Safety, Tolerability, and Biosignature of Humanized Prebiotics in Healthy Adults

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Interventional Study

Study Title: Safety, Tolerability, and Biosignature of Humanized Prebiotics in Healthy Adults

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I HAVE READ AND APPROVE THIS VERSION OF THE PROTOCOL.
[electronic signature accepted]

Principal Investigator: _____ **Date:** _____

Statistical Co-investigator: _____ **Date:** _____

Summary of Changes from Previous Version [If applicable]: N/A

Previous Version No.	Affected Section(s)	Summary of Revision(s)	Reason for Change(s)
6		REDUCE VOLUME OF BLOOD COLLECTION TO 10ML; CHANGE TO VIRTUAL CONSENT PROCESS; UPDATED DIET HISTORY QUESTIONNAIRE PLANS AND MEAL JOURNALING.	NO NEED FOR ADDITIONAL BLOOD; VIRTUAL CONSENT IS NEEDED DUE TO IDS REQUIREMENT TO PROVIDE SIGNED CONSENT IN ADVANCE OF DISPENSING NUTRITIONAL SUPPLEMENT
7		1. CHANGE GASTROINTESTINAL SYMPTOM SCALE FROM GSSC TO PROMIS 2. INCLUSION AND EXCLUSION CRITERIA WERE MADE CONSISTENT THROUGHOUT DOCUMENT AND ADDED SEVERE/PERSISTENT SYMPTOMS FROM THE PROMIS ASSESSMENT.	PROMIS IS A BETTER EVIDENCE-BASED TOOL FOR ASSESSMENT OF GASTROINTESTINAL SYMPTOMS (SPIEGEL, ET AL. AM J GASTROENTEROL. 2014)

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Statement of Compliance

This study will be conducted in accordance with the International Conference on Harmonization guidelines for Good Clinical Practice (ICH E6) and the Code of Federal Regulations on the Protection of Human Subjects (45 CFR Part 46). The statistical analysis plans will be consistent with guidance such as the CONSORT Statement [1] or STROBE Statement [2], ICMJE recommendations [3], the 2016 and 2019 statements of the American Statistical Association [4,5], and recommendations in Nature [6,7].[1] All personnel involved in the conduct of this study have completed human subjects' protection training.

[1] [1] www.consort-statement.org [2] www.strobe-statement.org [3] www.icmje.org [4] Wasserstein RL, et al. (2016), The ASA's Statement on p-Values, ***The American Statistician***, 70:2, 129-133 [5] Wasserstein RL, et al. (2019), Moving to a World Beyond p < 0.05, ***The American Statistician***, 73:sup1, 1-19 [6] Amrhein, et al. (2019) Scientists rise up against statistical significance, ***Nature*** 567, 305-307 [7] Editorial (2019) It's time to talk about ditching statistical significance: Looking beyond a much used and abused measure would make science harder, but better. ***Nature*** 567, 283-283.

Abbreviations and Definitions of Terms

Abbreviation/Acronym	Definition
AE	Adverse Event/Adverse Experience
CI	Confidence Interval
CRC	Colorectal Cancer
CRF	Case Report Form
CRP	C-Reactive Protein
eCRF	Electronic Case Report Form
FDA	Food and Drug Administration
GCP	Good Clinical Practice
GI	Gastrointestinal
GOS	Galacto-oligosaccharides
Gal	Galactose
Glc	Glucose
GSSC	Gastrointestinal Symptom and Severity Checklist
hGOS	GOS enriched with LacNAc; humanized GOS
HIPAA	Health Insurance Portability and Accountability Act
IB	Investigator's Brochure
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
ICF	Informed Consent Form
IL	Interleukin
INF	Interferon
IND	Investigational New Drug Application
IRB	Institutional Review Board
N	Number (typically refers to subjects)
NDA	New Drug Application
PI	Principal Investigator
QA	Quality Assurance
QC	Quality Control
SAE	Serious Adverse Event/Serious Adverse Experience
SD	Standard Deviation
SE	Standard Error
SOP	Standard Operating Procedures
TNF	Tumor Necrosis Factor
UC	Ulcerative Colitis
UP	Unanticipated Problem
WGS	Whole Genome Shotgun (Sequencing)

1. Protocol Synopsis

Study Title	Safety, Tolerability, and Biosignature of Humanized Prebiotics in Healthy Adults
Objectives	Objective 1. Validate 15 g/day dose safety and tolerability (including absence of GI adverse effects) of GOS and hGOS in healthy adults. Objective 2. Establish a biological signature of GOS and hGOS through assessment of prebiotics effects *vs* placebo (powdered corn syrup comprised of fructose, glucose, and an inert cellulose material that matched the consistency, color, sweetness, and taste of the prebiotics) on (i) abundance of beneficial gut bacteria and restoration of the gut microbiome saccharolytic potential, (ii) modulation of biomarkers of inflammation and (iii) evaluation of intestinal barrier function.
Target Population	Healthy adult individuals **Key Inclusion Criteria**: Individuals ages 18-55 with BMI of 18.5 to 32. **Key Exclusion Criteria**: • Formal diagnosis of IBD i(ncluding ulcerative colitis, Crohn's Disease) colorectal cancer, diabetes • Celiac disease or IBS diagnosed by a physician • Lactose intolerance as diagnosed by a hydrogen breath test • *C. difficile* infection within 2 months • Antibiotic consumption in the previous 2 months • History of gastric bypass surgery • Current tobacco smoker • Pregnant or breastfeeding • A response of 4 or 5 to questions 2 (abdominal pain), 6 (abdominal discomfort), 8 (constipation), 16 (diarrhea), 22 (abdominal distention), 26 (abdominal bloating), 31 (nausea), or 33 (vomiting).
Numbers of Participants	**Number to be recruited for screening:** 55-60 participants **Number of eligible participants enrolled:** 48 adult individuals
Clinical Phase	Pilot
Intervention	Daily oral administration of 10 - 15 g of Prebiotic galacto-oligosaccharides (GOS) or GOS enriched with lactosamine (LacNAc) for four weeks
Study Description	We will enroll 48 adult individuals (18-55 y.o.). Subjects will be assigned randomly to one of 3 groups (n = 16): GOS, hGOS or placebo (powdered corn syrup comprised of fructose, glucose, and an inert cellulose material that matched the consistency, color, sweetness, and taste of the prebiotics) to test safety and dose tolerability. The prebiotic treatment will last for 4 wk. Before the treatment (baseline) and during week 4 of the trial (after the prebiotic treatments), we will assess changes in quality and quantity in bacterial components of the gut microbiome pre- and post-treatment. Likewise, we will

	determine serum markers of inflammation, integrity of the intestinal barrier at baseline and at week 4. Participants will complete the Diet History Questionnaire (DHQ), a freely available food frequency questionnaire (FFQ) at baseline and will be asked to maintain a record of their daily diet by using a printed meal tracking diary. In addition, the PROMIS Gastrointestinal Symptom Scale will be used for potential side effect evaluation at baseline and each week for the duration of the study.
Outcome Measures	Dose safety and tolerability in **healthy adults**. Gut microbiome Inflammation Integrity of intestinal barrier
Study Duration	4 weeks
Subject Participation Duration	4-5 weeks
Estimated Time to Complete Enrollment	40 weeks
Statistical Analysis Plans	Data will be presented as mean ± SEM for variables that are normally distributed, or median (IQR) for variables not normally distributed. Group means will be compared by ANOVA and post hoc tests except when data is normally distributed (a nonparametric analysis of medians will be performed using the Kruskal-Wallis test for non-normally distributed data). Chi-square tests or Fisher's exact tests will be used for incidence data. For analysis of microbiome taxonomic and functional data we will carry out Mann-Whitney-Wilcoxon matched pair tests for pairwise comparisons between time points. Unpaired Mann-Whitney-Wilcoxon tests will be used for pairwise comparisons between treatment groups. We will apply the Kruskal-Wallis test when more than two groups are compared. *P* values will be corrected for the total number of comparisons with false-discovery rate (FDR).

2. Introduction: Background and Scientific Rationale

2.1. Background Information

An undefined three-point association exists between altered gut microbiota (dysbiosis), compromised gut barrier, and disease phenotype. This relationship is well documented in animal studies but not well examined in human studies; hence a 'triangulation' is inferred based on the association between two of these three factors. A recent review of the mechanisms, measurement, and clinical implications of a "leaky gut" in humans[1] stated that studies suggest that there are non-pathological situations that may be associated with increased intestinal permeability, and these relatively minor perturbations "can be reversed with dietary, non-pharmacological approaches". *Approaches to alleviate these conditions, including prebiotics and probiotics, in human interventions are lacking.*

Galacto-oligosaccharides (GOS) are prebiotics that have an FDA Generally Regarded As Safe (GRAS) status[2]. GOS modulate the intestinal microbiota[3, 4] and strengthen intestinal barrier function via modulation of goblet cell function[5, 6]. LacNAc is the major building block of gut glycoproteins and human milk oligosaccharides[7, 8], but its beneficial effects have not been studied as it could not be produced in enough quantities at reasonable cost until now. We have shown that short-term feeding of pure GOS increases expression of protective mucus-producing *MUC2,* decreases intestinal permeability, and reverses proportions of fecal β-galactosidases and β-glucosidases, reflecting increases in saccharolytic bacteria, including *Bifidobacterium*[9]. Likewise, GOS enriched with LacNAc, a building block of gut glycoproteins (humanized GOS, **hGOS**) decreases intestinal permeability in old mice. *Although LacNAc is a biological component of human breast milk and gut mucins, its use as a supplement has not been assessed and hence its GRAS status has not been granted yet by FDA. An IND exempt status has been obtained for this study.* GOS is also effective in the treatment of lactose intolerance[10, 11]. Therefore, GOS and hGOS represent an attractive approach to reducing intestinal permeability, improving the gut microbiome, and reducing inflammation in older adults. Our *long-term goal* is to establish the safety of pure prebiotic GOS and hGOS and their effectiveness in (i) reducing intestinal permeability and inflammation, (ii) restoring the saccharolytic potential of the gut microbiome, and (iii) alleviating mild gastrointestinal manifestations and self-reported food intolerances in older adults in long-term care. The objectives of this study are (1) to validate 15 g/day dose safety and tolerability of GOS and hGOS in **healthy adults**, and (2) to establish a biological signature of GOS and hGOS through assessment of prebiotics effects *vs* placebo on (i) abundance of beneficial gut bacteria and restoration of the gut microbiome saccharolytic potential, (ii) modulation of biomarkers of inflammation and (iii) evaluation of intestinal barrier function.

2.2.Supporting Pilot / Unpublished Data

Prebiotics significantly affect gut health and microbiota composition. The impact of recognized prebiotics, or functional foods that stimulate growth of gut native beneficial bacteria, on microbiome composition and functionality has been documented by us and others[3, 4, 11]. β(1-4) galacto-oligosaccharides (**GOS**) are complex carbohydrates that resist digestion in the upper GI tract, and thus arrive to the colon intact and consequently increase the abundance of specific primary and secondary metabolizers. Studies have reported the beneficial impact of GOS, alone or in combination with probiotics, on bowel function[12, 13], diarrhea and abdominal pain[10, 11, 14, 15], bloating and flatulence[16, 17] in adult individuals. N-Acetyl-D-lactosamine (**LacNAc,** Galβ1,4GlcNAc) is a biologically relevant molecule that is a building block of gut glycoproteins, and a precursor of several important blood group epitopes, including Lewis A, Lewis B, or sialyl Lewis A, which are involved in biological processes, including fertilization and pathogen recognition[18]. Of relevance for our study, repeating and variably branched lactose or LacNAc units, frequently with attached sialic acid and fucose monosaccharides, compose complex human milk oligosaccharides (HMOs), which are uniquely abundant in human breast milk but not in that of other mammals[19-21]. Most HMOs are not digested by human enzymes, acting as natural prebiotics for the development of a healthy gut microbiota in infancy[7, 8, 22]. While GOS is not a naturally occurring non-digestible carbohydrate, it does share structural similarities with LacNAc, with the latter containing nitrogen molecules attached to the disaccharide backbone. Despite the biological relevance of LacNAc, no studies have addressed its impact on human gut physiology and the microbiota since the chemical methods to generate LacNAc demand multi-step syntheses and are expensive and labor intensive. Likewise, current enzymatic methods use galactosyl-transferases, which require UDP-galactose as a glycosyl donor[23], and have high acceptor selectivity, yielding low efficiency with unnatural acceptors[24].

GOS and hGOS beneficially impact the gut microbiome, increasing beneficial bacteria (*Bifidobacterium* and *Akkermansia*) and saccharolytic potential of the microbiota and reduced intestinal permeability, modulating markers of inflammation[9]. Furthermore, GOS, but not hGOS, increased mucus in the intestinal lumen, suggesting different mechanisms of action. Our previous studies in mice have shown that short-term feeding of pure GOS increases expression of protective mucus-producing *MUC2,* decreases intestinal permeability, and reverses proportions of fecal β-galactosidases and β-glucosidases, reflecting increases in saccharolytic bacteria, including *Bifidobacterium*[9, 25].

In this trial, we will determine changes in the abundance of saccharolytic bacteria as described [9]. Briefly, we will use a reference study that mined 532 publicly available gut reference genomes and assigned them to four different groups (proteolytic, saccharolytic, lipolytic, and generalist bacteria) using metagenome analytical methods [26], and the metabolic reconstruction network AGORA [27]. In the case that the genus is not categorized in the mentioned studies, will refer to the Bergey's Manual of Systematic Bacteriology and previously published reports.

2.3.Scientific Rationale

The study will test two prebiotics: highly pure GOS and LacNAc-enriched GOS (hGOS). The intervention will include a dose of 15 g daily as previously reported[10]. Dose was selected based on previous literature reports[16, 17, 28-30] and the interventions conducted by our group to assess efficacy of GOS in lactose-intolerant individuals[10, 11, 31, 32]. The hGOS targeted dose will be composed of 15g of GOS enriched with 150 mg of LacNac (99:1), which is in agreement with the range found in human breast milk[33]. Based on the concentration detected in breast milk, an infant consumes between 54 and 243 mg of LacNAc per day, with the variation depending on stage of lactation and infant age and weight.

Participants will be assigned randomly to one of 3 groups (n = 16): GOS, hGOS or placebo (powdered corn syrup comprised of fructose, glucose, and an inert cellulose material that matched the consistency, color, sweetness, and taste of the prebiotics)[10, 32] to test safety and dose tolerability. The prebiotic treatment will last for 4 weeks, since we have shown in adult individuals that a 4-week period allows for the observation of changes to the gut microbiome[10, 11]. The doses of GOS, hGOS and placebo will be increased over 28 days beginning with 10 g/day and increasing to 15 g/day. Dose regiment will be 5 grams twice daily on days 1–10 followed by 7.5 grams twice daily on days 11–28. Doses will be in powder form and will be mixed with water or food and taken as directed by the dosing scheme.

High-sensitivity C-reactive protein (hs-CRP) is a recognized marker of systemic inflammation[34] and specific pro-inflammatory cytokines mediate interaction between immune cells and non-immune cells, contributing to the inflammatory status of the intestine[35, 36]. Based on previous studies with probiotics and prebiotics that report potential immunomodulatory effects[37-39], we decided to monitor hs-CRP, IL-1α, IL-1β, IL-2, IL-6, Il-8, IL-12, TNFα, and IFNγ for the duration of the clinical trial.

Before the treatment (baseline) and during week 4 of the trial (after the prebiotic treatment), we will assess changes in quality and quantity in bacterial components of the gut microbiome pre- and post-treatment. Likewise, we will determine serum inflammatory markers, and integrity of the intestinal barrier at baseline and at week 4. Subjects will also respond to the Diet History Questionnaire (DHQ), a freely available food frequency questionnaire (FFQ) and will be asked to keep daily diet records.

3. Objectives

3.1.Specific Aim 1

To validate 15 g/day dose safety and tolerability of GOS and hGOS in **healthy adults**.

3.2.Specific Aim 2

To establish a biological signature of GOS and hGOS through assessment of prebiotics effects *vs* placebo on (i) abundance of beneficial gut bacteria and restoration of the gut microbiome saccharolytic potential, (ii) modulation of biomarkers of inflammation and (iii) evaluation of intestinal barrier function.

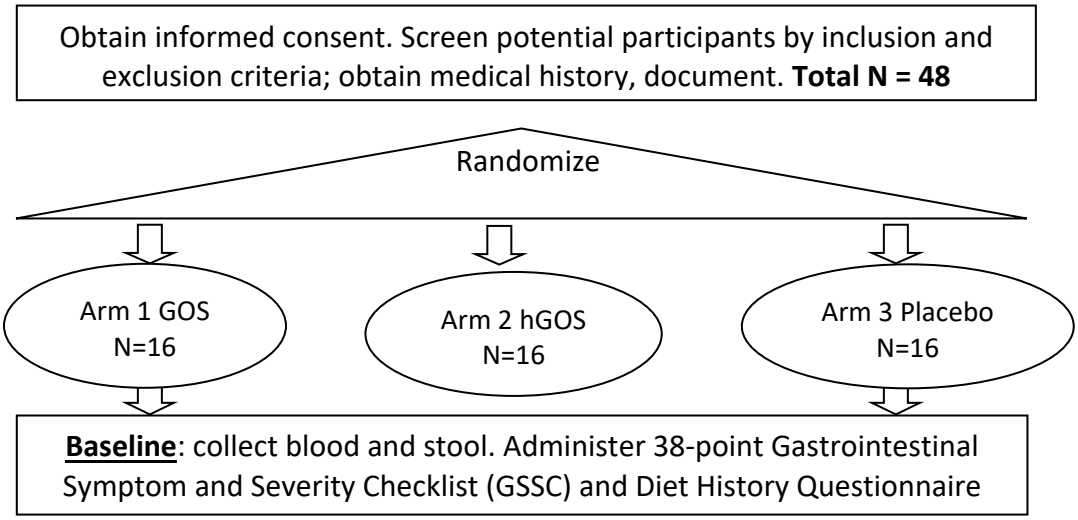
4. Study Design

This will be a controlled (GOS, hGOS versus placebo), interventional, longitudinal (baseline compared to end of study), prospective, single-site pilot study. Subjects will be assigned randomly to one of 3 groups (n = 16): GOS, hGOS or placebo. The treatment will consist in 10 - 15 g/day of GOS, hGOS or placebo (powdered corn syrup comprised of fructose, glucose, and an inert cellulose material that matched the consistency, color, sweetness, and taste of the prebiotics), which will be provided to participants as powder that can be added to any non- alcoholic beverage or sprinkled on food for intake. The intervention will last for 4 weeks, since we have shown in adult individuals that a 4 week period allows for the observation of changes to the gut microbiome. The study will end after the second time-point sample collection at 4 weeks.

Dose rationale: The intervention will include a dose of 5 grams twice daily on days 1–10 followed by 7.5 grams twice daily on days 11–28[10].Doses were selected based on previous literature reports[16, 17, 28-30] and the interventions conducted by our group to assess efficacy of GOS in lactose-intolerant individuals[10, 11, 31, 32]. The hGOS preparation will be composed of GOS enriched with LacNac at a concentration of 6.7-31µg/mL (<1% of the formulation), which is in the range found in human breast milk[33] and hence considered safe.

Flow diagram

Prior to Enrollment



Visit 1
Time Point

Weekly GSSC data collection

Visit 2
Time Point

<u>4 weeks</u>: collect blood and stool. Administer 38-point Gastrointestinal Symptom and Severity Checklist (GSSC) Questionnaire (FFQ)

Analyses: ***(a) Evaluation of inflammation and intestinal barrier function in serum; (b)***

4.1.Treatment Design

Dose rationale. The intervention will include a dose ranging between 10 to 15 g daily as previously reported[10]. Doses were selected based on previous literature reports[16, 17, 28-30] and the interventions conducted by our group to assess efficacy of GOS in lactose-intolerant individuals[10, 11, 31, 32]. The hGOS preparation will be composed of GOS enriched with LacNac at a concentration of 6.7-31µg/mL (<1% of the formulation), which is in the range found in human breast milk[33] and hence considered safe.

Regimen. The prebiotics dose will be 5 grams twice daily on days 1–10 followed by 7.5 grams twice daily on days 11–28. The placebo (powdered corn syrup comprised of fructose, glucose, and an inert cellulose material that matched the consistency, color, sweetness, and taste of the prebiotics) will be administered in a blinded matching packet at the same doses. If participants miss taking a dose on a given day, they will be directed to put the pouch for that day aside, and on the next day take the next numbered pouch.

4.2.Experimental / Observational Design

Subjects will be assigned randomly to one of 3 groups (n = 16): GOS, hGOS or placebo.

4.3.Measurement Design

Blood and stool samples will be collected at baseline (Day 0) and end of treatment (Day 28). Stool collection kits will be provided to participants in case samples cannot be obtained *during study visits.* Safety and tolerability will be determined based on the severity and/or frequency of side effect using a 38-point Gastrointestinal Symptom and Severity Checklist (GSSC). In addition, tolerability will be evaluated through occurrence of drop-out and adherence.

Measurements on blood (10 mL per visit for a total of 20 mL)**:**

(1) Fasting venous blood samples will be drawn and serum will be used to quantify zonulin using the Zonulin (IDK® Zonulin) (Serum) ELISA test (Immundiagnostik AG).
(2) Determination of levels of biomarkers of inflammation (IL-1α, IL-1β, IL-2, IL-6, Il-8, IL-12, TNFα, and IFNγ) as well as high-sensitivity C-reactive protein, a marker of low-grade inflammation (hsCRP) in serum. We will determine cytokine levels using the MCYTOMAG-70K (Milliplex) in the Advanced Analytics Core (AAC) of the CGIBD. **(ii)** hsCRP concentration will be measured via hsCRP ELISA (Sigma Aldrich).

Measurements in stool:

Determination of abundance of beneficial bacteria (*Bifidobacterium* and *Akkermansia* species) in the gut microbiome of treatment and placebo groups and the microbiome saccharolytic potential using WGS sequencing, the most accessible, cost-effective measure currently available.

4.4.Outcome Measures

Primary Outcome(s):

The expected outcome is to confirm a 15 g/day as a safe and tolerable dose of GOS and hGOS in healthy adult individuals using the 38-point Gastrointestinal Symptom and Severity Checklist (GSSC).

Secondary Outcome(s):

Definition of prebiotic biosignature characterized by:

- Increased abundance of saccharolytic bacteria (*Bifidobacterium*) and other beneficial bacteria (*Akkermansia*)
- Modulation of biomarkers of inflammation
- Improved intestinal barrier function.

Tertiary/Exploratory Outcome(s): N/A

Other Measures: Baseline Characteristics and Assigned Treatments: N/A

5. Study Participants

5.1. Number of Participants

Number to be recruited for screening: 55-60 participants will be recruited for screening.
Number of eligible participants enrolled: The targeted number of participants is 48. We expect that 20% of screened individuals will not be eligible or will be unwilling/unable to participate in the study. Participants will be adults (male and female, 18-55 years old with no formal diagnosis of IBD, IBS, UC, CRC, celiac disease, or diabetes mellitus) recruited at UNC Chapel Hill and within the general population in the surrounding area via email communications, social media, and flyers.
Subjects will not be diagnosed with specific GI conditions (IBD, IBS, UC, CRC) and with no history of celiac disease, diabetes mellitus or antibiotic consumption in the previous 2 months. Individuals with a non-GI related disease diagnosis might be included in the study.

5.2. Eligibility

Eligibility criteria are based on self-reported information from study participants.
Inclusion Criteria:

- Individuals must be between 18 and 55 years old.
- All participants will not be tobacco smokers and will be well-nourished according to standard anthropometric criteria with BMI between 18.5 and 32.
- Individuals must be able to give informed consent.
- Subjects willing and able to:
    - consume prebiotics or placebo preparations for a period of 4 weeks
    - record daily food consumption using hard-copy food diary provide at the baseline vist, and complete the Diet History Questionnaire (DHQ).
    - provide stool, and blood (via venipuncture) samples.
- Enrollment will not be restricted based on race, ethnicity, or gender. Subject population will reflect the population providing a broad selection of individuals to allow to enroll subjects from all races, ethnicities and genders as represented in North Carolina state.

Exclusion Criteria:

- Individuals diagnosed with Inflammatory Bowel Diseases (IBD), Ulcerative Colitis (UC), Colorectal Cancer (CRC) , or diabetes mellitus.
- Celiac disease or IBS diagnosed by a physician

- Lactose intolerance as diagnosed by a hydrogen breath test
- C. difficile infection or treatment within 2 months
- Individuals currently or within the past 2 months on prescribed antibiotics.
- Patients with a history of gastric bypass surgery.
- Any physical or psychological condition that, in the opinion of the investigators, would post unacceptable risk to the patient or raise concern that the patient would not comply with protocol procedures
- Women that are pregnant or breastfeeding will be excluded from the study.
- A response of 4 or 5 to questions 2 (abdominal pain), 6 (abdominal discomfort), 8 (constipation), 16 (diarrhea), 22 (abdominal distention), 26 (abdominal bloating), 31 (nausea), or 33 (vomiting) on the PROMIS Gastrointestinal Symptom Scale.

5.3.Strategies for Recruitment and Retention

Recruitment Strategy:

Subjects will be recruited using study flyers and/or asked to participate in the study via email or phone call. Only if the subject agrees to participate and meets enrollment criteria will they be enrolled in the study. Subjects will be screened by the Clinical Research Coordinator on the basis of inclusion and exclusion criteria laid out in this study. Only individuals with no formal diagnosis of IBD, IBS, UC, CRC, celiac disease, or diabetes mellitus will be selected as study participants based on a self-report. Also, individuals with a history of gastric bypass, current use of antibiotics or use of oral antimicrobials in the past 2 months will be excluded. The PI will review subject enrollment at weekly project meetings so that any deviation from the anticipated enrollment schedule will be detected quickly. All outreach and advertising activities will also be reviewed on a weekly basis.

Retention Strategy:

The PI and research team will hold weekly project meetings to monitor enrollment and ensure enrolled subjects complete all study procedures as intended. Likewise, brief weekly contacts (either by phone, email, or text message) with enrolled subjects will follow up regarding protocol adherence, gastrointestinal symptoms, and study satisfaction, in addition to any protocol-specific procedures. This approach provides the opportunity for early identification of possible reasons for study non-adherence or early discontinuation by the subject and to address such issues, if possible. If a subject decides to withdraw participation in the study, the date and reason will be recorded and stored in the RedCap database. On a weekly basis, the study coordinator will run a report on participants accrual and completion status for review by team to identify and resolve any problems or potential issues that arise during the course of the study.

Screen Failures:

During the screening period, inclusion/exclusion criteria for the study participation will be checked / tested. Subjects who meet all inclusion criteria and do not meet any exclusion criterion will be eligible to be randomized. Those who are not eligible for randomization or dosing will be considered as screening failures. In the case of screen failures, the reason will be recorded. We expect a 20% screen failure rate.

5.4.Consent Process

Potential participants will be approached via email or text message, and a virtual screening appointment will be set up over Zoom. The study will be explained to the subjects by Dr. Becker-Dreps or Clinical

Research Coordinator, Kelli Hammond. Potential participants who meet screening criteria and are still interested in the study will undergo consent procedures. Consent will be performed virtually using the Qualtrics platform. Potential participants will be allowed time to read the consent and all questions will be answered. Consent discussion will take place until the subjects are fully aware of all study details and all questions have been answered. There is no set time for consent discussion. Subjects will be told that they can withdraw from the study at any time without penalty. The subject and investigator and/or study coordinator will sign the consent form. An electronic version of the consent will be provided to the participants and printed copy will be provided to the subject at the time of the first study visit.

6. Study Intervention

6.1.Intervention – Test Article (if applicable)

Description: Prebiotic galacto-oligosaccharides (GOS) have an extensive history of safety and have been used for decades to improve overall host health via modulation of the gut microbiota[40]. Since GOS are complex carbohydrates resistant to digestion in the upper gastrointestinal tract that arrive to the colon intact, they increase the abundance of specific primary and secondary degraders expanding the probiome, autochthonous beneficial members of the intestinal microbiota[41]. Specific increases of *Bifidobacterium* (the "bifidogenic effect") hence are fundamental to the beneficial effect of the prebiotic that result in increased concentration of colonic short chain fatty acids (SCFAs), particularly butyrate[42]. Over 95% of the microbially generated SCFAs are absorbed by the colonic epithelium[43-45]. The three major SCFAs (acetate, propionate and butyrate) stimulate proliferation of normal crypt cells and intestinal barrier integrity. Butyrate and, to a lesser degree, propionate, have been reported to inhibit growth of colon cancer cell lines[46]. Furthermore, butyrate has potent immunomodulatory effects influencing activity of HDACs responsible for decreasing dendritic cells IL-12 and IL-6 cytokine secretions[47], and induces T_{REG} cells and IL-10-secreting T cells through Gpr109a on colonic dendritic cells and macrophages[48].

The prebiotic GOS and hGOS in this formulation are generated by the β-hexosyltransferase from *Sporobolomyces singularis*, optimized and heterologously expressed to generate high-purity GOS[49, 50]. Commercial GOS are a mixture of mono, di, and oligosaccharides in varying ratios. Although major advances that have led to standardization, and better characterization and reporting of such mixtures have been made, variability in the GOS composition impacts the reproducibility of studies and interventions as well as the ability to predict outcome. GOS are currently produced commercially by trans-galactosylation of lactose as donor and acceptor substrate by β-galactosidase enzymes (EC 3.2.1.23)[51]. Converting lactose into GOS by β-galactosidases is a kinetically controlled reaction, by the competition between hydrolysis and trans-galactosylation. During this conversion, the thermodynamically favored hydrolysis of lactose, which generates D-galactose and D-glucose, competes with the transferase activity that generates a complex mixture of various galactose-based di- and oligosaccharides of different structures[52]. Vivinal GOS is produced by *Bacillus circulans* β-galactosidase, Bimuno is a GOS blend derived from lactose conversion using *Bifidobacterium bifidum* NCIMB 41171 enzymes, and Oligomate is produced by the β-hexosyltransferase from the yeast *Sporobolomyces singularis*.

GOS may have a degree of polymerization (DP) up to 9. However, the enzymatic generation of GOS tends to produce low molecular weight oligosaccharides. As shown in Table 1, concentration of

disaccharides in commercial formulations can be as high as 52%. DP2 elements may differ in their structure. One seminal study[53] that reported the generation of GOS by *B. circulans* showed that the ß-(1-3), ß-(1-2), and ß-(1-6) linked galactosyl disaccharides (D1-D3) were formed by the trans-glycosylation reaction with the ß-galactosidase from *B. circulans*. Since lactose [the ß-(1-4) linked galactosyl-disaccharide] was already present in the initial reaction as substrate, the authors used a different reaction (containing *p*NP-Gal and glucose) to confirm lactose synthesis by the enzyme.

In a recent study[54], the analysis of 6 commercial GOS products using high-performance liquid chromatography (HPLC) size exclusion on a Rezex RSO-oligosaccharide Ag+ column showed that all tested prebiotics had the monosaccharides galactose (Gal) and glucose (Glc) together with DP2 (mostly lactose) up to DP5 oligosaccharides in detectable levels. Vivinal GOS, and two other GOS products had also detectable amounts of DP6 oligosaccharides. Interestingly, except for one GOS product, the DP2-DP4 fractions of the various products comprised >90% of all GOS components, with most products containing a majority of DP2, with exception of one GOS product that had the highest DP4, DP5, and DP6 content of all GOS products with very low amounts of Gal and Glc, indicating a DP-based purification step. This study is one of the most detailed studies on composition of GOS; however, it does not provide the names or sources of 6 of the 7 analyzed compounds. Another study that reported on impact of B-GOS (Bimuno, Clasado Limited) on the immune system of 40 elderly individuals stated that 52% of the product was DP2[29]. In contrast, RP-G28, a purified preparation reported <3.2% of DP2 oligosaccharides[11] (Table 1).

Table 1. Composition of prebiotic GOS commercial formulations

	RP-G28[11]	**Vivinal[54]**	**Bimuno*[29]**	**BIOLIGO® GL 700 ****	**Oligomate**
Degree of polymerization					
DP2	<3.2%	42.55 ± 0.22%	52%	17.5%	14.8%
DP3	56-59%	23.63 ± 0.20%	26%	38-52%	34.4%
DP4	26-30%	10.21 ± 0.23%	14%		
DP5		3.00 ± 0.17%	8%		
DP6	11-16%	0.39 ± 0.17%			
Glucose	<0.1%	18.52 ± 0.12%		22%	22.4%
Galactose	<0.1%	1.70 ±0.08%		0.8%	8.6%

*Bimuno = B-GOS (Clasado) = Clasado BioSciences Ltd. (St. Helier, Jersey, UK) = HOST-G904 (Therabiomics, Jersey, Channel Islands)
** BIOLIGO® GL 5700 GOS (Ingredion) = Purimune (GTC Nutrition, Golden, Colorado [2008])

To ensure the safety of the human volunteers we followed the FDA guidance for deriving the maximum recommended starting dose (MRSD) for clinical trials of new molecular entities in adult healthy volunteers. See: U.S. Department of Health and Human Services Food and Drug Administration. (2005). *Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers*. Rockville, MD: Center for Drug Evaluation and Research (CDER), 7.

The equation used is as follows:

HED (*mg*kg*$^{-1}$) = *Animal Dose* (*mg*kg*$^{-1}$) * *Animal* K_m/*Human* K_m

Where the K_m factor is a number based on body surface area. In our animal study we used a dose of 1,500 mg kg^{-1}, based on ~30 mg LacNAc fed to a ~20 g mouse per day[25]. We used a K_m factor of 3 for mice and 37 to represent a 60 kg human adult.

We can consistently produce hGOS containing concentrations that exceed 25 g/L of LacNAc with no significant variation between batches observed. Therefore, our approach is to normalize to reach the proposed dose levels using pure GOS. The active component is in power form that will be obtained after lyophilization. Concentration will be confirmed using previously developed HPLC protocols[33]

Acquisition: Dr. Jose Bruno-Barcena, Professor of Plant and Microbial Biology at North Carolina State University and Co-investigator in this proposal, will coordinate with the Golden LEAF Biomanufacturing Training and Education Center (BTEC) the cGLP production of the prebiotics galacto-oligosaccharides (GOS) and GOS enriched in N-acetyl-D-lactosamine (LacNac, humanized GOS, hGOS) for the clinical intervention planned for the study. BTEC has the capabilities, equipment, and expertise for the bioprocess cGLP production (See Appendix 1).

Formulation/Packaging/Labeling: GOS, hGOS, and placebo will be supplied as a sterile powder by BTEC, dried and portioned into daily dose pouches under sterile conditions at the NC Food Innovation Lab, Kannapolis, NC, . Pouches will be labeled and will serve to protect the preparations from light, air, and moisture. Product will then be transferred to the UNC Investigational Drug Services (IDS).

Protocol Number:
Study Acronym: GOS Pilot

Enrollment ID: Lot No.:
Subject Number: Subject's Initials:
Date Dispensed:
Contents: 56 pouches, 5 g each for the first 10 days followed by 7.5 grams each for days 11-28 of GOS, hGOS or matching placebo, plus two additional packets (one 5mg and one 7.5mg) for inadvertent loss or spillage.
Directions: Use only as directed. To be taken by mouth with food or beverage.
This product is an investigational product to be dispensed only by a qualified investigator.
Storage: Room Temperature 15-30 ℃ (59-86℉)
Caution: New drug limited by law to investigational (clinical trial) use only. Keep out of reach of Children.
Manufactured By: Golden LEAF Biomanufacturing Training and Education Center (BTEC), 850 Oval Dr, Raleigh, NC 27606
Distributed by:

Storage and Stability:

The powder prebiotics galacto-oligosaccharides (GOS), GOS enriched in N-acetyl-D-lactosamine (LacNac, humanized GOS, hGOS), and placebo can be safely stored in a dry area at room temperature and hold an expiration date of 2 years after production.

Preparation and Administration: Individuals will be provided with a total of 58 pouches numbered by day (#1 to #56) containing the appropriate daily dose of treatment or placebo. Pouches 57 and 58 will be extra pouches in case of loss or spillage. Participants will be instructed to consume one pouch twice aday. The premeasured powder can be added to hot or cold non-alcoholic beverages or sprinkled over food.

Modification: Individuals experiencing more than three of the following side effects a day: bloating, flatulence, belching, abdominal pain, discomfort, watery stool, and vomiting will be instructed on how to spread out the daily dose to minimize side effects. Participants experiencing these side effects be asked about frequency and severity. Severe side effects interfering significantly with subject's usual function will result in withdrawal from study. Weekly phone contacts will allow for timely report of adverse effects. Participants will also be given the choice to contact the study PI directly in case of emergencies.

Accountability: UNC Investigational Drug Services (IDS) will keep source documentation for accountability of the study treatments that will detail receipt, dispensing, and return of used and unused prebiotics and placebo. Treatment products will be stored in locked facilities until they are either returned to the PI at the end of the study. Prior to dispensing, sachets containing the treatment will be visually inspected. If the package appears to have tears or sign of tampering, the sachet will be rejected for use but retained until the end of the study. At the end of the study, the study team will determine dispensation of all remaining unused treatment packs at the study site. An accountability log will be kept, indicating the final disposition of study treatment. This log will be provided to the PI at the end of the study.

6.2.Assignment Procedures

Matching and Stratification:

N/A

Randomization, Concealment, and Blinding:

The Excel RAND function will be used for simple randomization of participants into the 3 different groups. The PI and designated study personnel are blinded to the randomization and are responsible for following the randomization and blinding procedures described in each clinical trial protocol.

Masking (Blinding):

This is a double-blinded study. The master randomization codes will be kept by IDS with appropriate security measures and access control. The research team will be unblinded once all of the samples have been collected and processed, before data analysis.

Unblinding. Circumstances for unblinding will be clearly established before the first patient begins treatment. Randomization procedures, if any, will be followed to ensure the code is broken only in accordance with the protocol. The study codes will be stored at the study site or may only be available directly from IDS. The code will only be broken in the case of an adverse event where it is necessary for the PI to know which treatment the individual is receiving before the individual can be treated. If it is

necessary to break the blind, the researcher will notify the IRB and document the event, together with the reasons for breaking the code.

6.3. Intervention – Procedural (if applicable)

Description:

(1) Subjects will receive enough prebiotic or control powder for the complete 28 days in pouches containing the exact daily dose. An additional pouch will be provided for both the 5g and 7.5g quantities in case of loss or spillage. The powder can be added to hot or cold non-alcoholic beverages or sprinkled over food.
(2) Subjects in the study will consume 5 grams of either GOS, hGOS or placebo twice daily on days 1–10 followed by 7.5 grams twice daily on days 11–28[10].

Training on Procedural Intervention:

Participants will be provided with detailed instructions of how to prepare and consume the prebiotics or control powder. Individuals will be informed on the potential side effects and how to avoid them by rationing the treatment over the day if needed or chosen.

6.4. Concomitant Therapy (if applicable)

New diagnosis and initiation of a new therapeutic including supplements during the course of the study should be disclosed. Antibiotic treatment during the course of the study will lead to discontinuation.

6.5. Rescue Medications and Procedures (if applicable)

N/A

6.6. Compliance Checks

Participant adherence will be monitored weekly via email and/or text messages through self-reporting of number of treatment pouches left.

6.7. Withdrawal / Discontinuation of Enrolled Participants

At baseline and every week after for the duration of the study, participant will answer a 33 question gastrointestinal symptom assessment with the PROMIS Gastrointestinal Symptom Scale (Spiegel, et al, Am J Gastrol, 2014). If a subject experience and increase in 2 levels over baseline in their response to questions 2 (abdominal pain), 6 (abdominal discomfort), 8 (constipation), 16 (diarrhea), 22 (abdominal distention), 26 (abdominal bloating), 31 (nausea), or 33 (vomiting) on the PROMIS Gastrointestinal Symptom Scale, they will be a) withdrawn from the study if the participant is in the first 10 days of their participation, or b) asked to go back to the lower dose of 5g sachets twice daily and reassessed in 3 days, if the participant has passed day 10 of participation. If participants are able to tolerate the lower dose, they will continue participating in the trial albeit consuming the lower dose of the prebiotic treatment. Likewise, if a participant develops a condition that is an exclusion criterion or participant noncompliance increases risk of decreased study integrity, the participant will be withdrawn from the study.

If 5 of the first 20 subjects enrolled experience a change of 2 levels on questions 2, 6, 8, 16, 22, 26, 31, 33 on the PROMIS Gastrointestinal Symptom Scale (bloating, flatulence, watery stools, abdominal pain) compared to baseline or higher adverse event leading to subject discontinuation, we will stop the study temporarily and request the medical monitor to do an unblinded assessment of the data. The medical monitor (unblinded) will assess subjects who discontinued due to the specified adverse effects to determine if they are all in one arm of the study or range across arms. The medical monitor will assess if there is a safety signal in a specific arm or treatment dose over the other that warrants the closure of the study. Since the medical monitor will be the person determining continuation or discontinuation of the study, the study team will remain blinded and unbiased.

6.8. Voluntary Withdrawal (Drop-Out) of Enrolled Participants

Participants may voluntarily withdraw from study at any time, for any reason, with no penalty or loss of rights. Participants that choose to withdraw from the study will be followed up (via text or email communication) to determine safety based on frequency and severity of side effects. Reasons for discontinuation will be documented in the study RedCap database.

7. Study Procedures and Schedule

7.1. Table of Events

Procedures		Screening –Day -14 to 0)	Baseline (Study Visit 1)	Phone Contact 1 (Day 0 + 7±2)	Phone Contact 2 (Day 0 + 14±2)	Phone Contact 3 (Day 0 + 21±2)	Final Visit (Day 0 + 28±2)	Follow-up Contact (Day 0 + 35±2)	Premature Discontinuation
Eligibility Assessment		X							
Informed Consent		X							
Medical History		X					X		X
Concomitant Medications		X					X		
Study Intervention			X	X	X	X	X		
Adherence Evaluation				X	X	X	X		
Assessment of Adverse Events				X	X	X	X	X	X
35-question PROMIS Gastrointestinal Symptom Scale (Appendix 1)			X	X	X	X	X	X	X
Clinical Laboratory	Phlebotomy		X				X		
Research Laboratory	Immunology - serum		X				X		
	Microbiome - stool		X				X		

Procedures		Screening –Day -14 to 0)	Baseline (Study Visit 1)	Phone Contact 1 (Day 0 + 7±2)	Phone Contact 2 (Day 0 + 14±2)	Phone Contact 3 (Day 0 + 21±2)	Final Visit (Day 0 + 28±2)	Follow-up Contact (Day 0 + 35±2)	Premature Discontinuation
	Intestinal barrier function - serum		X				X		

7.2. Enrollment/Pre-Screening (Day -14 to 0)

Screening to determine eligibility and if applicable, consent processes will be performed virtually on zoom. Within 2 weeks, blood sample collection, food questionnaire and stool collection kit will be provided on day 0.

7.3. Screening (Day -14 to 0)

Activities to be performed at screening:

(1) obtain consent,
(2) schedule study visits
(3) provide participant instructions

7.4. Visit 1 (Day 0, Baseline)

(1) schedule study visits/communications
(2) provide participant instructions
(3) collect blood sample for research only
(4) provide food questionnaire link and food diary booklet
(5) Gastrointestinal Symptom and Severity Checklist
(6) provide stool collection kit and mailing instructions for return
(7) provide intervention packets for 28 days

7.5. Phone Contact (Day 0 +7±2, Day 0 + 14±2, Day 0 + 21±2, and Day 0 + 35±2)

Information to be collected at days 7±2, 14±2 and 21±2:

(1) Follow-Up Phone Script
(2) Participant's compliance with intervention (adherence)
(3) Gastrointestinal Symptom and Severity Checklist - symptom rating
(4) Record adverse events as reported by subject

7.6. Final Visit (Day 0 +28±2)

Visit 2 (V2) will be the final visit of the study. The following will be conducted during this visit:

(1) Collect blood sample
(2) Provide food frequency questionnaire
(3) Provide stool collection kit and mailing instructions for return
(4) Gastrointestinal Symptom and Severity Checklist
(5) Record adverse events as reported by subject
(6) Record subject's compliance with intervention (adherence)
(7) Collect leftover intervention packets

7.7. Follow-Up Contact (Day 0 + 35±2)

Participants will be contacted at Day 0 +35±2 to monitor overall status and record adverse events.

(1) Follow-Up Phone Script
(2) Gastrointestinal Symptom and Severity Checklist - symptom rating
(3) Record adverse events as reported by subject

7.8. Premature Discontinuation

If a subject withdraws or the investigator discontinues subject participation after Visit 1, data from that visit will be included in the analysis of Objective 1, but not Objective 2

7.9. Collection and Management of Tissue Specimens

Sample Preparation:

Stool samples: After collection, samples should be immediately placed in the provided DNA/RNA Shield™ Fecal Collection Tube (Zymo Research Catalog No. R1101). The procedures for sample collection and stabilization should be carried out as quickly as possible. Samples can be stored at ambient temperature (4°C-25°C) for ≤ 2 years and frozen (< -20°C) indefinitely. In the Core facility, samples will be stored at -80°C until processed.

Blood samples: Samples will be collected on site using BD Vacutainer™ Venous Blood Collection Tubes: SST™ Serum Separation Tubes (FisherScientific Catalog No. 02-683-148) and stored at -80°C immediately after serum separation.

Record Keeping and Monitoring: The following procedures will be applied for maintaining sample accountability and traceability and for ensuring regulatory compliance.

1. Make sure the cryovial cap is secured tightly
2. Review the patient's Stool Sample Collection Log
3. Complete the cryovial labels

Patient #: _____

Visit # _____ and Date _____

Collection place: Home ☐ or Study Site ☐

4. Affix a label on each cryovial and put in plastic bag. Store the cryovials immediately in the -80°C freezer.
5. Complete the stool sample tracking log (only if sample is kept)

Sample Storage and Security:
Study personnel will receive, assess for integrity and relevant information, and store stool samples mailed by participants. Blood samples will be collected on site (V1 and V2) and placed immediately in ice and stored at – 80°C. Stool and blood samples will be stored in the UNC Microbiome Core in a designated -80°C freezer (serial #: 0160203301161024, located in Taylor Hall, room 312C). Only study personnel will have access to the blood and stool samples. All samples will be stored at UNC MCF until the study is completed.

8. Study Measurements and Evaluations

8.1. Outcome Measures for Evaluation of Feasibility / Tolerability

Tolerability will be assessed and recorded by the following parameters:

(1) Occurrence of drop-out
(2) Adherence/compliance
(3) Side effects (bloating, flatulence, belching, abdominal pain, discomfort, watery stool, and vomiting) will be monitored every week via text, email, or phone call using the PROMIS Gastrointestinal Symptom Scale.

8.2. Outcome Measures for Evaluation of Efficacy

N/A

8.3. Outcome Measures for Evaluation of Safety

The incidence and severity of AEs will be assessed during the study at visit 2 and every week via email, phone call, and/or text messages.

8.4. Other Outcomes in the Causal Pathway

N/A

8.5. Baseline Characteristics of the Participants

Apparently healthy adults 18-55 years old. All participants will be nonsmoking and well-nourished according to standard anthropometric criteria.

Enrollment will not be restricted based on race, ethnicity, or gender. Subject population will reflect the population in the long-term care facility providing a broad selection of individuals to allow to enroll subjects from all races, ethnicities and genders as represented in North Carolina state.

8.6. Variables Representing Treatment

A total of 45 individuals will be randomized 1:1:1 (placebo, GOS, hGOS) to treatment. Allowing for a 20% drop out rate, approximately 55 - 60 subjects will be enrolled.

9. Statistical Analysis Plans

a. Strategies that Apply to all the Specific Aims

(1) To help ensure replicability of the research, the analysis plans will be reviewed and finalized prior to collection of data (a priori). For the specific aim, the analysis plan specifies detailed steps for obtaining estimates of population parameters (e.g intervention safety) and for making inferences.
(2) Sensitivity analyses will be performed to assess the robustness of the major results as indicated by their sensitivity to reasonable perturbations of the choices of the methods and assumptions used. Any question about the optimal choice of methods and assumptions are best handled by relegating competing approaches to roles in the domain of sensitivity analyses. Results of the sensitivity analyses will be used to guide our level of trust in the main results. Best practices for dealing with incomplete data will depend on the documented causes of missing, censored, and coarsened values. The reasons for missing data values, drop-out, and protocol departures will have been documented in/with the database.
(3) The plan also includes outcome-dependent exploratory analyses to generate new hypotheses.
(4) Descriptive graphical and tabular methods will be used to characterize the participants, visualize the data, and examine relationships among variables.
(5) To indicate precision, all statistical estimates of population parameters will be tabulated along with corresponding confidence intervals (CI) or standard errors (SE). The CI will be interpreted as the set of potential values of the population parameter that are most compatible with the observed data.
(6) All hypothesis tests yielding p-values that are deemed to be not statistically significant will be reported as being inconclusive. The proposed statistical analysis strategy acknowledges that no p-value can reveal the plausibility, presence, truth, or importance of an association or effect--which is consistent with the statements of the American Statistical Association [4,5], the recommendations in Nature [6,7], and guidance, such as the CONSORT Statement [1], STROBE Statement [2], and ICMJE guidance [3].[2]]

[2] [1] www.consort-statement.org; [2] www.strobe-statement.org; [3] www.icmje.org; [4] Wasserstein RL, et al. (2016), The ASA's Statement on p-Values, ***The American Statistician***, 70:2, 129-133. [5] Wasserstein RL, et al. (2019), Moving to a World Beyond p < 0.05, ***The American Statistician***, 73:sup1, 1-19. [6] Amrhein, et al. (2019) Scientists rise up against statistical significance, ***Nature*** 567, 305-307. [7] Editorial (2019) It's time to talk about

9.1. Description of the Study Cohort

A table will be used to summarize the participant cohort characteristics (age, gender) as well as basic dietary information. Figures will be used to feature microbiome analysis data and inflammatory biomarkers.

9.2.Aim-Specific Plans

Plans for Aim 1 (A1): Validate 15 g/day dose safety and tolerability (absence of GI adverse effects) of GOS and hGOS in healthy adults.

Method. We will report descriptive statistics for demographics, basic medical history, prescription and over the counter, and dietary supplements, including means and standard deviations for continuous variables, and percentages and ranges for categorical data. We will report the number and percent of all individuals (intent to treat) who had any adverse event (prompted or in response to an open-ended question). We will also report the number and percent of events over time, as well as the severity of the event.

Statistical analysis for A1. Data will be presented as mean ± SD for variables that are normally distributed, or median (IQR) for variables not normally distributed. Binary/categorical outcomes will be reported as a percentage with confidence interval.

Plans for Aim 2: Establish a biological signature of GOS and hGOS through assessment of prebiotics effects *vs* placebo on:

(A2.1) Abundance of beneficial gut bacteria and restoration of the gut microbiome saccharolytic potential

Method. Determination of abundance of beneficial bacteria (*Bifidobacterium* and *Akkermansia* species) in the gut microbiome of treatment and placebo groups and the microbiome saccharolytic potential by Whole Genome Shotgun (WGS) sequencing[55]. For assignment of saccharolytic (SAC) and non-saccharolytic (NON_SAC) phenotypes we will use the reference study by Vieira-Silva *et al*.[26] as described[9].

Statistical analysis (A2.1). we will perform both exploratory analysis and statistical testing for changes in plasma cytokines. Data will be presented as mean ± SD. To find the change of microbiome abundance under difference circumstance, Mann-Whitney-Wilcoxon matched pair tests between baseline and 4 weeks follow-up will be performed for abundance of beneficial gut bacteria in each treatment arm. To compare the change of microbiome abundance in three arms, we will apply the Kruskal-Wallis test whether the change of microbiome abundance is the same among three groups. Due to multiple testing was proposed in A2.1, p values will be corrected for the total number of comparisons with a controlled false-discovery rate (FDR)

ditching statistical significance: Looking beyond a much used and abused measure would make science harder, but better. ***Nature*** 567, 283-283.

(A2.2) Modulation of biomarkers of inflammation

Rationale. High-sensitivity C-reactive protein (hs-CRP) is a recognized marker of systemic inflammation[34] and specific pro-inflammatory cytokines mediate interaction between immune cells and non-immune cells, contributing to the inflammatory status of the intestine[35, 36]. Based on previous studies with probiotics and prebiotics that report potential immunomodulatory effects[37-39], we decided to monitor hs-CRP, IL-1α, IL-1β, IL-2, IL-6, Il-8, IL-12, TNFα, and IFNγ for the duration of the clinical trial.

Method. Determination of levels of biomarkers of inflammation (IL-1α, IL-1β, IL-2, IL-6, Il-8, IL-12, TNFα, and IFNγ) as well as high-sensitivity C-reactive protein, a marker of low-grade inflammation (hsCRP) in serum. We will determine cytokine levels using the MCYTOMAG-70K (Milliplex) in the Advanced Analytics Core (AAC) of the CGIBD. hsCRP concentration will be measured via hsCRP ELISA (Sigma Aldrich).

Statistical analysis (A2.2). Similar to A2.1, we will perform an exploratory analysis of changes in plasma cytokines. Data will be presented as mean ± SD. To find the change of biomarker of interest under difference circumstance, paired t-test for matched pair between baseline and 4 weeks follow-up will be performed for levels of biomarkers in each treatment arm. To compare the change of biomarker level in three arms, we will use ANOVA to test whether the change of biomarker level is the same among three groups. Due to multiple testing, p values will be corrected for the total number of comparisons with a controlled false-discovery rate (FDR)

(A2.3) Evaluation of intestinal barrier function.

Method. Blood samples will be drawn and serum will be used to quantify zonulin using the Zonulin (IDK® Zonulin) (Serum) ELISA test (Immundiagnostik AG). Zonulin is a biomarker of impaired intestinal permeability, which has been associated with various disorders[56, 57].

Statistical analysis (A2.3). Since A2.3 has similar data and analytic goal as A2.2, the analytic approach will be the same as what we proposed in A2.2.

9.3. Planned Interim Analyses (If Applicable)

We will review safety data (adverse events) continuously, however, no formal interim analyses are planned.

9.4. Plans for Coping with Withdrawals and Loss-To-Follow-Up

Enrolled participants who discontinue treatment/or withdrawn from study will not be replaced. Allowing for a 20% drop out rate, approximately 55 - 60 subjects will be enrolled.

9.5. Sample Size Rationale

Tolerability: The sample size for tolerability was not determined from power analysis but based on multiple previous clinical trials conducted with GOS[58-61].

Microbiome: Our previous studies[10, 11, 31, 32] conducted with a different but equivalent GOS preparation (purified prebiotics) have demonstrated a consistent and statistically significant increased abundance of the targeted beneficial bacteria: *Bifidobacterium* and *Akkermansia*. The proposed pilot study will recruit

60 subjects. After estimated dropout rate of 20%, the final subjects that will complete the subject will be N=48, which is 16 for each arm. This sample size is chosen given limited funds available with the pilot award supporting this project.

Biomarkers of inflammation: Since this is the first study, we do not have the estimate of effect size so the power cannot be computed. Additionally, due to the limitation of the funding with n=20, we do not expect to have sufficient power to detect differences over time. In that case, we will perform an exploratory analysis of changes in plasma cytokines and biomarkers of inflammation and compare the summary statistics such as mean and standard error.

9.6. Missing data

We expect a 20% drop out during the proposed study. We assume the subjects will drop out randomly and all the missing data is missing completely at random (MCAR). In this case, we will implement complete-case analysis for the primary statistical analysis. However, we will also explore the possibility that the missing data is not MCAR. In the sensitivity analysis, we will compare the drop-out and non-drop group in terms of demographic characteristics and clinical factors. More specifically, we will perform two-group testing between the drop-out and non-drop group using either t-test, Wilcoxon test or chiq-square test depending on distribution and type of the variables of interest. If the missing data is indeed missing at random (MAR), we will perform multiple imputation method to generate the missing values with Rubin's rule to obtain the unbiased inference, which will be compared with results from the primary statistical results.

10. Safety Monitoring and Management

10.1. Risk / Benefit Assessment

Potential Risks: Subjects in the GOS and hGOS groups will consume 7.5-15g/day of the prebiotics or placebo preparations as a drink. Possible side effects of the GOS and hGOS groups may include mild to moderate bloating, gas (flatulence), belching, abdominal pain or discomfort, and vomiting. Individuals will be withdrawn from the study if treatment leads to severe side effects.

Potential Benefits: Substances defined as "prebiotics" (a substrate that is selectively utilized by host microorganisms conferring a health benefit per the latest definition of the International Scientific Association for Probiotics and Prebiotics, ISAPP) have a demonstrated beneficial effect in humans and animals. Galacto-oligosaccharides (GOS) in particular have been shown to alleviate symptoms of lactose intolerance and other minor gastrointestinal manifestations in adequate doses.

A compromised gut barrier, dysbiosis and inflammation are hallmarks of the aging gut with consequences that can range from mild GI manifestations like constipation, abdominal pain, and flatulence, to disorders of the gut-brain axis and other conditions not directly associated with the GI tract. This study will add to the knowledge of beneficial modulation of the microbiota to mediate restoration of the gut barrier and biomarkers of inflammation. A positive outcome from

this study will further advance research to improve nutritional conditions and overall health of older individuals in long-term care.

10.2. Assessment of Safety

Participants will be withdrawn if during the first 10 days of study participation, they experience a 2 level change in one or more of the most reported side effects (bloating, flatulence, watery stools, abdominal pain as assessed by questions 2 (abdominal pain), 6 (abdominal discomfort), 8 (constipation), 16 (diarrhea), 22 (abdominal distention), 26 (abdominal bloating), 31 (nausea), or 33 (vomiting) on the PROMIS Gastrointestinal Symptom Scale as compared to their baseline responses. Participants who have surpassed 10 days of study participation and experience a 2 level change in one or more of the most reported side effects (as described above) as compared to baseline, they will be asked to reduce the dose of study product to one sachet of 7.5 mg per day. The study product can be taken all at once or distributed between two time points. If the PROMIS Gastrointestinal Symptom Scale does not improve upon reducing the dose, the participant will be withdrawn from the study. If 5 or more of the first 20 subjects enrolled experience a 2 level change in the most commonly reported side effects (bloating, flatulence, watery stools, abdominal pain) compared to baseline or higher adverse event leading to subject discontinuation, we will stop the study temporarily and request the medical monitor to do an unblinded assessment of the data. The medical monitor (unblinded) will assess subjects who discontinued due to the specified adverse effects to determine if they are all in one arm of the study or range across arms. The medical monitor will assess if there is a safety signal in a specific arm or treatment dose over the other that warrants the closure of the study. Since the medical monitor will be the person determining continuation or discontinuation of the study, the study team will remain blinded and unbiased.

Participants who choose to withdraw from the study will be followed up (via text or email communication) to determine safety and efficacy outcomes. Reasons for discontinuation will be documented in the study RedCap database.

10.3. Unanticipated Problems, Adverse Events, Serious Adverse Events

Unanticipated Problems: An unanticipated problem is any incident, experience or outcome that meets all three OHRP criteria (1) unexpected (in severity, specificity, frequency, or nature), (2) related or possibly related to the research, and (3) suggests the research places subjects or others at greater risk than previously known or recognized.

Adverse Event (AE) Definitions: An AE is “any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related.” For the purposes of this definition, “untoward” means unfavorable, negative, or harmful. An AE is any event observed or reported that is associated with the intervention, without regard to causality.

Serious Adverse Events (SAE) Definition: An AE or suspected adverse reaction will be considered “serious” if, in the view of either the Investigator or Sponsor, it results in any of the following outcomes:

- Death;
- Is life-threatening (immediate risk of death);

- Requires inpatient hospitalization or prolongation of existing hospitalization;
- Results in persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions;
- Results in congenital anomaly/birth defect;
- Is an important medical event that may require an intervention to prevent one of the outcomes listed above.

Grading the Severity of Adverse Events and Events of 'Special Interest':

The severity or intensity of an AE describes the degree of impact upon the subject and/or the need for, and the extent of medical care required to treat the AE. The Investigator will use the adjectives MILD, MODERATE, or SEVERE to describe the maximum intensity of the AE. For purposes of consistency, these intensity grades are defined as follows:

MILD	Does not interfere with subject's usual function.
MODERATE	Interferes to some extent with subject's usual function.
SEVERE	Interferes significantly with subject's usual function.

Relatedness Definition:

The Investigator will assess relatedness for all AEs (serious and non-serious). Factors considered when assessing the relationship of an event to the investigational product:

(1) Alternative etiology – Is the event due to the underlying disorder being studied or to another known disorder?
(2) Known relationship – Has the event been observed before in patients treated with this investigational product or similar products?
(3) Temporal relationship – Is there a reasonable temporal relationship between the time of onset of the event and the administration of the investigational product?
(4) Concomitant medication – Is the event a known side effect of a concomitant medication?

Based on the previous factors we will consider the following descriptions when assessing relationship of an event to the investigational product:

Definite: There is a reasonable causal relationship between the investigational product and the AE, when the event responds to withdrawal of the investigational product (de-challenge) and recurs with re-challenge by administration of the investigational product.
Probable: There is a reasonable causal relationship between the investigational product and the AE. The event responds to de-challenge. Re-challenge not required.
Possible: There is a reasonable causal relationship between the investigational product and the AE. De-challenge is lacking or unclear.
Not Likely: There is a temporal relationship between the investigational product administration and the AE, but there is not a reasonable causal relationship between investigational product and the event.
Unrelated: There is not a temporal or causal relationship to the investigational product administration.

The Investigator will record causal relationship in the source documents, as appropriate, and report such an assessment in accordance with the SAE reporting requirements, if applicable.

Expectedness Definition: An assessment of expectedness will be performed for each AE and SAE. Previously reported expected effects of prebiotic administration include abdominal pain (cramps), bloating, flatulence (gas) and diarrhea (loose stools >3 times/day). During the study, GI side effects will be expected and only Aes that are meet the definition of severe AE or considered an SAE will be reported to the IRB or FDA.

AE and SAE Assessment, Follow-up Procedures: The Investigator will record all directly observed AEs and all AEs spontaneously reported by the study subject beginning from the time the subject begins use of the investigational product through 7 days after the last dose of the investigational product. Changes in health status after the subject has provided informed consent but before the subject begins use of the investigational product shall be recorded as changes in the medical history.
The Investigator will assess subjects at each visit for the occurrence of AEs. In order to avoid bias in eliciting AEs, subjects should be asked the following non-leading question:
"Have there been any changes in your health since your last visit?"

All AEs (serious and non-serious) reported by the subject must be recorded regardless of if a causal relationship with the investigational product is suspected. Each AE will be assessed to determine if it meets the criteria for a serious adverse event (SAE). If an SAE occurs, expedited reporting will follow local and international regulations as appropriate.

Reporting and Documentation Procedures: AE and SAE reporting periods will extend from the signature of the informed consent through the End of the Study Visit or until 7 days after the last dose of the investigational product. If an SAE occurs, Dr. Becker-Dreps will be notified within 24 hours of awareness of the event by the Investigator. This timeframe also applies to adding new information (follow-up) on previously forwarded SAE reports. In the rare event that the Investigator does not become aware of the occurrence of an SAE immediately, the Investigator will report the event within 24 hours of first awareness of the SAE and document the time of his/her first awareness of the SAE. For all SAEs, the Investigator will pursue and provide information to the Sponsor or designee in accordance with the timeframes for reporting specified above. This will generally include a description of the AE in sufficient detail to allow for a complete medical assessment of the case and independent determination of possible causality. Information on other possible causes of the event, such as concomitant medications and illnesses must be provided. All observed or volunteered AEs regardless of treatment group or suspected causal relationship to the investigational product(s) will be reported.

Participant Notification of New Information:

Participant will be notified via email, text message and/or phone call.

10.4. Safety Monitoring

Investigators will assess subjects at each visit and over the phone or via text (or email) each week of the study at days 0, 7±2, 14±2, 21±2, and 28±2 as well as day 7±2 post-intervention. If AEs as defined in this protocol are identified (a 2 level change in one or more of the symptoms as compared to baseline as assessed by questions 2 (abdominal pain), 6 (abdominal discomfort), 8 (constipation), 16 (diarrhea), 22 (abdominal distention), 26 (abdominal bloating), 31 (nausea), or 33 (vomiting) on the PROMIS

Gastrointestinal Symptom Scale). The treatment will be first be reduced to one 7.5g sachet per day (taken either at once or divided between two time periods if the participant has been participating for ≥ 10 days. The treatment will be discontinued if the participant is in the first 10 days of participation, and the AE will be recorded. Also, participants who report severe side effects (interfering significantly with subject's usual function) will be withdrawn from the study immediately. If 5 or more of the first 20 subjects enrolled experience a 2 level change in the most reported side effects (bloating, flatulence, watery stools, abdominal pain as assessed by questions 2 (abdominal pain), 6 (abdominal discomfort), 8 (constipation), 16 (diarrhea), 22 (abdominal distention), 26 (abdominal bloating), 31 (nausea), or 33 (vomiting) on the PROMIS Gastrointestinal Symptom Scale compared to baseline or higher adverse event leading to subject discontinuation, we will stop the study temporarily and request the medical monitor to do an unblinded assessment of the data.

10.5. Study Suspension / Early Termination of the Study

Reports of severe adverse events interfering significantly with daily function in >20% of participants will lead to early termination of the study. Premature termination of this study may also occur because of unexpected, significant or unacceptable risk to participants, incomplete or unevaluable data, determination of futility, a regulatory authority decision, change in opinion of the IRB, or intervention safety problems. All investigators in the study will be notified of events that may warrant early termination of the study.

11. Supporting Documentation and Operational Considerations

11.1. Regulatory, Ethical, and Study Oversight Considerations

11.1.1. Informed Consent Process

11.1.1.1. Consent/Assent and Other Informational Documents Provided to Participants

Consent forms describing in detail the study intervention, study procedures, and risks will be provided to the participant virtually and written documentation of informed consent is required prior to starting intervention/administering study intervention. The following consent materials are submitted with this protocol:

Adult Consent Form
Consent Addendum for Unencrypted Communication

11.1.1.2. Consent Procedures and Documentation

Informed consent is a process initiated prior to the individual's agreeing to participate in the study and continues throughout the individual's study participation. Consent forms will be approved by the Institutional Review Board (IRB), and the participant will be asked to read and review the document. The

consent process will occur virtually over zoom. The investigator will explain the research study to the participant and answer any questions that may arise. A verbal explanation will be provided suited to the participant's comprehension of the purposes, procedures, and potential risks of the study and of their rights as research participants. Participants will be able to carefully review the written consent form and ask questions before signing. The participants should have the opportunity to discuss the study with their family or surrogates or think about it prior to agreeing to participate. The participant will sign the informed consent document electronically prior to any procedures being done specifically for the study. Participants must be informed that participation is voluntary and that they may withdraw from the study at any time without prejudice. The participants will be sent an electronic copy and given a printed copy of the informed consent document for their records. The informed consent process will be conducted and documented in the source document (including the date), and the form signed before the participant undergoes any study-specific procedures.

11.2. Study Discontinuation and Closure

This study may be suspended or prematurely terminated if there is sufficient reasonable cause. Written notification, documenting the reason for study suspension or termination, will be provided by the suspending, or terminating party to study participants, investigator, funding agency, and regulatory authorities. If the study is prematurely terminated or suspended, the Principal Investigator (PI) will promptly inform study participants, the Institutional Review Board (IRB), and sponsor and will provide the reason(s) for the termination or suspension. Study participants will be contacted, as applicable, and be informed of changes to study visit schedule.

Circumstances that may warrant termination or suspension include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to participants
- Demonstration of efficacy that would warrant stopping
- Insufficient compliance to protocol requirements
- Data that are not sufficiently complete and/or evaluable
- Determination that the primary endpoint has been met
- Determination of futility

Study may resume once concerns about safety, protocol compliance, and data quality are addressed, and satisfy the sponsor, and the IRB.

11.3. Confidentiality and Privacy

Participant confidentiality and privacy is strictly held in trust by the participating investigators, their staff, and the sponsor. This confidentiality is extended to cover testing of biological samples in addition to the clinical information relating to participants. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without the prior written approval of the sponsor.

All research activities will be conducted in as private a setting as possible.

The study monitor, other authorized representatives of the sponsor, representatives of the Institutional Review Board (IRB), regulatory agencies, or pharmaceutical companies supplying the study product may inspect all documents and records required to be maintained by the investigator. The clinical study site will permit access to such records.

The study participant's contact information will be securely stored at the site for internal use during the study. At the end of the study, all records will continue to be kept in a secure location for as long a period as dictated by the reviewing IRB, Institutional policies, or sponsor requirements.

Study participant research data, which is for purposes of statistical analysis and scientific reporting, will be transmitted to and stored in RedCap. This will not include the participant's contact or identifying information. Rather, individual participants and their research data will be identified by a unique study identification number. The study data entry and study management systems used by clinical sites and by research staff will be secured and password protected. At the end of the study, all study databases will be de-identified and archived.

Certificate of Confidentiality (if applicable)

To further protect the privacy of study participants, a Certificate of Confidentiality will be issued by the National Institutes of Health (NIH). This certificate protects identifiable research information from forced disclosure. It allows the investigator and others who have access to research records to refuse to disclose identifying information on research participation in any civil, criminal, administrative, legislative, or other proceeding, whether at the federal, state, or local level. By protecting researchers and institutions from being compelled to disclose information that would identify research participants, Certificates of Confidentiality help achieve the research objectives and promote participation in studies by helping assure confidentiality and privacy to participants.

11.4. Future Use of Stored Specimens and Data

Data collected for this study will be analyzed by the investigators of this study and stored in RedCap. After the study is completed, de-identified, sequencing microbiome data will be submitted to the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI), for use by other researchers including those outside of the study. Permission to transmit data will be included in the informed consent.

With the participant's approval and as approved by local Institutional Review Board (IRB), de-identified biological samples will be stored at the UNC Microbiome Core. These samples could be used to research the gut microbiome and intestinal permeability, and other conditions for which older adult individuals are at increased risk.

During the study, an individual participant can choose to withdraw consent to have biological specimens stored for future research. However, withdrawal of consent with regard to biosample storage may not be possible after the study is completed.

11.5. Key Roles and Study Governance

Principal Investigator

Sylvia Becker Dreps, MD, MPH
UNC – Chapel Hill
590 Manning Drive, CB # 7595 Chapel Hill, NC 27599-7595
(984)-974-4888; (919) 943-7445
sbd@email.unc.edu

Overall structure of the study team

Dr. Sylvia Becker Dreps, MD, MPH is the Principal Investigator in the proposed studies.

Dr. Andrea Azcarate-Peril, PhD is a collaborator in the proposed studies and will assist with data analysis of de-identified data.

Dr. Vivan Lee MD (Co-investigator) is a clinical gastroenterologist with experience in patients with a broad spectrum of gastrointestinal conditions including colon polyps, gastrointestinal bleeding, gastrointestinal infections, heartburn, abdominal pain, dyspepsia, IBD, constipation and diarrhea. She will serve as a medical monitor if needed during the study.

Ms. Kelli Hammond (Clinical research coordinator) will assist with day to day study activities, consent process, participant contact, and data entry.

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Dr. Roach, Ph.D. (Co-investigator, Bioinformatician) and Dr. Kia, Ph.D. (Co-investigator, Bioinformatics, and biostatistics specialist) will provide support on data analysis and interpretation.

11.6. Safety Oversight

Safety oversight will be under the direction of the study's principal investigator..

11.7. Clinical Monitoring

Clinical site monitoring is conducted to ensure that the rights and well-being of trial participants are protected, that the reported trial data are accurate, complete, and verifiable, and that the conduct of the trial follows the currently approved protocol/amendment(s), with International Conference on Harmonization Good Clinical Practice (ICH GCP), and with applicable regulatory requirement(s).

- The Medical Monitor or designee will perform monitoring of the study in cooperation with the Investigator, including review of AE reports.
- When additional information is required, the Medical Monitor will contact the Investigator or designee.

11.8. Quality Assurance and Quality Control

The site will perform internal quality management of study conduct, data and biological specimen collection, documentation, and completion. A quality management plan will be developed to describe a site's quality management.

Quality control (QC) procedures will be implemented beginning with the data entry system and data QC checks that will be run on the database will be generated. Any missing data or data anomalies will be communicated for clarification/resolution.

Following written Standard Operating Procedures (SOPs), the PI will verify that the clinical trial is conducted and data are generated, and biological specimens are collected, documented (recorded), and reported in compliance with the protocol, International Conference on Harmonization Good Clinical Practice (ICH GCP), and applicable regulatory requirements (e.g., Good Laboratory Practices (GLP), Good Manufacturing Practices (GMP)).

The investigational site will provide direct access to all trial related sites, source data/documents, and reports for the purpose of monitoring and auditing by the sponsor, and inspection by local and regulatory authorities.

11.9. Data Handling and Record Keeping

11.9.1. Data Collection and Management Responsibilities

Data collection is the responsibility of the clinical trial staff at the site under the supervision of the site investigator. The investigator is responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported.

All source documents should be completed in a neat, legible manner to ensure accurate interpretation of data.

Hardcopies of the study visit worksheets will be provided for use as source document worksheets for recording data for each participant enrolled in the study. Data recorded in the electronic case report form (eCRF) derived from source documents should be consistent with the data recorded on the source documents.

Clinical data (including adverse events (AEs), concomitant medications, and expected adverse reactions data) and clinical laboratory data will be entered into RedCap a 21 CFR Part 11-compliant data capture system provided by UNC Chapel Hill. The data system includes password protection and internal quality checks, such as automatic range checks, to identify inconsistent, incomplete, or inaccurate data. Clinical data will be entered directly from the source documents.

11.9.2. Study Records Retention

Study documents should be retained for a minimum of 2 years after the last approval of a marketing application in an International Conference on Harmonization (ICH) region and until there are no pending or contemplated marketing applications in an ICH region or until at least 2 years have elapsed since the formal discontinuation of clinical development of the study intervention. These documents should be retained for a longer period, however, if required by local regulations. No records will be destroyed without the written consent of the sponsor, if applicable. It is the responsibility of the sponsor to inform the investigator when these documents no longer need to be retained.

11.10. Protocol Deviations

A protocol deviation is any noncompliance with the clinical trial protocol, International Conference on Harmonization Good Clinical Practice (ICH GCP), or Manual of Procedures (MOP) requirements. The noncompliance may be either on the part of the participant, the investigator, or the study site staff. As a result of deviations, corrective actions are to be developed by the site and implemented promptly. These practices are consistent with ICH GCP:

- 4.5 Compliance with Protocol, sections 4.5.1, 4.5.2, and 4.5.3
- 5.1 Quality Assurance and Quality Control, section 5.1.1
- 5.20 Noncompliance, sections 5.20.1, and 5.20.2.

It will be the responsibility of the site investigator to use continuous vigilance to identify and report deviations within 2 working days of identification of the protocol deviation, or within 2 working days of the scheduled protocol-required activity. Protocol deviations will be sent to the reviewing Institutional Review Board (IRB) per their policies. The site investigator will be responsible for knowing and adhering to the reviewing IRB requirements. Further details about the handling of protocol deviations will be included in the MOP.

11.11. Publication and Data Sharing Policy

This study will be conducted in accordance with the following publication and data sharing policies and regulations:
National Institutes of Health (NIH) Public Access Policy, which ensures that the public has access to the published results of NIH funded research. It requires scientists to submit final peer-reviewed journal manuscripts that arise from NIH funds to the digital archive PubMed Central upon acceptance for publication.
This study will comply with the NIH Data Sharing Policy and Policy on the Dissemination of NIH-Funded Clinical Trial Information and the Clinical Trials Registration and Results Information Submission rule. As such, this trial will be registered at ClinicalTrials.gov, and results information from this trial will be submitted to ClinicalTrials.gov. In addition, every attempt will be made to publish results in peer-reviewed journals. Data from this study may be requested from other researchers 10 years after the completion of the primary endpoint by contacting Dr. Becker-Dreps.
In addition, this study will comply with the NIH Genomic Data Sharing Policy, which applies to all NIH-funded research that generates large-scale human or non-human genomic data, as well as the use of these data for subsequent research.

11.12. Conflict of Interest Policy

The independence of this study from any actual or perceived influence, such as by the pharmaceutical industry, is critical. Therefore, any actual conflict of interest of persons who have a role in the design, conduct, analysis, publication, or any aspect of this trial will be disclosed and managed. Furthermore, persons who have a perceived conflict of interest will be required to have such conflicts managed in a way that is appropriate to their participation in the design and conduct of this trial. The study leadership in conjunction with the North Carolina Translational and Clinical Sciences Institute and UNC Chapel Hill has established policies and procedures for all study group members to disclose all conflicts of interest and will establish a mechanism for the management of all reported dualities of interest.

12. Additional Considerations

This section should include a description of any additional considerations not currently covered in this protocol template, such as particular institutional or IRB-related requirements.

<Insert text>

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14. List of Appendices

This section should include a listing of any tables, questionnaires, investigator brochure, device manual, subject 'handouts', etc that should accompany the study protocol.

Appendix 1: 38-point Gastrointestinal Symptom and Severity Checklist (GSSC)

Appendix 2: Food frequency questionnaire.

Appendix 3: [include version number and date, and a short description.]