University of Pennsylvania

Functional Research on Emulsifiers in Humans (FRESH)

Regulatory Sponsor:	*James D. Lewis, MD, MSCE* *Department of Medicine and Department of Biostatistics and Epidemiology* *720 Blockley Hall* *215-573-5137*
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Administrative Change:	

CONFIDENTIAL

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List of Abbreviations

CRF – case report form
CHPS - Center for Human Phenomic Science (CHPS)
IRB – Internal Review Board
EC – Ethics Committee
CMC – carboxymethylcellulose

Study Summary

Title	Functional Research on Emulsifiers in Humans
Short Title	FRESH Study
Protocol Number	828422
Phase	Not applicable
Methodology	Controlled feeding experiment
Study Duration	161 days
Study Center(s)	1
Objectives	Establish a tractable and physiologic means of administering and measuring CMC consumption and its metabolic impact in healthy volunteers. Examine extent to which CMC consumption impacts human gut microbiota composition, gene expression, and/or localization. Explore effects of CMC consumption on a range of inflammatory and metabolic parameters that characterize metabolic syndrome.
Number of Participants	20
Diagnosis and Main Inclusion Criteria	Healthy volunteers
Study Product, Dose, Route, Regimen	Participants will be randomly assigned to receive 0 or 15 gm per day of carboxymethylcellulose in 6 divided doses baked in chocolate brownies and served in sorbet
Duration of administration	11 days
Reference therapy	None
Statistical Methodology	Determination of the effect of CMC consumption on the fecal and mucosal microbiome composition and inflammatory and metabolic parameters. Comparison of microbial metabolites across diets (with CMC versus without CMC).

1 Introduction

This document is a protocol for a human research study. This study is to be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Institutional research policies and procedures.

1.1 Background

The human intestine is home to some 100 trillion microorganisms. The density of bacterial cells in the colon has been estimated at 10^{11} to 10^{12} per ml, which makes the colon one of the most densely populated microbial habitats known on earth. The genome size of this pool of intestinal microbes is estimated to exceed the size of the human nuclear genome by two orders of magnitude[1]. There are far more total cells in the gut microbial population than human cells in our bodies--indeed, it has been stated that "there are more of them in us than us"[2 1, 3].

The great majority of these gut bacterial species have not been cultured outside the human host, so most species are little studied. Recent advances in scientific methods have allowed for new surveys of the intestinal microbiota using DNA sequencing of uncultured communities[1, 4, 5] or microarray-based methods[6]. Many of these studies used 16S ribosomal DNA sequences to query the different bacterial taxa, since 16S sequences are similar enough to allow amplification of most bacterial species with single primer pairs, but diverse enough to allow "finger printing" of taxa to relatively fine taxonomic levels[7]. Such studies have revealed that major bacterial phyla in the human gut include *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and various minor groups. Other recent studies extended this analytical approach by sequencing fragments of bulk genomic DNA from intestinal microbiota instead of 16S DNA[3, 8-11]. Metagenomic analysis allows inferences to be made about the community taxonomy and the metabolic pathways in the intestinal microbiota inferred from the genes present. For example, the Gill et al. study[10] disclosed that the intestinal microbiota contribute unique pathways for metabolism of glycans, amino acids, xenobiotics, methanogenesis, vitamins, isoprenoids, and other compounds.

When maintained in a stable manner, at an appropriately safe distance from gut epithelial cells, the microbiota provides benefits to the host, especially in terms of energy harvest, pathogen exclusion, and promotion of immune development. In contrast, disturbance of the microbiota-host relationship can drive chronic gut inflammation that can promote an array of chronic inflammatory diseases including metabolic syndrome. While host genetics are an important determinant of the host-microbiota relationship, the dramatic increase in incidence of numerous chronic inflammatory diseases, including inflammatory bowel disease (IBD) and metabolic syndrome over the last 60 years amidst relatively constant genetics, and highlights the importance of understanding how non-genetic factors such as diet might alter the microbiota-host relationship to promote gut inflammation. Consequently, we and others have focused on how differences in macronutrient consumption influence the microbiota. However, dietary patterns can also be characterized by constituents other than macro and micronutrients. Post-mid-20th century dietary changes include consumption of an array of synthetic compounds that are added to many processed foods. The biologic effect of these food additives are not well described, particularly

their potential to influence microbiota composition and/or its interaction with the host. One particularly disconcerting class of compounds are synthetic dietary emulsifiers including polysorbate 80 (P80)and carboxymethylcellulose (CMC). These detergent-like compounds, which are incorporated into a variety of processed foods, are not well-absorbed, thus allowing them to interact with the microbiota and gut mucosa. We have recently shown that, in mice, consumption of P80 and CMC, alters microbiota composition, have pro-inflammatory potential and promote microbiota encroachment into the colonic mucosa, low-grade inflammation, and metabolic syndrome[1]. However, it is not known whether these compounds have similar effects in humans. Addressing this critically important question is the central goal of this study. Our initial focus will be on CMC, which, despite not having been tested in humans and not widely studied in animals, has "generally regarded as safe (GRAS)" status from the US FDA, allowing it to be widely and unrestrictedly used in a broad variety of processed foods.

1.2 Investigational Agent

CMC will be purchased from Modernist Pantry who supplies CMC to small bakeries throughout the United States.

1.3 Preclinical Data

This proposal seeks to address the post-mid-20th century increased incidence of chronic inflammatory diseases of the intestine. Crohn's disease and ulcerative colitis, collectively referred to as the inflammatory bowel diseases (IBD), are debilitating diseases that may afflict up to 3 million Americans [12]. Like many immune-mediated diseases, the incidence of IBD is increasing in the US and elsewhere at rates far in excess of that expected by genetic drift. A milder but much more common form of gut inflammation referred to as "low-grade" inflammation, is defined by modestly elevated pro-inflammatory gene expression amidst a lack of histopathologically-evident inflammation [13]. Such low-grade inflammation associates with, and may promote, metabolic syndrome, which is a group of interrelated metabolic disorders including obesity, insulin resistance, hyperglycemia, hyperlipidemia, and hepatic steatosis [14]. Humanity is facing an epidemic of metabolic syndrome, and its downstream consequences, including type 2 diabetes, cardiovascular disease, and liver dysfunction that threaten to overwhelm the world's healthcare systems and economies thus making it a top public health priority in dire need of investigation. Our research suggests that IBD, metabolic syndrome, and other chronic inflammatory diseases may share common underlying causes, including a disturbed host-microbiota relationship. In accord with this notion, recent studies demonstrate that infections in infants increase likelihood of developing both IBD [15] and obesity [16].

Thus, better understanding of factors that can alter the host-microbiota relationship is of paramount importance. Many candidate factors can be reasonably envisaged to have impacted the microbiota (e.g. antibiotics, polymers such as BPA, pollutants, enhanced sanitation, etc.). Recent studies suggest that some commonly used dietary emulsifiers, which are detergent-like molecules that are incorporated into most processed foods to improve texture and stability, may be one specific contributor to the increased prevalence of chronic inflammatory diseases [17, 18]. We reported that feeding mice CMC and P80 results in a microbiota that encroaches upon the epithelium and has inherently greater potential to activate host pro-inflammatory signaling [19]. Such alterations promote

chronic low-grade inflammation and a metabolic syndrome-like phenotype in mice and increased incidence of severe colitis in mice that are genetically pre-disposed to this disorder. Investigation of these commonly consumed but poorly studied synthetic compounds to determine their impact on human biology, in a manner analogous to our mouse studies, is of the utmost significance to human health.

1.3.1 Risk-Benefit Balance

The risks associated with participating in this controlled feeding experiment are low. The diets will be constructed to meet all nutritional requirements. The CMC will be purchased from Modernist Pantry and is the same as that which is sold to small bakeries throughout the United States. Thus, consumption of the study diet entails no greater risk than consumption of a typical American diet outside of the study procedures.

Participants will undergo phlebotomy and sigmoidoscopy with biopsy. There is a small potential risk for complications of sigmoidoscopy, including bleeding and perforation. The risks of these complications are estimated to be less than 1% for bleeding and less than 0.1% for perforation. Patients with contraindications to biopsy will be excluded. The risks of phlebotomy include bleeding, infection, phlebitis, and fainting. As with all research, there is a small risk of loss of confidentiality. Participants may also experience some discomfort associated with eating a limited diet for 14 days. Participants may also feel tired and/or discomfort from staying in the hospital for the 11 day inpatient stay.

In summary, the risk of serious or severe adverse events is likely extremely low. While there is no direct benefit expected for the participants, the balance of the participants' risk vs. the knowledge to be gained is believed to be favorable.

2 Study Objectives

The specific aims for our proposal are:

1. Establish a tractable and physiologic means of administering and measuring CMC consumption and its metabolic impact in healthy volunteers.

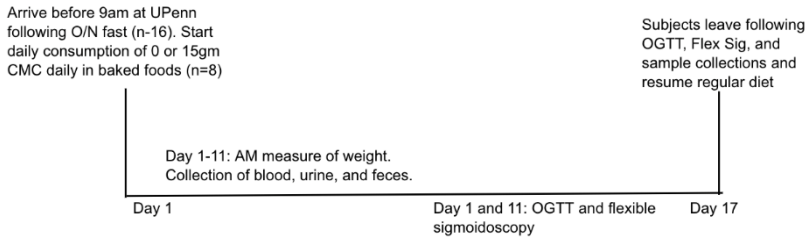
2. Examine extent to which CMC consumption impacts human gut microbiota composition, gene expression, and/or localization.

3. Explore effects of CMC consumption on a range of inflammatory and metabolic parameters that characterize metabolic syndrome.

3 Study Design

3.1 General Design

Figure 1. Schematic Study Outline



This is a randomized double-blind, controlled feeding experiment examining the effect of CMC on the stool microbiota composition, gene expression, and microbiota localization with respect to the epithelium. In addition, we will assess the impact of CMC exposure in the diet on markers of metabolic syndrome including response to oral glucose tolerance testing and other plasma metabolite measurements.

Healthy volunteers will be recruited for a 11-day in-patient study. All participants will receive 3 full days of freshly prepared emulsifier-free food to begin the study. This will be called the “washout period”. Participants will then be randomized into an “emulsifier-free” arm and a “CMC” arm.

Both arms will continue the same diet except that the “CMC” arm’s brownie and sorbet will have CMC added to achieve a level of 15 gm per person per day consumption, which are the levels of emulsifier consumption that can readily be achieved by humans who eat a large amount of processed foods. If the average American eats 2kg of processed food per day containing 2% emulsifier (the limit of individual emulsifiers), this would amount to 40g of emulsifiers per day. Serial collection of fecal, blood, urine and mucosal biopsy samples will define our ability to reliably quantitate CMC consumption by assay of biological fluids and preliminarily investigate the extent to which CMC consumption by humans impacts the gut microbiota and its interaction with the host parameters related to inflammation and metabolism.

3.2 Primary Study Endpoints

This is not an efficacy study. There are no pre-specified efficacy or safety endpoints for this study. Rather, we will use an unbiased approach to determine the composition of the microbiota and metabolome.

4 Participant Selection and Withdrawal

4.1 Inclusion Criteria

1. Participant is capable of giving informed consent
2. Participant is age 18 to 60 years

4.2 Exclusion Criteria

1. Diagnosis with IBD, celiac disease, or other chronic intestinal disorders. Since we are interested in assessing the impact of diet on the microbiome in the absence of pathologic inflammation, we will exclude participants with chronic intestinal abnormalities.

2. Baseline bowel frequency less than every 2 days or greater than 3 times daily. Normal bowel frequency is every 3rd day to 3 times per day.[20-23] Although unknown, stool frequency could be related to the microbiome composition.[24-26] Furthermore, change in diet could alter baseline stool frequency, potentially causing diarrhea, particularly in those with high baseline stool frequency, or severe constipation in those with low stool frequency. To avoid the need for use of antidiarrheal medications or laxatives, which themselves could alter the microbiome composition, these patients will be excluded.
3. Current smoker. What effect smoking has on the microbiome of the gut is unknown. Furthermore, because our hospital is a smoke-free environment, volunteers will not be able to smoke. Thus, inclusion of smokers would increase the risk of early withdrawal from the study.
4. Body Mass Index (BMI) <18.5 or >40 at screening. Volunteers with BMI below normal[27] will be excluded to prevent inclusion of participants with a subclinical systemic disease that may influence the gut microbiome. Volunteers with severe obesity will be excluded as obesity may be associated with altered gut microbiome composition.
5. More than two of the criteria for metabolic syndrome:
    - A waist circumference greater than 35 inches (89 centimeters) for women and 40 inches (102 centimeters) for men at screening.
    - A diagnosis of diabetes mellitus or baseline HbA1c > 6.4% or a fasting glucose level of greater than 100mg/dL
    - Systolic blood pressure >130 mmHg or diastolic blood pressure >85 mmHg or treated with medications for hypertension at screening.
    - Fasting triglycerides >149 mg/dl or treated with medications for hypertriglyceridemia
    - Fasting HDL cholesterol <40 mg/dl in men or <50 mg/dl in women or treated with medications for hypercholesterolemia
6. Known substance abuse disorder or consumption of illicit drugs or alcohol in the 24 hours prior to admission to the Center for Human Phenomic Science (CHPS).
7. Prior bowel resection surgery other than appendectomy. It is unknown how prior bowel resection surgery may influence the microbiome composition, hence we will exclude these participants.
8. Contraindication to flexible sigmoidoscopy and biopsies. Patients with suppressed white blood count may be at increased risk of systemic infection following sigmoidoscopy with biopsies. As such, participants with a WBC less than 3,500 or an absolute neutrophil count of less than 1,000 will be excluded. Patients with thrombocytopenia or with a coagulopathy may be at increased risk of bleeding complications after colonoscopic biopsies. As such, patients with a platelet count of less than 100,000 or an INR greater than 1.2 will be excluded from the study.
9. Estimated GFR<60ml/min/1.73m^2 based on measured serum creatinine concentration
10. Pregnant and lactating women. To avoid any risk to an unborn fetus or new born baby from changing the mother's diet, pregnant and lactating women will be excluded.

11. Use of antibiotics in the 6 months prior to Visit 2. A small proportion of bacteria may require 6 months to recover after treatment with antibiotics.[28]
12. Use of antacids, NSAIDs, or dietary supplements in the week prior to Visit 2. NSAIDs have been associated with C. difficile colitis, although whether this is causative and whether this is mediated through changing the fecal microbiota composition is unknown.[29] Antacids could potentially alter the gut microbiota by changing the acid milieu or by altering fecal transit time. For our study purposes, multivitamins will not be considered dietary supplements.
13. Use of laxatives or anti-diarrhea medications in the two weeks prior to Visit 2.
14. Use of anticholinergics in the week prior Visit 2.
15. Use of narcotics in the week prior to Visit 2.
16. HIV infection, AIDS, or other known conditions resulting in immunosuppression - we will determine this by direct participant query; no formal testing will be done.
17. Allergies or intolerance to the components of the study diets.
18. Participant has experienced diarrhea within the two weeks prior to Visit 2. Diarrhea is defined as a change in bowel habits with an increased frequency or loose stools such that the stool could not be lifted with a fork.
19. Refusal to use a medically accepted method of birth control while participating in this study, such as a barrier method, hormonal contraceptives, implanted birth control devices, permanent methods (such as a vasectomy), and/or abstinence.
20. Vegans and Vegetarians.
21. Student or employee of any one of the investigators.
22. Anyone who cannot receive study payment (ie: visa)
23. Any condition that the investigator feels may limit the volunteer's ability to complete the study protocol.

4.3 Participant Recruitment and Screening

Our goal is to have 16 participants complete this study; however, it is anticipated we may need to screen up to 50 participants to meet our target enrollment.

Participants will be recruited through local and web-based advertising. We may also contact participants who have been enrolled in other studies under the same PI in the past if they have consented to future contact. Other participants may contact the research coordinator by phone or email in response to local paper flyers or web based advertisements (i.e.: craigslist). All advertisements will be approved by the local IRB before being used.

Potential participants will be pre-screened via telephone for eligibility prior to the screening visit. At the screening visit, potential participants will be screened for eligibility before being enrolled in the study. Potential participants will be directly queried regarding exclusion criteria. If there are questions regarding eligibility, relevant information from the participant's medical record will be obtained, with appropriate permission for release of records as necessary to comply with HIPAA. In the absence of any concerns, the participant's medical records will not be requested or reviewed as part of this study.

4.4 Early Withdrawal of Participants

4.4.1 When and How to Withdraw Participants

Participants may be withdrawn from the study at any time if the investigator or the participant believes that the participant's safety will be jeopardized by continued participation, or if the participant does not follow study procedures. Likewise, participants will be withdrawn from the study if they withdraw consent.

5 Study Intervention

All foods will be prepared within the CHPS's metabolic kitchen without emulsifiers (unless specifically added). All participants will follow the same Western style diet (i.e. the only difference being portion size). The macronutrient percentages of calories for the study diet will be 55% carbohydrate, 30% fat, and 15% protein. The diet will be composed of two menus that will be consumed on alternating days. Water, coffee and tea will be provided as desired. Participants will have access to additional food outside of the meals provided, however, the entire serving of the previous meal must have been consumed. The additional food provided outside of the scheduled meals will consist of the food from the same Western style diet provided for each meal.

For the three days prior to admission, participants will be eating an emulsifier free diet at home. Food will be provided by the CHPS metabolic kitchen. After admission to CHPS, no emulsifiers will be consumed prior to dinner on Day 1. Thus, accounting for the 8 hours of nil per os status prior to admission**,** all participants will have approximately 80 hours of emulsifier free washout time prior to administration of the food containing CMC.

Participants will be randomly assigned to receive 0 or 15 gm per day of CMC (8 participants in each arm of the study**).** Beginning with the dinner meal on Day 1, all participants will consume one serving of brownie or sorbet (15 gm) per day that contain 0 or 2.5 gm CMC per serving. The brownie and sorbet servings will be provided at all three scheduled meals and scheduled snacks. Prior to eating any other food on the study menu, participants must consume the brownie and sorbet servings.

5.1 Randomization procedures

The double-blind randomization order will be generated by Dr. Hongzhe Li. Because of the small sample size, we will utilize block randomization with a block size of 4 participants.

5.2 Prior and Concomitant Therapy

Participants will be allowed to take their usual outpatient medications. In order to facilitate this, participants will bring these medications with them to the CHPS on the day of admission and they will be administered under the supervision of the nursing staff during the course of their in-patient stay. Participants will be allowed to take acetaminophen as needed for minor ailments, such as a headache. Consumption of concomitant medications will be recorded in the study records daily. Participants will not be permitted to take any medications listed in the exclusion criteria during their participation in the study, prior to discharge from CHPS.

5.3 Supervision of access to food outside of the study protocol

In order to prevent access to food outside of the study protocol, participants will be accompanied by a member of the research staff whenever they leave the CHPS. When sufficient staff is available to supervise the participant, participants will be allowed to walk outside.

5.4 Activity Level

Once a participant is determined to be eligible, they will be given a Fitbit Flex. Participants will be required to wear this Fitbit Flex for at least 3 days prior to admission to CHPS to record the number of steps they take daily. The study team will calculate the average number of daily steps the participant took during the 3 days prior to admission. During the inpatient stay, participants will be required to attain within 10% of the average number of daily steps that they took in the 3 days prior to admission. To achieve this, participants will be escorted on walks outside of the inpatient unit by a coordinator, as needed.

6 Study Procedures

The sequence of study procedures is summarized in Section 6.2. The study procedures section is divided into two parts, one describing the collection methods of samples for metabolic and microbial profiling and a second describing the sequence of events.

6.1 Sample collection methods for metabolic and microbial profiling

6.1.1 Urine sample collection and storage

There is increasing evidence that the composition of the gut microbiome influences the metabolic profile of blood, urine, and other tissues.[30-32] Urine will collected at the post-screening visit and during the inpatient stay. During the post-screening visit, urine will be collected following an overnight fast. During the inpatient stay, urine samples will be collected daily prior to 09:00h after an overnight fast. Up to 15 mL of urine will be obtained for each sample, aliquoted into three 5ml aliquots, and frozen at minus 80°C for use in metabolomic studies.

6.1.2 Blood sample collection and storage

Blood will be collected after an overnight fast prior to breakfast at the screening visit, at the post-screening visit, and on Days 1-4, 8, 10, and 11 of the inpatient study. Approximately 16cc of blood will be collected for metabolomics at each collection. Plasma will be separated from the blood samples. The plasma will be stored frozen at minus 80°C for use in metabolomic studies. Peripheral blood monocytes (PBMCs) will also be collected from the same samples and stored at minus 80°C for potential future use in cell phenotyping studies.

Participants may be contacted and asked to return 1 month after discharge for a follow-up blood collection.

6.1.3 Stool sample collection

Stool will be collected from the inpatient participants daily. Only the first sample of each day will be aliquoted and frozen. Subsequent samples (if available) will be weighed and the weight will be recorded. Each stool specimen that is collected will be processed in the same manner. The participant will be provided with a collection device to be placed in the commode that allows for separation of urine and feces, and keeps the feces separated from the toilet water. After they have finished voiding their first stool of the day, the specimen will be immediately processed according to the following protocol by the CHPS nurses. Using vials with small spoons attached to the lid, a small amount of stool will be scooped on to the spoon from the surface of the stool sample and leveled off with a tongue depressor. The weight of the sample will be recorded. A minimum of seven samples will be obtained from one stool collection. All samples will be labeled with the subject ID #, and the date of collection from the volunteer. The core sample will be labeled the same way. The aliquoted samples and the core sample will be immediately frozen at minus 80°C. The nurses will rate the sample based on the Bristol Stool Chart. Residual stool samples will be stored into up to five 15ml coring vials. Subsequent stools will be weighed in the collection device, rated on the Bristol Stool Chart, and then discarded.

In order to have a baseline stool sample prior to study diet, participants will be required to collect a stool sample prior to the post-screening visit. Participants will also collect a stool sample on the day prior to admission to CHPS. Study staff will provide the participant with collection materials and instructions for collecting the stool at home and bringing it in to study staff. Immediately after voiding the sample, the participant will put a small amount of stool into a spoon-top vial containing ethanol. They will label this vial and the collection device containing the remainder of the stool sample with the date of collection, and will rate the stool on the Bristol Stool Chart. They will then arrange a drop-off time with the study coordinator within 48 hours of sample collection. The study coordinator will bring this stool sample to the study lab on the 9th floor Biomedical Research Building (BRB). Lab personnel will aliquot the stool sample into a minimum of 7 spoon-top vials.

Participants may be asked to provide a stool sample 1 month after discharge. In addition, follow-up stool samples (which may be collected every 3 months for up to 5 years after the participant's last study day) may be collected and will be processed in the same manner as the inpatient samples. The collection kit may be shipped to the participant's home and the participant will either return the stool sample to study staff or ship it to Dr. Gary Wu's lab at the University of Pennsylvania.

6.1.4 Sigmoidoscopy to obtain mucosal biopsy samples

On days 1 and 11, each participant will undergo a sigmoidoscopy to obtain biopsies from the area of approximately 15 cm from the anal verge, which correlates with approximately the rectosigmoid junction. No bowel preparation will be utilized prior to the sigmoidoscopy since it is unknown what affect the bowel preparation may have on the fecal microbiome or the mucosally adherent microbiome. No sedation medications will be administered.

16 biopsies obtained from the distal 20 cm of the colon will be collected; 4 placed in Carnoy solution for non-denaturing confocal microscopy, 4 flash frozen in liquid

nitrogen, 4 placed in RNAlater, and 4 will be kept fresh for immediate cell isolation studies. Additional biopsies may be taken (up to a total of 20), to replace lost/dropped/unusable biopsies (i.e., if the biopsy is too small), for quality control and/or for methods testing.

6.1.5 Rectal Swabs

On Day 1, prior to the flexible sigmoidoscopy, participants will undergo a rectal swab for assessment of the mucosally-associated gut microbiota.

Rectal swabs will be obtained during the physical examination by the CHPS nurse practitioner or study investigator. Four total swabs will be collected per participant. Three (3) sterile swabs will be inserted 3 cm into the rectum, turned 360 degrees, removed, placed into a sterile tube, and frozen at -80 degrees celcius until analysis. One of the swabs will be delivered immediately to Dr. Wu's laboratory for anaerobic culture. Finally, one (1) swab will be waved in the air and collected as a control.

6.1.6 Food Satiety questionnaire

On days 2, 3, 5, 6, 9, and 10, following lunch, participants will complete a standard food satiety questionnaire. A standardized 150mm VAS approach to measuring satiety and hunger as described by Hill and Blundell and modified by Doucet will be employed. [81, 82] The following questions will be asked at 12:15h before lunch is served. Participants will be given their meal at 12:30h and will finish eating their meal by 13:00h. They will complete the questionnaire again at 13:00h. The questions should be asked in the participant's room with all lights on and with no food in the room.

Questions to be asked are as follows.

1) How strong is your desire to eat (very weak to very strong)?
2) How hungry do you feel (not hungry at all to as hungry as I have ever felt)?
3) How full do you feel (not full at all to very full)?
4) How much food do you think you could eat (nothing at all to a large amount)?
5) How preoccupied are you with thoughts of food (no thoughts to very preoccupied?
6) What is your urge to eat (no urge to strong urge)?

6.1.7 Oral glucose tolerance test

On day 1 and 11, participants will complete a 2.5 hour oral glucose tolerance test. This test will be completed prior to breakfast following a 12 hour fast. At 08:00h, a catheter will be placed in an antecubital or forearm vein for blood sampling, with the arm placed in a heating pad to promote arterialization of venous blood. After 20 min of acclimatization to the catheter placement, baseline blood samples will be taken at -5 and -1 min prior to the ingestion of 75 grams anhydrous oral glucose over a 5 min period starting at t = 0 at ~08:30h. Subsequent blood samples will be collected at t = 15, 30, 60, 90, and 120 min post-ingestion. All samples will be collected on ice into chilled EDTA tubes with protease inhibitor cocktail containing DPP4-inhibitor added immediately, centrifuged at 4 degrees C, aliquoted in ice, and stored at minus 80 degrees C until biochemical analysis. A total of 4 ml of blood will be collected at each

test. Glucose, free fatty acids, insulin, glucagon, and GLP-1/GIP will be the substances tested.

6.1.8 PROMIS scale

On days 1 and 11, participants will complete the PROMIS scale for abdominal pain and gas/bloating. This should be completed prior to eating study food that day. On Days 1 and 11, this can be completed during the oral glucose tolerance test.

6.2 Sequence of study activities

Visit Number	Visit 1	Visit 1.1	Visit 2	Visit 2.1	Visit 3											Visit 4	Visit 5	Visit 6
Study Day	**-60 to -9**	**-7**	**-4**	**-3 to -1**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**24**	**48***	**107***
Food pick up			X															
Emulsifier free diet				X														
Randomized diet					X	X	X	X	X	X	X	X	X	X	X			
Satiety Questionnaire						X	X		X	X			X	X				
Record Calories Consumed					X	X	X	X	X	X	X	X	X	X	X			
DHQII					X													
Fitbit Data Collection			X	X	X	X	X	X	X	X	X	X	X	X	X			
Walks (as needed)					X	X	X	X	X	X	X	X	X	X	X			
Weight	X		X		X	X	X	X	X	X	X	X	X	X	X			
Waist Circumference	X																	
CBC	X																	
CMP	X														X			
PT and INR	X																	
HbA1c	X																	
Fasting Lipid Panel	X																	
Vital Signs	X		X		X	X	X	X	X	X	X	X	X	X	X			
Physical Exam	X				X													
Medical History	X																	
24 hour recall x3		X																
Urine Collection	X		X		X	X	X	X	X	X	X	X	X	X	X			
Stool Collection			X		X	X	X	X	X	X	X	X	X	X	X		X	X
Stool Kit Pick Up	X		X												X		X	
Stool Kit Return			X		X												X	X
Blood Collection			X		X	X	X	X				X		X	X		X	
PROMIS scale					X										X			
Oral glucose tolerance test					X										X			
Rectal swabs					X													
Sigmoidoscopy with biopsy					X										X			
Monitoring adverse events					X	X	X	X	X	X	X	X	X	X	X	X		
Concomitant medications	X		X		X	X	X	X	X	X	X	X	X	X	X			
AE Follow Up Phone Call																X		
Follow Up Questionnaire																	X	X

*Fitbit will be distributed at Visit 2 (4 days prior to admission to inpatient CHPS).

*Urine pregnancy test will be conducted for females at screening and Day 1 of inpatient procedures.

6.2.1 Pre-screening and Informed Consent

Each potential participant will be pre-screened for eligibility over the phone. Additionally, inclusion and exclusion criteria may be reviewed with the potential participant during the pre-screening visit. Those who appear to meet all eligibility criteria will then be introduced to the study. Full explanation of the study and the general procedures will be explained thoroughly to the participant. Potential participants will be given an opportunity to ask questions and have them answered by study staff.

Potential participants will sign and date written consent to participate *prior* to screening visits. No steps for screening or beyond will occur prior to informed consent. The consent process will be completed in English.

A copy of the signed consent form will be given to the participant. The original signed consent form should be placed in the participant study file. A second copy should be placed in a confidential study folder containing copies of consents for all study participants. A third copy will be provided to the CHPS.

6.2.2 Screening (Visit 1)

Screening evaluation should be completed no more than 60 days prior to admission to the CHPS. After providing informed consent, the participant's medical history will be collected to assess for exclusion criteria, including vital signs and demographic data. Demographic data collected will include age, sex, race, ethnicity, lifetime weight history, current and recent medication use, and past medical and surgical history. A physical exam will also be conducted.

In addition, blood will be drawn for CBC, comprehensive metabolic panel, lipid panel, Hba1c, coagulation parameters following an overnight fast. Women will complete a urine pregnancy test.

During the screening process, participants will be presented with the study diets to assure that they are able to consume this for a 14 day period. Screening will not proceed further unless participants agree that they can follow the study diet, regardless to which they are assigned.

Demographics
- Birth date
- Sex
- Race and ethnicity
- PMH & concurrent medical problems

Current medications and dose
Tobacco use
Alcohol use
Exclusion criteria lab work
- CBC
- Comprehensive metabolic panel (CMP)
- PT and INR
- Fasting lipid panel
- Urine pregnancy test
- Hba1c

Physical exam

6.2.2.1 Physical examination

The physical exam will document any baseline abnormalities as well as the baseline BMI, waist circumference and a seated blood pressure measurement.

6.2.2.2 Three Day Dietary Recall (Visit 1.1)

Data on recent dietary patterns will be collected for 7 days immediately prior to Visit 2. The participant will complete a diet diary during the week prior to Visit 2 that will be used to facilitate the dietary recall. Note that the last 24 hour recall for the day immediately prior to Visit 2 can be completed on the same day as Visit 2.

Three day dietary recalls will be performed by the CHPS bionutritionists. Dietary assessments are to be performed interactively using the Nutrition Data System program (University of Minnesota) that includes prompts for questions depending on the food item entered as part of the interview. Using this software, the diet information will be summarized according to the macronutrient composition (daily consumption of calories, protein, fat, carbohydrates and dietary fiber). Additionally, the data on fat and other macronutrients can be further subcategorized, such as according percent of calories from saturated, monounsaturated, and polyunsaturated fats.

6.2.2.3 Dietitian Evaluation

Caloric requirements will be estimated by the dietitian in the CHPS prior Visit 2 using standard methods to estimate metabolic requirements.[33] This will be completed prior to Visit 2 so that the CHPS metabolic kitchen can be prepared for the dietary needs of the participant prior to providing 3 days of outpatient emulsifier free food.. Participants who gain or lose weight will not have their food quantity adjusted to prevent further weight loss or gain as the effect of emulsifiers on body weight is one of the measures that the study will evaluate.

6.2.3 Post Screening Visit (Visit 2)

Post screening should be completed 4 days prior to admission to the CHPS.

6.2.3.1 Fitbit Distribution

Once participants are determined to be eligible, they will be given a Fitbit Flex to measure their activity level each day in the 4 days prior to admission. A study coordinator will set up the Fitbit Flex for the participant. The participants will be sent home with the Fitbit Flex and instructions on how to use, charge, and wear their Fitbit every day and through their return on the day of admission.

6.2.3.2 Sample Collection

Eligible participants will have blood, urine, and stool samples will be collected prior to beginning the emulsifier free diet. Blood and urine will be collected following an overnight fast (see section 6.1.1 and 6.1.2) at Visit 2. A stool sample will be collected by the participant at home either the day prior to Visit 2 or the morning of Visit 2. Participants will bring the stool sample to the visit. Study staff will provide the participant with collection materials and instructions for collecting the stool at home and returning it to study staff (See section 6.1.3).

6.2.3.3 Emulsifier Free Diet

Participants will be given food prepared by the CHPS kitchen. All participants will receive 3 full days' worth of freshly prepared emulsifier-free food at Visit 2. Participants will consume the emulsifier-free food exclusively for the three days prior to admission to CHPS. Other than the prepared study meals, participants may have water, coffee and tea with no added sugar or dairy as desired. No other food or drink is allowed.

6.2.3.4 Vital signs and Weight

Vital signs and weight will be recorded. Weight should be recorded with the participant wearing undergarments and a hospital gown prior to the first meal of the day. The patient should urinate prior to being weighed. Weight will be measured in triplicate. The participant should not be able to see the readout on the scale and should not be told their weight.

6.2.4 Day 1 of Inpatient Procedures (Visit 3.1)

All participants will arrive at the CHPS in the morning, having not eaten since 11:59PM the evening prior. All of the below procedures for day 1 will be the same for both study arms.

6.2.4.1 History and physical exam

Vital signs (blood pressure should be done sitting) and weight will be recorded. Weight should be recorded with the participant wearing undergarments and a hospital gown prior to the first meal of the day. The patient should urinate prior to be weighed. Weight will be measured in triplicate. The participant should not be able to see the readout on the scale and should not be told their weight.

A complete physical exam and medical history will be completed to assess for abnormalities present at baseline and to confirm absence of intercurrent illnesses that would make the participant ineligible. Because the participants may be anxious on the first day of the inpatient stay, we will not use blood pressure on Day 1 as an exclusion criteria as long as the blood pressure at screening met the inclusion criteria (section 4.2).

6.2.4.2 Adverse Events

Patients will be queried for adverse events.

6.2.4.3 Urine pregnancy Test

A urine pregnancy test will be repeated for all women.

6.2.4.4 Concomitant medications

Consumption of concomitant medications will be recorded in the study records. Participants will not be permitted to take any medications that are listed in the exclusion criteria during the study.

6.2.4.5 Oral Glucose Tolerance Test

Participants will complete a 2.5 hour oral glucose tolerance test. This test will be completed prior to breakfast following a 12 hour fast.

6.2.4.6 Sample collection

Blood, urine, and feces samples will be obtained. Up to 15 ml of urine samples will be collected prior to 09:00h after an overnight fast starting at midnight. Approximately 16cc of blood will be collected for metabolomics at each collection following an overnight fast prior to breakfast. Participants will be provided a stool collection kit to collect a stool sample on the day prior to admission or on the morning of admission. The first stool sample will be weighed, aliquoted and frozen. Participants will collect stool from all subsequent bowel movements. This stool will be weighed, the weight recorded and discarded. In addition, a stool sample will be collected by the participant at home either the day prior to Visit 3 or the morning of Visit 3. Participants will bring the stool sample to the visit. Study staff will provide the participant with collection materials and instructions for collecting the stool at home and returning it to study staff (See section 6.1.3).

6.2.4.7 Rectal Swabs

Prior to the flexible sigmoidoscopy, each participant will undergo a rectal swab. (See section 6.1.5).

6.2.4.8 Flexible Sigmoidoscopy

Following the oral glucose tolerance test, each participant will undergo a sigmoidoscopy to obtain biopsies from the area of approximately 15 cm from the anal verge.

6.2.4.9 Food Frequency Questionnaire

We will collect information on the participant's usual diet utilizing the Diet History Questionnaire II (DHQ II), a food frequency questionnaire (FFQ) developed by the Risk Factor Monitoring and Methods Branch (RFMMB) of the National Cancer Institute (NCI). The original DHQ I was designed based on cognitive research findings, to be easy to use. It had 124 food items and included both portion size and dietary supplement questions. The DHQ II has a food list that has been updated based on more recent dietary data and consists of 134 food items and 8 dietary supplement questions. There are four different versions of DHQ II. These four versions differ by time frame (the past year versus the past month) and whether or not questions are included about portion size. We will utilize the questionnaire that asks about the past month and includes questions about portion sizes for this study since we are interested in most recent intake.

6.2.4.10 Recording of diet

Participants will begin their randomized diets following the oral glucose tolerance test and flexible sigmoidoscopy at dinner. Participants will be instructed to consume only the food provided and no other food. Participants must eat all of the brownie and servings of sorbet provided. Participants will have access to additional food outside of the meals provided, however, the entire serving of the previous meal must have been consumed. The amount of food consumed will be recorded on the case report forms.

6.2.4.11 Timing of meals

The first meal will be served following the oral glucose tolerance test or after the flexible sigmoidoscopy. All scheduled snacks and meals will be provided following the oral glucose tolerance test except during the flexible sigmoidoscopy. Lunch will be served at approximately 12:30h and dinner at approximately 18:00h. A snack will be provided at 15:00h and 21:00h. Beginning at the dinner meal, all participants will consume one serving of brownie or sorbet (15 gm) per day that contain 0 or 2.5 gm CMC per serving. Participants will be expected to finish each meal by the time of their next meal. There will be no limitation on the time to consume snacks. All food must be consumed by midnight.

6.2.4.12 Activity Level Maintenance

Participants will be required to attain within 10% of the average number of daily steps that they took in the 3 days prior to admission. To achieve this, participants may be escorted on walks outside of the inpatient unit by a coordinator, as needed.

6.2.4.13 PROMIS Scale

Participants will complete the PROMIS scale prior to eating their first meal.

6.2.5 Day 2-10 of Inpatient Procedures (Visit 3.2-3.10)

All of the below procedures for day 2-10 will be the same for both study arms.

6.2.5.1 Vital Signs and Weight

Vital signs and weight will be recorded. Weight should be recorded with the participant wearing undergarments and a hospital gown prior to the first meal of the day. The patient should urinate prior to being weighed. Weight will be measured in triplicate. The participant should not be able to see the readout on the scale and should not be told their weight. The number of bowel movements during the prior day will also be recorded.

6.2.5.2 Adverse Events

Participants will be queried for adverse events.

6.2.5.3 Concomitant medications

Consumption of concomitant medications will be recorded in the study records. Participants will not be permitted to take any medications that are listed in the exclusion criteria during the study.

6.2.5.4 Sample collection

On days 2, 3, 4, 8, and 10 blood will be obtained. Approximately 16cc of blood will be collected for metabolomics after an overnight fast prior to breakfast. Urine and feces samples will also be obtained. Up to 15 ml of urine samples will be collected prior to 09:00h after an overnight fast starting at midnight. The first stool sample will be aliquoted and frozen.

6.2.5.5 Recording of diet

Participants will continue on their randomized diets. Participants will be instructed to consume only the food provided and no other food. Participants must eat all of the brownie and servings of sorbet provided. Participants will have access to additional food outside of the meals provided, however, the entire serving of the previous meal must have been consumed. The amount of food consumed will be recorded on the case report forms.

6.2.5.6 Timing of meals

Breakfast will be served at 09:00h. Lunch will be served at approximately 12:30h and dinner at approximately 18:00h. A snack will be provided at 10:30h, 15:00h and 21:00h on Days 2-9. Participants will be expected to consume snacks by the next meal with the last snack consumed by midnight on Days 2-9. On Day 10, the 21:00h intervention snack will be provided in addition to the intervention and meal at 18:00h. Participants will be expected to finish each meal by the time of their next meal or snack. Lunch should be finished within 30 minutes. On Day 10, all food must be consumed by 19:30h. No food or beverage other than water is to be consumed between midnight and breakfast on Days 2-9 and between 19:30h and breakfast on Day 10.

6.2.5.7 Satiety Questionnaire

On days 2, 3, 5, 6, 9 and 10, participants will complete a standard food satiety questionnaire at 12:15h before lunch and at 13:00h following the completion of lunch.

6.2.5.8 Activity Level Maintenance

See Day 1 of inpatient procedures.

6.2.6 Day 11 of Inpatient Procedures (Visit 3.11)

All of the below procedures for day 11 will be the same for both study arms.

6.2.6.1 *Vital Signs and Weight*

See Day 2-3 of inpatient procedures.

6.2.6.2 Adverse Events

Participants will be queried for adverse events.

6.2.6.3 Concomitant medications

Consumption of concomitant medications will be recorded in the study records. Participants will not be permitted to take any medications that are listed in the exclusion criteria during the study.

6.2.6.4 Oral Glucose Tolerance Test

Participants will complete a 2.5 hour oral glucose tolerance test. This test will be completed prior to breakfast following a 12 hour fast.

6.2.6.5 Sample collection

Blood, urine, and feces samples will be obtained as described above. Up to 15 ml of urine samples will be collected prior to 09:00h after an overnight fast starting at midnight. Approximately 16cc of blood will be collected for metabolomics after an overnight fast prior to breakfast. The first stool sample will be aliquoted and frozen.

6.2.6.6 Flexible Sigmoidoscopy

Following the oral glucose tolerance test, each participant will undergo a sigmoidoscopy to obtain biopsies from the area of approximately 15 cm from the anal verge.

6.2.6.7 Recording of diet

Participants will be given their scheduled, randomized diets following the oral glucose tolerance test except during the flexible sigmoidoscopy. Participants will be instructed to consume only the food provided and no other food. Participants must eat all of the brownie and servings of sorbet provided. Participants will have access to additional food outside of the meals provided, however, the entire serving of the previous meal must have been consumed. The amount of food consumed will be recorded on the case report forms.

6.2.6.8 Timing of meals

The first meal will be served following the oral glucose tolerance test. All scheduled snacks and meals will be provided following the oral glucose tolerance test except during the flexible sigmoidoscopy. If the flexible sigmoidoscopy directly follows the oral glucose tolerance test, participants will be given their final meal before discharge.

6.2.6.9 Activity Level Maintenance

Participants will not be walked to maintain activity level.

6.2.6.10 PROMIS Scale

Participants will complete the PROMIS scale prior to eating their first meal.

6.2.6.11 Discharge

Participants will be discharged following the completion of the oral glucose tolerance test and the flexible sigmoidoscopy, and the collection of blood, urine, and stool or by 5pm. They will be given post-flexible sigmoidoscopy instructions. An AE log and a participant payment form will be completed. Participants will then be escorted out of the hospital.

6.3 Post-study follow-up

There will be three additional visits following the completion of inpatient visits.

6.3.1 Adverse Event Follow Up Phone Call (Visit 4)

The research team will contact the participant by phone once during the week following the completion of the inpatient procedures to assess for any new or unresolved adverse events.

6.3.2 One Month Follow-Up Sample Collection (Visit 5)

Participants will provide a stool sample 1 month (+/- 1 week) after discharge. Participants will return for a blood collection 1 month after discharge. Participants will complete a post study follow up questionnaire at this visit.

6.3.3 Three Month Follow-Up Sample Collection (Visit 6)

In addition, follow-up stool samples (which may be collected every 3 months for up to 5 years after the participant's last study day) may be collected and will be processed in the same manner as the full study. Participants may provide a stool sample every three months (+/- 2 weeks). Participants will complete a post study follow up questionnaire at this visit.

6.4 Sample preparation, processing, and storage at the University of Pennsylvania

6.4.1 Aliquoting and processing of samples for archiving

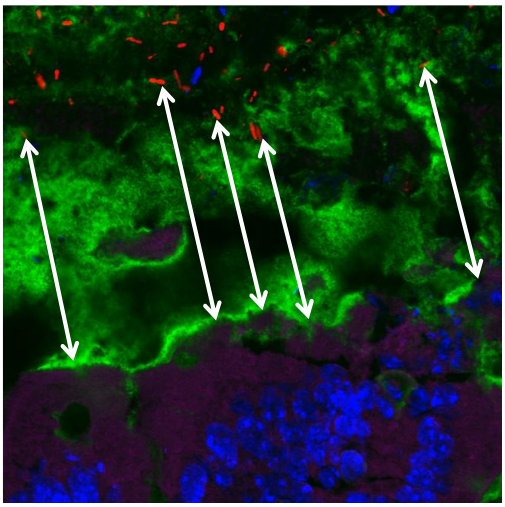


Figure 2. Assessment of microbiota encroachment. Carnoy-fixed biopsies are stained with probes to label bacteria (red), mucus (green), nuclei (blue) or actin (purple). The location of the 5 closest bacteria are measured. 5 fields per sample are analyzed.

Stool samples are aliquoted into spoon-capped vials labeled with a barcode that is a unique identifier, the date and a protocol number, usually 6 per sample, for archiving. Any residual stool is aliquoted for specific immediate demands by investigators and/or removed from the collection vessel into up to five 15 ml coring vials, which is also labeled with a similar barcode. Each sample is then scanned, and placed in an -80^{o}C freezer in an appropriate freezer box with its own separate barcode. The freezer box barcode is scanned at the same time. This provides a computerized list of where each sample has been stored which can then be matched with other information that is collected on this sample in the LabVantage lab management system. Blood samples are processed for either serum or plasma, depending on the preservatives added at the time of sampling. Once in the lab, the blood is centrifuged to clear the plasma/serum and the supernatant is measured out in 300-500 ul aliquots into polypropylene cryotubes. Each aliquot is labeled in the same manner as the stool aliquots and immediately placed in a barcoded box in an -80^{o}C freezer to be archived for later use. Finally, when retrieving a stored sample the box and aliquot barcodes are

scanned and compared to a list of samples needed to ensure the correct material is being removed for use.

6.4.2 Assay for CMC

Levels of CMC in feces and urine will be quantitated by co-investigator Andrew Patterson at The Penn State University via NMR-based approaches developed by Tajima and colleagues[34, 35].

6.4.3 Microbiome characterization

Microbiota localization. For each subject, 4 distal biopsies, collected pre/post CMC and immediately preserved in Carnoy fixative, will be assayed by fluorescence microscopy to determine bacterial localization relative to the epithelium. Specifically, for each specimen, we will examine 5 high-powered fields, selected arbitrarily while blinded to the accompanying clinical data. We will use staining protocols developed by Gunnar Hansson and colleagues [36, 37] that we have ourselves used extensively in our recent publications [19, 38]. For each field, we will measure the distance of the 5 closest bacteria per field as illustrated in **Figure 2.** The 25 distances (5 per field x 5 fields) will then be averaged to produce the "average closest bacteria" and compared between groups as reported in our mouse-based studies [19, 38]. **2) Microbiota composition.** Each fecal sample will be subjected to 16S, metagenomics, and meta-transcriptomic sequence-based analysis as previously described. Sequencing via the Illumina platform (Miseq for 16S, NextSeq500 for metagenomics/transcriptomic). Data will be processed and analyzed via the Qiime platform and R software, in a manner similar to our previous publications [19, 38-40]. **3) Microbiota pro-inflammatory potential**. For each fecal sample collected, we will measure fecal levels of bioactive flagellin and LPS as reported in our previous publications [19, 38, 40]. Briefly, for each sample, we will prepare a soluble surface extract to be generated by bead-beating followed by centrifugation. Such samples will be diluted and then applied to cells engineered to express receptors for TLRs 4 or 5 and produce peroxidase under the control of a NF-κB responsive promoter - our initial publication using this technique describe an extensive set of control studies we have utilized to validate this approach [41]. These assays, standardized with E. coli flagellin and LPS will report the levels of bioactive LPS and flagellin, which we have shown are broadly reflective of a fecal specimens ability to drive pro-inflammatory gene expression upon injection into mice [41]. Comparison between groups will use t-test, repeated measures ANOVA or analogous non-parametric tests.

6.4.4 Microbial DNA Sequencing

DNA will be prepared in the PennCHOP Microbiome Center. Samples will be sent to the center de-identified and coded with a study number. We will perform 16S rRNA and ITS gene sequencing to evaluate the bacterial and fungal microbiota, respectively, in rectal swab samples. Isolated DNA will be quantified using the Picogreen system and 50 ng of DNA will be amplified. Pyrosequencing will be carried out using barcoded primers as previously described[42]. For pyrosequencing of bacteria, primers annealing to the V1V2 region of the 16S bacterial gene will be used. The development of the ITS1 fungal primers is described in[42]. For pyrosequencing, we will

use the Roche/454 Genome Sequencer Junior. Sequence data will be processed using QIIME[43]. If warranted by our preliminary data, we may analyze samples further using a metagenomic approach, in which DNA samples are nebulized, ligated to linkers, and subjected to pyrosequencing (IlluminaHiSeq). This allows enumeration of the types of genes present in a sample. To determine bacterial and fungal load, respectively, we will determine 16S and 18S gene copy number from the rectal swab samples. The qPCR methods including the details of the primers as well as PCR cycling conditions have been previously described[44].

6.4.5 Metabolic profiling based on plasma samples

Fasting glucose and oral glucose tolerance (on day 1 and 11), day 11 sera will be assayed for a standard comprehensive metabolic panel (includes albumin, urea, calcium, creatinine, glucose, and liver enzymes AST/ALT) performed at Penn's clinical lab. Additionally, sera will be assayed for a range of hormones and cytokines using a customized bead-based assay designed to include immune inflammatory and metabolic markers of obesity and type 2 diabetes (includes IL-8, IL-6, TNF□, lipocalin-2, MCP1, insulin, leptin, ghrelin, ESR, CRP) all of which are known to be elevated in metabolic syndrome (performed at GSU). Additionally, fecal samples will be assayed for lipocalin-2 by ELISA.

6.4.6 Long term sample storage and reuse of samples

All specimens remaining after the study is complete will be retained for possible future use unless specifically requested by the participant that the specimen be destroyed or it is deemed by the investigators that the specimens are no longer needed. Future studies, including genetic testing, may be conducted with residual samples.

7 Statistical Plan

7.1 Sample Size Determination

We will recruit the minimum number of subjects to provide adequate statistical power for the aims of the project. For aim 1, statistical power will depend on the prevalence of detectable CMC in the feces of participants at the end of follow-up. We anticipate that in the CMC treated group, the prevalence will be 100% and in the untreated group the prevalence will be 0%. However, with 8 subjects per group, we will have 89% power to detect a difference if the true prevalence of detectable CMC after 2 weeks of a high dose CMC and CMC free diet are 90% and 10%, respectively. For aim 2, power calculation were based on preliminary data in which the difference in mean distance of the nearest bacteria to the epithelium between patients with and without diabetes was 19.13 um. The within group standard deviation (SD) for patients without diabetes was 7.17. The within group SD for those with diabetes was even smaller. With a sample size of 8 subjects per group and assuming a within group SD of 7.17, we will have 90% and 80% power to detect a difference in the distance of the nearest bacteria to the epithelium between the treatment groups (CMC vs. no CMC) that is 35% and 44% smaller than the difference observed between patients with and without diabetes, respectively. In Aim 3, the study will have 80% power to detect a difference between

groups equal to 1.51 units of SD. For paired analyses within treatment groups, there will be 80% power to detect a difference of 1.15 units of SD.

7.2 Overview of Statistical Methods

Descriptive statistics (mean, SD, median, range, counts, and percentage) will be used to describe and compare (t-test or rank sum test for continuous variables and Fishers exact test for categorical variables) the baseline characteristics, usual diet, and immediate pre-enrollment diet between the treatment arms.

7.2.1 Determining the effect of CMC on fecal and mucosal microbiome composition

We will first compare the bacteria taxa in each fecal stool communities sampled at baseline, after the 3 day emulsifier free period and during the intervention period(total of 96 stool samples and 16 biopsy samples per treatment arm – 8 participants on each diet) to determine within-diet and between-diet variation prior to the administration of antibiotics. The analyses will be conducted separately for microbiomes in fecal samples and also in colonic mucosa samples. We will employ both of our methods (PCoA and proportion of the community represented by different taxa) to summarize the data at each time point. Besides comparing the microbiome among the treatment groups at day 11, we will also perform repeated measurement analysis of variance (RM-ANOVA) to study the time effects on microbiome and potential time and diet interaction effects for our data on fecal samples. The outcomes of such RM-ANOVA are the significant principal components summarizing the bacterial taxa.

7.2.2 Comparison of metabolites between diets with and without CMC

The first analysis will compare the abundance of fecal and plasma metabolites between the two dietary groups. As assignment to the diet will be based on randomization, the primary analysis will be an unadjusted t-test to compare the concentration of the various metabolites measured in the plasma and feces. As there will be multiple comparisons, we will report both nominal (p values) and q values accounting for the multiple testing.

7.2.3 Effects of CMC consumption on a range of inflammatory and metabolic parameters that characterize metabolic syndrome

Weight change will be compared between the treatment groups from day 1 to day 11 using a t-test. If there is evidence of confounding despite randomization, additional analyses will use linear regression adjusted for change in weight from day 1 to day 11 and BMI at day 0.

We will also assess changes to each subject regarding satiety (from the questionnaire re food adequacy/sufficiency). These data are on visual analogue scale so will be compared using unpaired t-test for between group comparisons or paired t-tests for within group comparisons.

The area under the glucose and insulin concentration curves during the oral glucose tolerance test at day 1, day 11 and the change from day 1 to day 11 will be compared between groups using a t-test. The $\Delta I_{30}/\Delta G_{30}$ will be used as a measure of

beta cell function[45] while homeostasis model assessment of insulin resistance (HOMA-IR) will be used to estimate insulin resistance[46] and compared between the groups with a t-test at the same time points.

Sera will be assayed for a range of hormones and cytokines using a customized bead-based assay designed to include immune inflammatory and metabolic markers of obesity and type 2 diabetes (includes IL-8, IL-6, TNFα, lipocalin-2, MCP1, insulin, leptin, ghrelin, ESR, CRP) all of which are known to be elevated in metabolic syndrome (performed at GSU). Additionally, fecal samples will be assayed for lipocalin-2 by ELISA. These data will be compared using unpaired t-test for between group comparisons or paired t-tests for within group comparisons.

8 Safety and Adverse Events

Adverse Event

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

- results in study withdrawal
- is associated with a serious adverse event
- is associated with clinical signs or symptoms
- leads to additional treatment or to further diagnostic tests
- is considered by the investigator to be of clinical significance

Serious Adverse Event (SAE)

Adverse events are classified as serious or non-serious. A ***serious adverse event*** is any AE that is:

- fatal
- life-threatening
- requires or prolongs hospital stay
- results in persistent or significant disability or incapacity
- a congenital anomaly or birth defect
- an important medical event

Important medical events are those that may not be immediately life threatening, but are clearly of major clinical significance. They may jeopardize the participant, and may require intervention to prevent one of the other serious outcomes noted above. For example, drug overdose or abuse, a seizure that did not result in in-patient hospitalization or intensive treatment of bronchospasm in an emergency department would typically be considered serious.

All adverse events that do not meet any of the criteria for serious will be regarded as ***non-serious adverse events***.

Adverse Event Reporting Period

The study period during which AEs must be reported is normally defined as the period from the initiation of any study procedures to the end of the study treatment follow-up. For this study, the study treatment follow-up is defined as 7 days following the last administration of study diet.

Preexisting Condition

A preexisting condition is one that is present at the start of the study. A preexisting condition should be recorded as an adverse event if the frequency, intensity, or the character of the condition worsens during the study period.

General Physical Examination Findings

At screening, any clinically significant abnormality should be recorded as a preexisting condition. At the end of the study, any new clinically significant findings/abnormalities that meet the definition of an AE must also be recorded and documented as an AE.

Post-study Adverse Event

All unresolved AEs that are categorized by the investigator as definitely or probably related to participation in the study should be followed by the investigator until the events are resolved, the participant is lost to follow-up, or the AE is otherwise explained. At the last inpatient evaluation, the investigator will instruct each participant to report any subsequent event(s) that the participant, or the participant's personal physician, believes might reasonably be related to participation in this study. The investigator will notify the study sponsor of any death or AE occurring at any time after a participant has discontinued or terminated study participation that may reasonably be related to this study. The sponsor should also be notified if the investigator should become aware of the development of cancer or of a congenital anomaly in a subsequently conceived offspring of a participant that has participated in this study.

Abnormal Laboratory Values

A clinical laboratory abnormality should be documented as an adverse event if <u>any one of the following</u> conditions is met:

- The laboratory abnormality is not otherwise refuted by a repeat test to confirm the abnormality
- The abnormality suggests a disease and/or organ toxicity
- The abnormality is of a degree that requires active management; e.g. change of dose, discontinuation of the drug, more frequent follow-up assessments, further diagnostic investigation, etc.

Hospitalization, Prolonged Hospitalization or Surgery

Any AE that results in hospitalization or prolonged hospitalization should be documented and reported as an SAE unless specifically instructed otherwise in this protocol. Any condition responsible for surgery should be documented as an AE if the condition meets the criteria for an AE.

Neither the condition, hospitalization, prolonged hospitalization, nor surgery are reported as an AE in the following circumstances:

- Hospitalization or prolonged hospitalization for diagnostic or elective surgical procedures for a preexisting condition. Surgery should ***not*** be reported as an outcome of an AE if the purpose of the surgery was elective or diagnostic and the outcome was uneventful.

8.1 Recording of Adverse Events

At each contact with the participant, the investigator must seek information on AEs by specific questioning and, as appropriate, by examination. Information on all AEs should be recorded immediately in the source document, and also in the appropriate adverse event case report form (CRF). All clearly related signs, symptoms, and abnormal diagnostic procedures results should be recorded in the source document, though should be grouped under one diagnosis.

All AEs occurring during the study period must be recorded. The clinical course of each event should be followed until resolution, stabilization, or until it has been determined that the study treatment or participation is not the cause. SAEs that are still ongoing at the end of the study period must be followed up to determine the final outcome. Any SAE that occurs after the study period and is considered to be possibly related to the study treatment or study participation should be recorded and reported immediately.

8.2 Reporting of Serious Adverse Events

8.2.1 Study Sponsor Notification by Investigator

An SAE must be reported to the study sponsor by telephone within 24 hours of the event. An FDA Medwatch 3500A SAE Form must be completed by the investigator. The investigator will keep a copy of this form on file at the study site. Report SAEs by phone to:

James D. Lewis, MD
Phone - (215) 573-5137 or (856) 906-3173

In the event that Dr. Lewis cannot be reached report SAEs to
Gary Wu, MD
Phone - (215) 898-0158
or
Lisa Nessel
Phone – (215) 573-6003
Or
Brittaney Bonhomme
Phone – (215) 898-2625

At the time of the initial report, the following information should be provided:

- Study Name
- Participant number
- A description of the event
- Date of onset
- Current status
- Whether study treatment was discontinued
- The reason why the event is classified as serious
- Investigator assessment of the association between the event and study treatment

Within the following 48 hours, the investigator must provide further information on the SAE in the form of a written narrative. This should include a copy of the completed Medwatch 3500 A SAE Form and any other diagnostic information that will assist the understanding of the event. Significant new information on ongoing SAEs should be provided promptly to the study investigator.

8.2.2 Ethics Committee/Internal Review Board Notification by Investigator

All reports of SAES (including follow-up information) must be submitted to the Ethics Committee (EC)/Internal Review Board (IRB) within 10 working days. Copies of each report and documentation of EC /IRB notification and receipt will be kept in the Clinical Investigator's binder.

9 Data Handling and Record Keeping

9.1 Confidentiality

Information about study participants will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability Act of 1996 (HIPAA). Those regulations require a signed participant authorization informing the participant of the following:

- What protected health information (PHI) will be collected from participants in this study
- Who will have access to that information and why
- Who will use or disclose that information
- The rights of a research participant to revoke their authorization for use of their PHI.

In the event that a participant revokes authorization to collect or use PHI, the investigator, by regulation, retains the ability to use all information collected prior to the revocation of participant authorization.

Analytical data may be handled at Georgia State University and/or University of Pennsylvania. All data related to this trial will be recorded using the patients' assigned unique study number. Data will be reported only in a confidential manner such that the personal identity of any subject will not be identifiable. All personal identifiers will be stored on REDCap (Research Electronic Data Capture). REDCap is a secure, web-based application designed to support data capture for research studies and secure

sensitive data providing physical security of machines by the Penn Medicine Academic Computing Services, saving all data on to both the database and webservers located on private networks, as well as only providing access to data within the application. Only limited data sets will be shared outside of REDCap. Limited data sets will include dates, but not direct identifiers. All study data will be maintained under a double locked system, such as a locked closet within a locked office or on a password protected computer in a locked office. At the end of the study these data will be electronically archived on a password protected computer or other electronic storage device.

9.2 Source Documents

Source data is all information, original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in source documents. Examples of these original documents, and data records include: hospital records, clinical and office charts, laboratory notes, memoranda, participants' diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate and complete, microfiches, photographic negatives, microfilm or magnetic media, x-rays, subject files, and records kept at the pharmacy, at the laboratories, and at medico-technical departments involved in the clinical trial.

9.3 Case Report Forms

The study case report form (CRF) is the primary data collection instrument for the study. All data requested on the CRF must be recorded. All missing data must be explained. If a space on the CRF is left blank because the procedure was not done or the question was not asked, write "N/D". If the item is not applicable to the individual case, write "N/A". All entries should be printed legibly in black ink. If any entry error has been made, to correct such an error, draw a single straight line through the incorrect entry and enter the correct data above it. All such changes must be initialed and dated. DO NOT ERASE OR WHITE OUT ERRORS. For clarification of illegible or uncertain entries, print the clarification above the item, then initial and date it.

9.4 Records Retention

Study documents and records will be retained for at least 2 years after the last participant has completed the study.

10 Study Monitoring, Auditing, and Inspecting

The investigator will permit study-related monitoring, audits, and inspections by the EC/IRB, the sponsor, government regulatory bodies, and University compliance and quality assurance groups of all study related documents (e.g. source documents, regulatory documents, data collection instruments, study data etc.). The investigator will ensure the capability for inspections of applicable study-related facilities (e.g. pharmacy, diagnostic laboratory, etc.).

An independent safety officer has been assigned the role of monitoring adverse events. Dr. Yu-Xiao Yang will serve in this role. The safety officer will receive quarterly

reports of all adverse events. In addition, the officer will receive reports of serious adverse events within 72 hours of the investigators becoming aware of the event. If a serious adverse event occurs, the safety officer may request to be unblinded to further investigate, if necessary. A biostatistician would generate a list of AEs by arm which only be viewed by the safety officer, if necessary, to ensure all study staff remain blinded. Additionally, the safety officer will have the authority to review the study documents at any time and for any reason to assure the safety of the participants.

Participation as an investigator in this study implies acceptance of potential inspection by government regulatory authorities and applicable University compliance and quality assurance offices.

11 Ethical Considerations

This study is to be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Institutional research policies and procedures.

This protocol and any amendments will be submitted to a properly constituted independent EC/ IRB, in agreement with local legal prescriptions, for formal approval of the study conduct. The decision of the EC/IRB concerning the conduct of the study will be made in writing to the investigator and a copy of this decision will be provided to the sponsor before commencement of this study.

All participants for this study will be provided a consent form describing this study and providing sufficient information for participants to make an informed decision about their participation in this study. See Attachment A for a copy of the Informed Consent Form. This consent form will be submitted with the protocol for review and approval by the EC/IRB for the study. The formal consent of a participant, using the EC/IRB-approved consent form, must be obtained before that participant is submitted to any study procedure. This consent form must be signed by the participant or legally acceptable surrogate, and the investigator-designated research professional obtaining the consent.

12 Study Finances

12.1 Funding Source

This study is financed through a grant from the National Institutes of Health.

12.2 Conflict of Interest

Any investigator who has a conflict of interest with this study (patent ownership, royalties, or financial gain greater than the minimum allowable by their institution, etc.) must have the conflict reviewed by a properly constituted Conflict of Interest Committee with a Committee-sanctioned conflict management plan that has been reviewed and approved by the study sponsor prior to participation in this study. All University of Pennsylvania investigators will follow the University conflict of interest policy.

12.3 Participant Stipends or Payments

Participants will be compensated $3100 for completion of the entire inpatient stay. Participants who do not complete the full inpatient stay will receive only $100 for each 24 hours of inpatient stay completed. Participants who return to CHPS upon admission with their Fitbit Flex will be given the option to keep the Fitbit Flex. Participants who complete visit 5 (blood and stool collection) will be compensated an additional $150. Participants who complete visit 6 (3 month follow-up stool collection) will be compensated an additional $50. Thus, participants who complete visits 1-6 will be compensated a total of $3300.

13 Publication and Data Sharing Plan

Any publications emanating from these data must acknowledge the funding source.

Neither the complete nor any part of the results of the study carried out under this protocol, nor any of the information provided by the sponsor for the purposes of performing the study, will be published or passed on to any third party without the consent of the study regulatory sponsor. Any investigator involved with this study is obligated to provide the regulatory sponsor with complete test results and all data derived from the study.

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15 Attachments

16 Protocol Signature Page

I will provide copies of the protocol, any subsequent protocol amendments and access to all information furnished by the sponsor to study personnel under my supervision. I will discuss this material with them to ensure that they are fully informed about the investigational drug and study protocol.

I agree to conduct this clinical trial according to the protocol described herein. I also agree to conduct this study in compliance with applicable federal, state and local regulations, *Guidelines for Good Clinical Practice (GCP)*, and with the requirements of my Institutional Review Board. I understand that I may not implement this protocol without first receiving written IRB approval.

Furthermore, I understand that I may not make changes to this protocol. (The only exception being action needed to remove a subject from immediate harm, with subsequent notification to the study PI and IRB).

Investigator's Signature: _____

Date: _____

Investigator's Name: _____
(Please Print)