, BUN, Cr)
- XN metabolites: ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry
- Plasma endotoxin concentrations: Limulus amebocyte lysate assay
- Plasma markers (concentrations of soluble CD14, intestinal fatty acid-binding protein, and lipopolysaccharide-binding protein): ELISA
- Plasma Toll-like receptor (TLR2 and TLR4) activators: HEK293 reporter cells
- Circulating systemic levels of pro-inflammatory cytokines in the plasma: flow cytometry-based multiplex assay

5. Medical Records Review

The release of medical records will be signed at the Randomization visit once the participant has been assigned to a group. Medical records review will attempt to describe the participant sample's CD characteristics in better detail such that subgroups may be evaluated separately in *post*-hoc analyses. Medical records will be assessed for:

- Location of lesion (ileal, proximal, central, and/or distal colon)
- History of abdominal mass in instances where an abdominal exam is unable to be performed
- As possible with the available data on diagnostic reports, we will compute the Crohn's Disease Endoscopic Index of Severity (CDEIS) as proximally as possible to participant enrollment:

Crohn's Disease Endoscopic Index of Severity (CDEIS)	
Endoscopic Finding	**Scoring**
Deep ulcerations	0 if absent or 12 if present
Superficial ulcerations	0 if absent or 6 if present
Length of ulcerated mucosa (0-10 cm)	According to length in cm
Length of diseased mucosa (0-10 cm)	According to length in cm

18b Retention

Retention of study participants will be achieved through proactive and timely communication, and through detailed attention to professionalism and safety. Additional retention strategies include the

use of stipends dispensed for clinical screening ($50) and each clinical visit completed ($190 each) following enrollment for up to a total of $1000.00.

19 Data Management

The investigators are responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported. All source documents will be completed in a neat, legible manner to ensure accurate interpretation of data. The investigators will maintain adequate case histories of study subjects, including accurate case report forms (CRFs), and source documentation.

Each participant will be assigned a unique alpha-numeric ID upon screening. All blood and urine aliquot tubes, stool collection vials and associated paperwork will be marked using the unique ID to protect patient confidentiality. All data resulting from study visits will be collected on standardized case report forms (CRFs). Data will be transferred from CRFs to a secure REDCap database for data management. REDCap is a secure, web-based application that supports electronic data capture for research studies. REDCap features include: 1) intuitive data entry features; 2) audit trails for tracking data manipulation and export; 3) user-based privileges that support HIPAA compliance; 4) seamless data export to common statistical packages; and 5) procedures for importing data from external sources. The REDCap database will be accessible only by the study team. Data will be destroyed 3 years after completion of the study. Computer files will be deleted from the server and paper files will be destroyed using a professional document shredding service.

All completed forms will be kept in locked files to which only project personnel have access. These files will also be in locked rooms. Data from paper forms will be manually entered into REDCap and kept as electronic files. Electronic files will be password protected with access given to study personnel only.

Staff training will be standardized and will follow a data entry SOP. The REDCap database has been designed to be identical to the case report forms (CRF). The PI will review CRFs each week to evaluate completeness and compliance with the protocol and will conduct random data reviews monthly to assure accuracy of electronically stored data on REDCap in relation to CRFs. The PI will be ultimately responsible for data quality control issues.

20a Statistical methods

Analysis of laboratory toxicology measures:

Laboratory measures (including red blood cell count, white blood cell count, hematocrit (%), hemoglobin, mean corpuscular volume, AST, ALT, creatinine-estimated glomerular filtration rate (eGFR), blood urea nitrogen (BUN), and creatinine) will be analyzed by first assessing descriptive statistics including mean, median and standard deviation. All distributions will be tested for skew and transformed (e.g., natural log transformed) as needed to reduce the influence of skew in subsequent analyses. All parameters will be evaluated by formally comparing means from baseline

to each time point by unpaired, 2-sided t-tests, as well as for trends over time by applying linear mixed ANOVA models with time point as a repeated factor.

As an element of the evaluation for safety of the intervention, the % laboratory newly abnormal (for values within the clinically normal range at baseline) per clinical laboratory reference ranges for each lab parameter will be reported at each 2-week time point and the % compared between XN and placebo groups by Fisher's exact test.

Assessment for changes in the distribution of each lab parameter will be performed by presenting means and confidence intervals (CI) for the change in each group, as well as the mean CI for the difference between groups; significance will be tested using 2-sided, unpaired t-tests (or, if inspection of distributions indicates strong non-normality, with a non-parametric Wilcoxon signed rank test) of the mean values for XN compared to placebo.

Assessment for overall *increases* or *decreases* in parameters of interest will be tested for linear trends of the means by linear mixed ANOVA with time point as a repeated factor, which may be suggestive of evidence for cumulative toxicity if trending in a clinical deleterious direction.

Analysis of inflammatory cytokines, gut permeability biomarkers, and endotoxemia:
Given the small sample size of these clinical trials, results related to changes in inflammatory cytokines (IL-1β, IL-2, IL-6, IL-8, IL-10, TNF-α, and IL-12p70), gut permeability biomarkers (CD14, intestinal fatty acid binding protein) and markers of endotoxemia (LPS, LPS-binding protein) are considered preliminary, and thus our principle assessment will be measurement of effect size as *Cohen's d* statistic between XN- and placebo-treated groups at each 2-week interval compared to baseline, with the primary comparison being between baseline and week 8. We will also perform formal statistical comparisons as unpaired, 2-sided t-tests (or, if inspection of distributions indicates strong non-normality, with a non-parametric Wilcoxon signed rank test) of the means at relevant time points. The following findings will be considered evidence of possible effects: 1. Clinically and statistically significant effects for changes in cytokines of interest and/or 2. Calculation of a Cohen's d value for effect size estimation greater than 0.5 (with larger d indicating a larger potential effect size).

Assessment for overall *increases* or *decreases* in parameters of interest will be tested for linear trends of the means by linear mixed ANOVA with time point as a repeated factor. Trends will be considered significant if $p<0.05$ for the model overall.

Analysis of XN, its metabolites, and BAs to determine a small molecule signature of longer-term oral treatment with XN:
We will identify and quantify XN and XN-derived metabolites in plasma, stool, and urine samples. Bile acid profiles will also be determined in the stool samples. The metabolite profile data will then be used for correlation with the time-resolved gut microbiome analysis. This approach will allow us to determine links between metabolite profiles and gut microbiome compositions.

Determining differential abundance between treatments: High-level exploratory analyses will be performed through metrics such as alpha and beta diversity and ordination techniques. Further, differential abundance analysis for 16S and metagenomics data will be conducted through negative binomial models, with specifications to deal with inflation of zero counts, such as those implemented in statistical R packages edgeR and DESeq2 or through a compositional Bayesian data analysis approach, such as the approach implemented in the R package ALDEx2.[20-22] Exploratory data analysis will first be done to evaluate which modeling technique is appropriate for the data generated based on assumptions made by each modeling approach. For MS-based omics data, we use a suite of robust statistical approaches for: 1) processing the data to identify outliers, and 2) identifying statistically different proteins and metabolites between comparative samples. As a first step to statistical testing, we use the Statistical Procedure for the Analysis of molecular abundance Normalization Strategies (SPANS) approach to simultaneously evaluate several MS-based omics data set normalization strategies to determine the most appropriate normalization factor in the context of the experimental design.[23-26] For individual data streams, there are several strategies for identifying molecular features that statistically classify patients, depending on the structure of the data and the phenotype data being used for classification. For the determination of statistically relevant features, if the features follow the standard normal distribution, then we utilize standard ANOVA methods; otherwise, a non-parametric version, such as Kruskal-Wallis or sum-rank test, can be utilized.[27]

Longitudinal regression modeling:

We will apply systems biology tools to quantify how an individual's inflammation state, as measured by calprotectin, changes over time in association with XN metabolite status and microbial taxa, their genes, gut metabolites, or BAs. To link the time dependence of inflammation to these various parameters, we will follow our prior work and construct compound Poisson generalized linear models (CPGLMs) that allow us to test for differences in temporal trends in the relative abundance of specific OTUs, genes, metabolites, or BAs as a function of various experimental parameters.[28] CPGLMs include a point mass at zero as well as a weighted mixture of Poisson and gamma distributions. These features enable accurate modeling of the sparse (zero-inflated) but otherwise continuous and non-normal data usually produced during microbiome and metabolome investigations.[28] CPGLMs also correctly account for multiple sources of variation due to different experimental parameters (e.g., XN metabolite status, inflammation state, time, and sex).[29] We will construct CPGLMs that model how the temporal variation of each OTU, gene, or metabolite (i.e., response variables) relates to these experimental parameters. We will adopt a stepwise approach to model construction and optimal models will be identified. Models will include interaction terms that consider co-dependencies among the experimental parameters. Random intercepts and time slopes for each individual will be included in the model to account for within-individual correlation of a response variable's abundance over time. Response variables that manifest as significantly different slopes as a function of XN metabolite status are those variables that putatively interact with XN metabolites. Related analyses of model slopes will resolve parameters that elicit dependencies with inflammation state, as well as parameters that vary

in accordance with the XN metabolite-inflammation interaction. We will also test models that include a lag effect between response variables and these parameters to resolve, for example, taxa whose temporal change in abundance predicts subsequent inflammation changes. We have extensive experience using GLMs and related techniques for the study of microbiome-phenotype associations.[20,22,30] Recognizing that the proposed CPGLMs are complex and may suffer from a lack of power, we may consider an alternative approach which avoids models that attempt to accommodate the data structure through zero-inflated distributional adjustments and instead consider a compositional data analysis approach, where the data is transformed and normalized using the Aitchison simplex, normalized through e.g. centered log ratio adjustment, and followed by fitting with traditional GLMs and accounting for other covariates and covariance structures. This approach would retain higher statistical power because there no longer would be an attempt to resolve the zero-inflation problem. This approach has been shown to perform better in terms of differential expression with small sample/replicate sizes.[31] An alternative, more well-powered approach to variable/feature selection would be to perform lasso or ridge regression via elastic net, which is known to operate well in small sample relative to large feature scenarios.

Time-resolved alterations of the gut microbiome by 16S rRNA gene sequencing and metagenomics analysis:

Microbiome data generation:
We will profile the structure and functional capacity of the microbiome using 16S rRNA and shotgun metagenomic DNA sequencing. Following our prior work, we will extract whole genomic DNA from each stool sample using a QIAGEN PowerFecal DNA kit.[26] We will then PCR amplify the V4V5 region of the 16S gene and sequence amplicons on an Illumina MiSeq at OSU's Center for Genome Research and Biocomputing.[32] We will produce 300 bp, paired-end sequences and target a sequence depth of 50,000 reads per sample. We will also generate metagenomes from the same DNA extracts using NexteraXT library preparation and the CGRB's Illumina HiSeq 3000 (150 bp paired-end sequences). We will target a sequence depth of 15 million reads per sample, which our prior work showed was more than sufficient for resolving associations between IBD and microbiome taxonomy and functional capacity.[28,33]

Microbiome data analysis:
We will analyze 16S sequences to profile gut microbiome structure. The DADA2 software pipeline will quality filter sequences, assemble contigs between paired ends, resolve unique sequence variants (i.e., OTUs), and taxonomically annotate these sequences using the RDP database.[34] The Phyloseq R package will quantify the alpha- and beta-diversity of microbiome samples.[35] Non-parametric tests (e.g., PERMANOVA) will measure differences in α- and β-diversity and β-dispersion across patient groups and over time. We have experience developing and applying methods for the analysis of microbiome data.[26,33,36-38] Metagenomic analysis will complement 16S assessments of taxonomy and quantify the functional capacity of microbiome samples. After quality controlling metagenomes using Shotcleaner, which follows Human Microbiome Project guidelines to trim adapters, filter low-quality sequences, and informatically subtract host genomic DNA from metagenomes, we will apply several strategies for the analysis of metagenomes.[39] First, we will assemble paired-end metagenomes using the Automatic Tool for Local Assembly Structures (ATLAS), an open-source tool for NGS data analysis developed in-house.[40] ATLAS transforms raw sequence data into functional and taxonomic data at the microbial population level and

provides genome-centric resolution by integrating genome binning. The full ATLAS protocol is developed and maintained as open-source software on GitHub and is implemented in Snakemake for modular, flexible, and reproducible workflows. Individual protein sequence databases will be created based on a combined metagenome that will serve as references for paired metaproteome analysis. To complement these assemblies and produce as comprehensive an assessment of microbiome structure and functional capacity as possible, we will also conduct analyses at the level of individual, unassembled reads. Specifically, we will analyze quality-controlled metagenomes using MetaPhlan2 to measure the abundance of specific microbial taxa in each metagenome.[41] We will also use ShotMAP to assign reads into KEGG Orthology groups based on homology.[33] Briefly, ShotMAP predicts coding sequences from unassembled metagenomic data using Prodigal and applies RAPsearch or DIAMOND to rapidly identify reads that show evidence of homology to KEGG Orthology groups. Our prior statistical simulations surrounding this technique highlight its accuracy and our prior applications of these tools resolved taxa and microbiome metabolic modules that predict immune activation in mouse models of IBD and flare-ups in IBD patients.[28,33]

20b Subgroup and/or adjusted analyses.

No subgroup analyses are planned. If significant differences in disease severity (CDAI-measured) become evident between XN- and placebo-treated groups, ANCOVA will be performed for all tests of mean differences (i.e., pro-inflammatory cytokine, gut permeability and endotoxemia-related parameters), adjusting for baseline CDAI score between groups.

20c Methods for imputation and/or consideration of protocol non-adherence.

To evaluate the statistical relevance of missing data, for example when the data is absent in the control group and present in the treatment group, we will utilize a G-test.[25] However, with multiple comparisons comes the problem of increased likelihood of a significant result by random chance. To counter this problem, we use either the Dunn-Sidak or Bonferroni correction to adjust p-values.[42] If the data have a linear relationship, then we use partial least squares discriminant analysis (PLS-DA) and rank the molecules by p-value in a pair-wise comparison to the control group.[43,44]

Monitoring

21a Data monitoring

A Data Safety and Monitoring Board (DSMB) independent of the study team will be created for the proposed trial and will consist of: a clinical investigator with IBD experience, a clinician with IBD practice experience, a biostatistician, and at least one other experienced clinical researcher/trialist. The DSMP will review all AE reports according to IRB policy, monthly reports of recruitment, retention and overall study progress; and attend quarterly meetings. Protocol amendments can be suggested by members of the independent monitoring committee at any time, and additional meetings of the DSMB can be called by any member at any time. The PI and study coordinator will also participate in quarterly meetings with the committee to provide additional information and/or clarify reports.

21b This study will be stopped prior to its completion if the intervention is associated with adverse effects that call into question the safety of the intervention, if any new information becomes

available during the trial that necessitates stopping the trial; or if other situations occur that might warrant stopping the trial. The ultimate decision to stop the trial may be made by the DSMB, NCCIH, and/or the FDA.

The trial will be halted if >25% of the total sample experience moderate or greater AEs or laboratory abnormalities requiring them to withdraw.

The DSMB will provide a final recommendation to the PI to halt the study or amend the study protocol, and will develop a response plan, in consultation with the NUNM IRB and the NCCIH Program Officer.

If immediate changes are required for participant safety, enrollment and other study activities will not continue until the modifications/amendment is approved by the IRB.

22 Harms

Adverse Event Monitoring and Reporting

Adverse event (AE) monitoring will occur using a standardized multi-system AE questionnaire based on the National Cancer Institute's Common Terminology Criteria for Adverse Events v4.0 (2009). The Multi-System Adverse Event questionnaire will be administered at each study visit. However, an AE can also be reported to the study staff at any time and collected via a "Standardized Adverse Events Questionnaire: Spontaneous Reporting" form. The "Spontaneous Reporting" form captures the source of the AE report, the severity/grade of the report, any clinical actions taken, and the current state of the AE. Following a spontaneous report, the study team, sometimes in coordination with a participant's physician, will develop a response plan, which is then also documented.

Serious AEs will be reported to the PI within the same business day; all other AEs within 48 hours. Serious AES, and any AE that in the discretion of the Clinical Investigator may require a modification to medications, will be reported to primary care doctors via phone contact between the Clinical Investigator and the PCP within the same business day, if possible, or the following. All other AEs will be reported to the PCPs in a summary report from each clinical visit within 1 week of scheduled visits. Adverse events will be dealt with directly, discussed with co-investigators and advisors, and adjudicated by the DSMB as related, possibly related, or unrelated to the study procedures.

In accordance with NUNM IRB AE reporting guidelines, SAEs (grades 3-5) will be reported to the NUNM IRB within 24 hours of PI awareness. Unanticipated AEs of any grade that are both related to research, result in a change to risk/benefit, or require protocol and/or consent modifications will be reported to the NUNM IRB within 10 working days hours of PI awareness. All Anticipated AEs regardless whether they are classified as severe or related to the study intervention will be reported at the time of annual continuing review.

All adverse events will be recorded in the Adverse Event Log.

The DSMB will meet quarterly to conduct continuous review of data and participant safety. The DSMB will review quarterly safety reports, and adjudicate adverse events as required. The DSMB will recommend amendments to the protocol, changes in study procedures, changes to the data collection plan or study forms, or study termination due to safety or other issues as needed.

The PI will have final responsibility for all reporting to the NUNM IRB, DSMB and/or NCCIH PO as required.

Clinical Management Plan/ Plans for Ensuring Necessary Medical Intervention

Participant safety is of the greatest concern in clinical trials of experimental compounds, even when naturally derived and available in food. Of particular concern is any threat to the clinical stability of a person with CD. For this reason, with their consent, we will notify participant's primary care and/or medical specialist of their patient's participation in the trial. We will inform their medical providers should it become necessary to communicate with them about any laboratory abnormalities, critical values and/or AEs should they be discovered at baseline or throughout the clinical trial.

We will maintain the ability to administer additional blood-based or stool-based testing for acute or viral infection as circumstances require.

Potential Risks to Participants

Risks associated with any dietary supplement include gastrointestinal symptoms such as gas, bloating, digestive upset and change in bowel movement frequency or consistency. Also, there is a chance of identifying a previously unknown allergy, and therefore a risk of rash, breathing difficulty and serious allergic reaction.

We will ask about symptoms at each study visit and any side effects will be recorded. We may ask the participant to discontinue the study product if we believe it would be in their best interest.

There will be six blood draws during this study. There is a small chance that the needle will cause some discomfort, bruising, redness or bleeding at the needle puncture site. Occasionally, individuals may feel lightheaded or faint during or immediately after a blood sample is collected. There is a rare risk of swelling around the vein or of infection.

We will do everything we can to keep records private, yet there is always a small risk of a breach of confidentiality of personal health information. These risks have been addressed and minimized as much as is possible by keeping data in locked cabinets and on password protected and encrypted computers, and by using ID numbers instead of names on all data collected.

There is a chance that some participants may confuse research study visits for routine clinical care.

23 Auditing

The NUNM IRB, DSMB, and NCCIH Program Officials will receive copies of all study monitoring/audit or inspection reports within 14 day of PI receipt.

Table 4		
Data type	**Frequency of review**	**Reviewer**
Subject accrual (including compliance with protocol enrollment criteria)	Weekly	PI
	Monthly	Study team
	Quarterly	Independent Monitors
Status of all enrolled subjects, as of date of reporting	Weekly	PI
	Quarterly	Independent Monitors
Data entry quality control checks on 15% of charts	Monthly	PI
Adherence data regarding study visits and intervention	Weekly	PI
	Monthly	Study team
	Quarterly	Independent Monitors
	Annually	NCCIH
SAEs (unexpected and related)	Per occurrence	PI, Independent Monitors NIH/NCCIH
SAEs (expected or unrelated)	Per occurrence	PI
	Annually	Independent Monitors, NIH/NCCIH
Unanticipated Problems	Weekly	PI
	Per Policy	IRB

Ethics and dissemination

24 Research ethics approval

The protocol for this trial will continue to undergo refinement until it's final approval by the IRBs of National University of Natural Medicine (NUNM).

25 Protocol amendments

All protocol amendments, other than minor administrative changes as defined by the NCCIH Guidance on Changes in Clinical Studies in Active Awards will be submitted in a prospective manner to NCCIH except when necessary to protect the safety, rights, or welfare of subjects. Prior to submission to NCCIH the proposed changes will be reviewed and approved by the Independent Monitoring Committee. IRB approval will not be sought until after NCCIH approval of the protocol amendment has been obtained.

26a Consent of assent

Upon initial determination of eligibility, candidates will be invited to an in person Clinical Screening Visit conducted by the Study Coordinator. Written informed consent will be the first activity of this visit. Candidates will have adequate time to review the written informed consent document (or have it read to them, according to their preference) which clearly indicates the voluntary nature of their participation and provides recourse should they feel their safety or privacy was violated in any way. Candidates will have an opportunity to ask any questions of the Coordinator or the site PI.

Should we identify hypertension (BP>140/90 mmHg) during the visits a participant will be provided with the Referral for Medical Management form. If candidates do not have a primary care provider, resources for local, free or inexpensive medical services will be provided to them.

26b Additional Consent for Re-Use of Samples

The informed consent form for full trial participation includes a statement of consent to re-use the biologic samples collected for these trials for subsequent analyses related to the Aims of the proposed trials, should they become necessary. Participants will have the autonomy to provide, or refuse, additional consent for this purpose. A small volume (<7 mL) of blood and urine will be collected and frozen at -70C for the purposes of potential future analyses.

27 Confidentiality

Throughout the study, measures to ensure the privacy of information on study participants will be maintained. All study investigators and staff are certified in Good Clinical Practices (GCPs), Human Subjects Research (HSR) and Responsible Conduct of Research (RCR) and have received training in HIPAA regulations. Participants and staff will be informed of the confidentiality of information and assured that data will be used only for statistical purposes and group analyses in which the individual cannot be identified. No data beyond what is stated in the Informed Consent will be sought without authorization from the participant. Information on illnesses and hospitalizations will not be sought from hospitals or doctors without a signed medical release from the subject. Conversely, no information on any individual will be released to anyone other than study personnel without a signed medical release from the participant, or where appropriate, the next of kin or a physician in case of a life-threatening emergency to the subject.

Each participant will be assigned a unique alpha-numeric ID upon screening. All blood and urine aliquot tubes, stool collection vials and associated paperwork will be marked using the unique ID to protect patient confidentiality. All data resulting from study visits will be collected on standardized case report forms (CRFs). Data will be transferred from CRFs to a secure REDCap database for data management. REDCap is a secure, web-based application that supports electronic data capture for research studies. REDCap features include: 1) intuitive data entry features; 2) audit trails for tracking data manipulation and export; 3) user-based privileges that support HIPAA compliance; 4) seamless data export to common statistical packages; and 5) procedures for importing data from external sources. The REDCap database will be accessible only by the study

team. Data will be destroyed 3 years after completion of the study. Computer files will be deleted from the server and paper files will be destroyed using a professional document shredding service.

All completed forms will be kept in locked files to which only project personnel have access. These files will also be in locked rooms. Data from paper forms will be manually entered into REDCap and kept as electronic files. Electronic files will be password protected with access given to study personnel only.

28 Declaration of interests

PIs Bradley and Stevens have received grant support and product donations from Metagenics, Inc. who will be encapsulating the finished product used in this trial. Neither Drs. Bradley or Stevens hold stock in Metagenics, Inc. or its parent company and have no direct financial ties to the outcome of the proposed clinical research.

29 Access to data

Only the PIs and biostatisticians will have access to study data. All investigators will be masked until the initial data analysis plan has been completed and then PIs will be unmasked as to group assignments. Upon creation of a linkage code document, that document will be physically stored in a signed, sealed envelope by the NUNM Director of Operations (Ms. Heather Schiffke) until it is unsealed at the completion of analyses. A copy of the linkage document will also be created electronically and maintained on the password protected computer, in a password protected file, by the Director of Operations.

30 Ancillary and post-trial care

Unanticipated problems will be recorded in the data collection system throughout the study. The PI will record all reportable events with start dates occurring any time after informed consent is obtained. If participants believe they have suffered an AE related to study procedures, they are welcome to contact us at any time and AEs will be adjudicated. At each study visit, the investigator will inquire about the occurrence of AE/SAEs since the last visit. Events will be followed for outcome information until resolution or stabilization.

31a Dissemination policy

All data produced by the project will be deposited into the appropriate public repository no later than the time of first publication describing the data.

We will use the UCSD Metabolomics Workbench (http://www.metabolomicsworkbench.org/) as a medium for sharing metabolomics datasets and results. We will make available our metabolomics data, including raw data, platform information and vendor software version, and associated analytical and biological metadata as well as the final data results matrices, consisting of known (identified) and/or unknown (unidentified) metabolites. In the case of known metabolites, we will provide the InChIKey and/or PubChem compound ID (if these are available). Other compound identifiers (e.g. KEGG ID, ChemSpider ID) will be translated to the

corresponding InChIKey and PubChem compound ID using the UC Davis Chemical Translation Service. In the case of unknown metabolites, we will provide a local identifier and other annotations such as measured m/z value, chromatographic parameters (LC retention times and/or GC retention indices), and tandem mass spectra to enable tracking of the unknown metabolite(s) across different experiments. The results matrix will also list the units of measurement (MS peak height, MS peak area, and if available pmol/ml, ng/sample etc.). We will share our data no later than on acceptance of the first publication of the findings from the respective data set(s). For activity-based proteomics data, we will use PRIDE or the MassIVE data repository at UCSD.

Nucleic acid sequence data will be submitted to the NCBI Short Read Archive.

Gene expression data will be submitted to Gene expression Omnibus at NCBI, NIH.

Microbiome metadata will be deposited into DbGap in formats that are in accordance with commonly used standards, such as MIMARKS. Metagenomic nucleic acid sequence data will additionally be deposited in MG-RAST at Argonne National Laboratory, along with associated metadata. We will also make microbiome result summary files (e.g., tables cataloging: sample metadata, taxon or protein family abundances across samples, taxa or protein families that stratify microbiome samples) publicly available through github. Additional project data and relevant protocols for which no public repository exists will be made available upon request.

In addition, results obtained from our proposed studies will be shared with the entire scientific community through presentations at national and international meetings and through publications in peer-reviewed scientific journals.

We will make available any unique research resource produced under this grant for use at other academic or non-profit institutions at no cost except for standard shipping expenses and if applicable the cost of producing the materials.

Material transfers will be made in accordance with the Materials Transfer Agreements (MTAs) of Oregon State University, Pacific Northwest National Laboratory, and the National University of Natural Medicine as appropriate. Should any intellectual property arise which requires a patent, we will ensure that the technology (materials and data) remains widely available to the research community in accordance with the NIH Principles and Guidelines.

All project publications will be deposited in PubMed Central at the time of publication.

31b Authorship Eligibility

All investigators who actively participate in the conduct of the proposed research, and participate in the writing, analyses, interpretation and/or detailed editing of the resultant manuscripts will have opportunity to be included as an author.

31c. Public access to protocol, participant-level data and/or statistical code

We will publish our trial protocol shortly after finalization with the IRB, FDA and NCCIH OCRA. We will deposit relevant data to public data warehouses as detailed above. We will make our data and statistical code available to journal editors upon request for open access publications and/or to confirm our results.

Appendices

32 Informed consent materials

Please see the informed consent documents enclosed as Appendices.

33 Biological specimens

At each bi-weekly clinical visit, we will collect blood (and heparin plasma), 24 hr-urine, and stool. Urine and stool collection kits will be distributed during each clinical visit for returning the following clinical visit. Participants will be instructed on how to collect and transport all samples. 24-hr urine collection containers will be returned to the clinical research center for aliquoting and storage. Stool samples will be collected and aliquoted at home the morning of each visit. Blood collection will occur via standard phlebotomy by a trained phlebotomist from the antecubital fossa of the arm or the back of the hand.

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