**Assessing Gut Microbiota Mediated Health Outcomes of Whole Wheat and Its Major Bioactive Components**
**Study Protocol**
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## I. Objectives

The use of functional foods or their bioactive components to manage chronic disorders has become popular in recent years. One dietary product that can be tapped for its health benefits is whole grain. Observational studies suggest an inverse relationship between the intake of whole grain and obesity, prediabetes, some cancers, and all-cause mortality. A growing body of clinical evidence (mostly small randomized clinical trials) demonstrates positive effects of whole grain on certain risk factors for chronic diseases and cardiometabolic abnormalities; however, outcomes of clinical interventions have revealed substantial interpersonal variation in the health benefits, with a subset of individuals not exhibiting any measurable benefits. Whole grains/whole wheat contains significant amounts of bioactive phytochemicals in addition to its well-recognized fiber content. Outcomes of many studies support that whole grains/whole wheat provide favorably improve human health by the direct effects of these phytochemicals and/or their microbial metabolites. Thus, the ***objectives*** of this study are to investigate the gut microbiota-mediated effect of whole wheat consumption, the most widely consumed whole grain, on the fecal and plasma metabolomes, metabolic health, and gut health in a cohort of adults with prediabetes. We ***hypothesize*** that gut microbial metabolism of whole wheat and its major bioactive components is a determining factor of human health benefits; and by understanding the gut microbiome--mediated benefits of whole grain, we can truly harness its benefits for metabolic health in humans.

## II. Background and Rationale

***A. Controversy in the benefits of whole grain/wheat bread consumption***. Bread is consumed by billions of people worldwide. This key dietary component comprises ~10% of energy intake in adults.[1] Wheat is the most commonly used cereal for baking bread, and whole wheat contains germ and bran, which are rich in dietary fiber, proteins, phytochemicals, B-vitamins, lipids, iron, magnesium, and zinc.[2, 3] While these components are considered ‘‘healthy,’’ they are often removed in the milling process of refined flour. It is well-known that dietary fiber in whole wheat plays an important role in human health. However, given the largely underestimated phytochemical contents in whole wheat, the role of phytochemicals during its consumption is understudied and therefore should be addressed.[4, 5] The groups of reported phytochemicals in whole wheat include alk(en)ylresorcinols (ARs), benzoxazinoids, phytosteroids, sphingolipids, lignans, flavonoids, phenolic acids, fatty acids and glycolipids, tocols, carotenoids, oxylipins, and other minor components. Animal and *in vitro* studies[6-8] have shown that diets containing whole wheat or wheat bran can reduce the fasting blood sugar by ~20 %; suppress high-sucrose diet-induced hyperinsulinemia by more than 50%; and wheat ARs (C15:0 – C25:0) may play important role in type II diabetes mellitus (T2DM) prevention by effectively inhibit glycerol-3-phosphate dehydrogenase and prevent triglyceride accumulation, as well as suppress adipocyte lipolysis and hormone-lipase activities. In addition, sinapic acid from whole wheat consumption has been shown to ameliorate hyperglycemia through inducing high GLUT4 expression and increased glucose uptake via the phospholipase C -protein kinase C signals in diabetic rats.[9] Meanwhile, scientific evidence regarding the health benefits of whole-grain consumption in humans is contradictory despite accumulating observational studies and randomized control trials (RCTs). Several studies suggest that whole grain consumption is beneficial to human health based on associations that significantly reduce all-cause mortality;[10] cancer risk, [11] cardiovascular diseases,[12] T2DM, and metabolic syndrome; and an improvement in glycemic control. [1, 13, 14] Conversely, some studies showed improvement in only a handful of clinical markers [15-17]. And, large-scale interventional crossover trials show no significant effect on these disease-risk markers.[15, 18, 19]

***B. Gut microbes as a key mediating factor in individual phytochemical response***. Food is generally processed by our gastrointestinal system primarily to supply energy and key functional elements to the body. Dietary components, such as carbohydrates, proteins, and fats are respectively catabolized into monosaccharides, amino acids, and fatty acids that serve as fuel to maintain organ function and promote cellular growth and recycling. Furthermore, the biological organization of the mammalian superorganisms, the gut microbiota, introduces a complex noneukaryotic compartment that also interplays with food components and key regulatory physiological processes of the host. It is now well-known that gut microbiota interacts with the diet and impacts host physiology and metabolism.[20] However, there are many recent reports regarding a large inter-individual variation in the response to different dietary components including phytochemicals,[21] and these findings have indicated that the gut microbial composition could be used to identify those subjects who would benefit from dietary interventions. [1, 13, 14] Circulating concentrations of phytochemicals, such as the flavonoids naringenin and hesperitin, psoralens, and lignans, can vary widely among individuals even in controlled feeding studies.[22, 23] Previous reports have demonstrated that gut bacteria can hydrolyze glycosides, glucuronides, sulfates, amides, and esters,[21] and they can also carry out reduction, ring-cleavage, demethylation, and dehydroxylation reactions.[24-26] The hydrolysis of glycosides and glucuronides typically results in metabolites that are more biologically active than the parent compounds. In contrast, further bacterial degradation and transformation of aglycones can lead to the production of more or less active compounds, depending on the substrate being metabolized and the products formed. One of the most familiar examples of interindividual differences in the microbiome-dependent dietary effects relates to isoflavone metabolism. For instance, equol is a well-known microbial metabolite from the soy isoflavone daidzein, and the proportion of equol-producing individuals is reported to be approximately one-third to one-half of the human population.[21] Host genetics may contribute to interindividual differences in equol production in humans; however, the reasons for such differences in the ability to harbor the equol-producing bacteria remain essentially unknown. Interindividual differences in gut microbial community composition also affect the metabolism of other phytochemicals and ultimately human exposure to specific bioactive compounds.

***C. Gut microbial diversity may explain the personalized protective effects of whole wheat against prediabetes.*** As evident by the sharp incline in the prevalence of prediabetes, blood glucose levels are rapidly increasing in the worldwide population. It is estimated that impaired glucose tolerance will affect at least 37% of the adult population in the US alone.[27] Prediabetes, characterized by chronically impaired blood glucose responses, is a significant risk factor for type II diabetes mellitus (T2DM), with up to 70% of prediabetics eventually developing the disease.[28] It is also linked to other manifestations, collectively termed metabolic syndrome, including obesity, hypertension, non-alcoholic fatty liver disease, hypertriglyceridemia, and cardiovascular disease.[29] Thus, maintaining normal blood glucose levels is considered critical for preventing and controlling metabolic syndrome.

***D. Summary***. This overview demonstrates that consumption of whole grain foods has been associated with decreased risk of obesity, prediabetes, and type 2 diabetes.[1, 30-32] However, mechanisms mediating the protective effect of whole grains are unclear, although glycemic response and insulin sensitivity are the most common mechanisms suggested by epidemiological studies. [33-36] An early report has raised the possibility of gut microbiome-mediated effects linking cereal fiber consumption with decreased risk of T2DM;[37] this knowledge gap continues to persist. It was suggested that modifications in gut microbiota, gut fermentation, and influences of yet unknown molecular factors such as phytochemicals may affect insulin sensitivity and further development of type 2 diabetes. Recent results suggest a link between changed gut microbiota and varied glycemic responses.[1, 13, 14] Furthermore, subjects with T2DM had depleted *Bifidobacteria*,[38], and also the level of glucose tolerance was associated with certain gut bacteria.[39] Another review concluded that despite convincing epidemiological evidence, *the lack of controlled clinical studies to support or explain the mechanisms underlying the protective effect of whole grains against type 2 diabetes*.[40] Thus, this application ***aims to uncover*** the role of gut microbiota and their metabolites of whole wheat bread (WWB) components in mediating blood glucose excursions in prediabetes. We will

specifically compare the gut microbiota composition of prediabetes subjects who exhibited improved glucose metabolism following the WWB diet with those who responded least to this dietary intervention to determine if differences in gut bacteria could explain the beneficial effect of WWB in some individuals.

## III. Anticipated Results

We expect to achieve >95% compliance (based on in-house observations of test meal consumption, returned foods, and circulating biomarkers of WWB intakes, e.g., alkylresorcinol concentrations of 19:0, 21:0, and 23:0) without any adverse effects or changes in body mass or energy intakes.

Based on prior studies,[13] we expect a >25% within-trial decrease in postprandial blood glucose by WWB in high-responders compared to WB. While a statistically significant between-treatment effect is also expected, our study is designed to define 'high-responders' vs 'low-responders' towards precision nutrition rather than achieve group mean differences by bread treatment.[14] In addition, we expect reduced serum endotoxin during WWB intervention, as metabolic endotoxemia is known to initiate insulin resistance in obese persons.[41, 42] These benefits are expected to occur through an anti-inflammatory mechanism that attenuates gut-derived endotoxin translocation[43] or an antimicrobial activity of WBB that depletes pyrogenic Gram-negative bacteria. In support of the former, we expect WWB will decrease small intestinal and colonic permeability,[44] based on decreased urinary excretion of lactulose/mannitol and sucralose/erythritol. WWB may also alter, at least in a subset of the study participants, the gut microbial composition, as reported previously in both healthy adults as well as in persons with MetS (e.g. an increase of *Lachnospira, Faecalibacterium, Prausnitzii,* and *Prevotella copri* abundance and a decrease in *Enterobacteriaceae* abundance in the WWB group[45, 46]). It is also possible to detect selective antimicrobial activities of WWB (possibly due to its rich phytochemicals)[16, 47] and consequentially decreased LPSproducing Gram-negative bacteria. If these findings are consistent in a subset group of study participants who have larger changes of glycemic response post WWB intervention, they will collectively suggest a mechanism, at least in part, that the multi-facet health benefit of WWB consumption is gut microbiome– dependent. Meanwhile, with our combined targeted and untargeted metabolomics approaches, we expect a group of known (e.g., energy metabolism metabolites and amino acids)[48, 49], and unknown metabolic features may change significantly in fecal and blood metabolome between WWB and WB intervention. Taken together, these studies will establish a solid foundation for determining health and metabolic effects on the human host based on the composition and microbial metabolic capability of gut microbes.

## IV. Pitfalls and Alternatives

*Recruitment.* We acknowledge the challenge to recruit adults with prediabetes for a nutrition study during this unusual time of pandemic. Consistent with our previous studies,[50-52] we will consult the recruitment office of the OSU CCTS for assistance. They maintain a participant database (>126K registrants) and provide marketing support for study recruitment (e.g. social media, printed flyers, e-newsletters reaching ~100K faculty/staff and students at OSU).

All proposed procedures are well-established and frequently utilized by our research team. We, therefore, do not expect potential technical problems. However, we acknowledge the challenge of the potential interpersonal variability of dietary responses to WWB among the enrolled study participants, and there may be difficulties to identify sufficient "high-responders" and "low-responders" based solely on the glycemic responses. If needed, we will combine other health parameters, such as HOMA-IR, serum endotoxin, and select inflammatory biomarkers, to define response indicators for their gut microbiome dependency.

## V. Significance of the Research and Potential Benefits

Whole grain, especially whole wheat food, is a recognized, important part of healthy dietary patterns due to its high abundance of fibers, protein, and bioactive phytochemicals. However, from both clinical trials and observational studies, controversy persists to clearly define the advantage of having incorporating whole wheat into the diet to manage metabolic disorders including prediabetes. Gut microbial composition and their metabolism, as recently emerged in many exciting studies, may play a critical role in determining the health benefits of having whole wheat in a regular diet in diverse populations. Therefore, our study is ***significant*** because it will provide critical data to help resolve the controversy by studying the gut microbiome-mediated, personalized effects of whole wheat intake in persons with prediabetes. Specifically, the integration of our multi-omics workflow coupled with studies in humans and in vitro will advance an understanding of the metabolic signatures of gut bacteria function to control the risk of prediabetes/glucose intolerance

## VI. Procedures

### A. Overview of Study Design

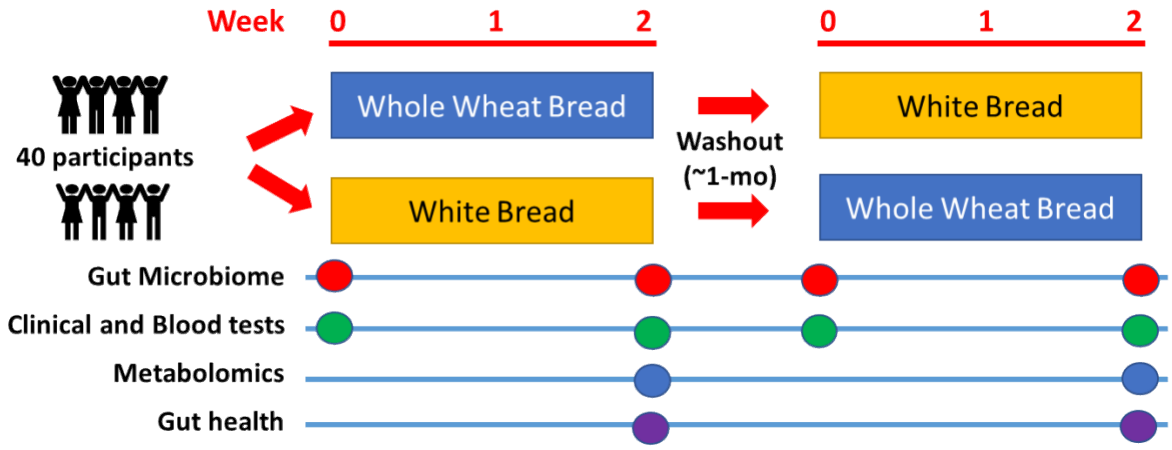


**Figure 1**. The schematic of the proposed clinical study with WWB intervention. Each dot represents a time point for its corresponding analysis. Measures for **gut microbiome (red dots)** involve participants collecting their own fecal samples during the preceding 24-hours. Investigators will then conduct 16S rRNA sequencing of fecal/gut bacteria to determine phylogenetic composition. **Clinical and blood tests (green dots)** entail measures of anthropometrics, blood pressure, routine blood chemistries (glucose, insulin, lipids), circulating inflammatory proteins (C-reactive protein, interleukin-6 and -8, tumor necrosis factor-α, myeloperoxidase, mRNA expression of pro-inflammatory genes from isolated peripheral blood mononuclear cells (Toll-like receptor-4, MyD88, p65, interleukin-6 and -8, tumor necrosis factor-α, myeloperoxidase, monocyte chemoattractant protein-1. Serum endotoxin will also be assessed. Measures of **metabolomics (blue dots)** will be performed from collected fecal and serum samples to quantify >200 metabolites from >25 metabolic pathways. In addition, short chain fatty acids will be evaluated from fecal samples. **Gut health (purple dots)** entails participants ingesting a solution containing non-digestible sugars and collecting their own urine for 24-hours. Urinary excretion of sugars is then measured to determine region-specific intestinal permeability.

We plan to conduct a 2x2 *randomized, controlled, crossover trial* in persons with prediabetes (n = 40; 1:1 M/F) to assess 2-wk consumption of white bread (WB) vs whole wheat bread (WWB) on cardiometabolic and gut microbiome parameters (**Figure 1**). Participants will be recruited from the Columbus, OH area,

consistent with earlier successful studies conducted by the Bruno lab. Participants will be block-randomized in 4-unit blocks to receive WB or WWB (128 g/day; 4 bread slices/d, according to the USDA "My Plate" recommendation and other similar studies)[14, 53, 54] for 2-wks. During each study arm, participants will be provided standardized test meals containing WB or WWB, these foods will be prepared and packaged in the Human Nutrition Metabolic Research Kitchen (Evans Laboratory) to be devoid of whole grain products, probiotic-containing foods (e.g., yogurt), or fermented foods that are known to alter gut bacteria populations. Before and after each 2-wk study arm, a fasting blood sample will be obtained for metabolomic and clinical measures. Blood pressure and anthropometrics also will be assessed on days 1, 7, 14, and participants will collect a fecal sample to provide to the study team during their visits on day 1 and day 14. Lastly, on day 14, participants will simultaneously complete an oral glucose tolerance test and gut permeability test, which involves ingesting a glucose solution that also contains non-digestible sugar probes prior to collecting complete 24-hour urine samples for assessments of upper and lower gut permeability (described below). Upon completing these procedures, participants will undergo >2-weeks wash-out before repeating the study identically, but with crossover to the alternate bread diet.

**B. Study Subjects**

***1. Enrollment Criteria.*** Male and female adults with prediabetes (18-65 y), and having no history of liver or cardiovascular disease, overt diabetes, or cancer will be enrolled. They are expected to have fasting blood glucose between 100 to 125 mg/dL and not taking any medications to manage hyperglycemia. To improve participant homogeneity, we will target persons having a body mass index (BMI) of 30-35 kg/m$^2$ who are more likely to be afflicted with prediabetes. *Major exclusion criteria*: significant dietary restrictions (e.g. vegetarian, gluten-intolerant, lactose intolerant) or food allergies (e.g., nuts dairy, egg, soy, fish); user of dietary supplements, prebiotics, or probiotics; aerobic exercise >5 h/wk; smoker; alcohol consumption (≥2 drinks/d); pregnancy or fertility treatments; usage of antibiotics or antifungals within 3-mo prior to participation; chronically active inflammatory or neoplastic disease in the three years prior to enrollment; chronic gastrointestinal disorder, including diarrhea, inflammatory bowel disease, and Celiac disease; myocardial infarction or cerebrovascular accident in the 6 months prior to participation; coagulation disorders; chronic immunosuppressive medication usage.

***2. Recruitment***. We will recruit participants through posted flyers, e-mail, electronic and newsprint advertisements (e.g. campus student and faculty/staff newspapers, local and regional newspapers; see attachment "*Advertisements*"), and word of mouth. The posted advertisements will instruct interested participants to call the study center (Bruno Laboratory, Department of Human Sciences) or complete an online pre-screening survey to obtain further information. During the phone-in hours, a trained individual (i.e. project coordinator or graduate assistant) will be available to describe the study and determine preliminary qualification by conducting a scripted phone interview (e.g. do you take dietary supplements?, do you smoke?; see "*Phone Script"* attachment). The individual will record answers and assess whether the person calling is likely or not to be an acceptable study participant. If the caller and the interviewer agree that the caller should participate, the prospective participant will be invited to a screening meeting, where the study will be fully described, and the individual will be provided a consent form to complete prior to any involvement in the study procedures. Data collected during this phase will include participant's age, health status, physical activity, and contact information (see "*Informed Consent"* attachment).

***3. Power Calculation and Data Analysis.*** Dr. Mo (biostatistician*; co-PI and Dept of Biomedical Informatics*) performed a power analysis. To ensure that the order by which each participant completes each trial is randomized, a random sequence generator (http://www.random.org/sequences/) will be used to determine the order by which each participant will undergo the two trials. We will primarily compare the

within-subject changes (WWB endpoint vs. WB endpoint) between high-responders and low-responders. We anticipate to have 6-12 high-responders and 5-10 low-responders of the total 40 study participants to achieve 80% power to detect a 1.6-fold difference in the changes of postprandial glucose levels (AUC from 0 to 180 minutes) between high-responders and low-responders, at the expected coefficient of variation (CV) 0.5 at a significant level of 0.05.

Mixed effect modeling will be performed, diet and time will be included as fixed factors while participant serves as a random factor. False discovery rate (FDR) will be used to control type I error rate for microbiome and metabolome data, while the Bonferroni method will be applied for other measures (e.g., inflammatory markers). Data will be first normalized before analysis. Spearman correlations will be calculated to define associations between microbes, inflammatory genes, metabolites that show significant changes in highresponders.

To test whether the changes in microbes after WWB intake associate with the biological response, logistic regression will be fitted to each of the potential microbes or as a combination. We will also couple the logistic regression with receiver-operating characteristic (ROC) analysis to evaluate the specificity and sensitivity of the microbes that predict participants' biological response, and the area under the curve (AUC) of the microbial model will be displayed. Data analysis will be performed in R (open resource) or SAS (SAS, Inc; Cary, NC). The sample size was calculated in PASS 20 (NCSS, LLC; Kaysville, Utah).

**C. Measurement/Instrumentation**

All procedures will take place in the Human Nutrition Program located in Evans Laboratory. Infrastructure that is present includes a metabolic research kitchen, a private office to meet with participants, a clinical research laboratory for performing phlebotomy and research procedures, and a separate biochemical laboratory for processing and preserving biological specimens.

***1. Anthropometric Parameters and Blood Pressure.*** At screening, day 1, 7, and 14, participants will rest for 15 minutes prior to determining blood pressure using an automated blood pressure cuff. BMI will be calculated from height determined from a wall-mounted stadiometer and weight from a calibrated scale.

***2. Blood Chemistries.*** Fasting plasma glucose and lipids (total cholesterol, HDL-C, triglyceride) will be measured by clinical assay (Pointe Scientific) at screening, day 1, and day 14. Fasting insulin will be measured by ELISA (ALPCO) at day 1 and day 14. Postprandial glucose (0-180 min at 30 min intervals) following an oral glucose tolerance test will also be measured on day 14. At the beginning and the end of each intervention arm, we will define the effects of WWB on inflammatory responses. We will assess intestinal inflammation by measuring fecal calprotectin and myeloperoxidase (i.e. indices of neutrophil inflammatory responses) by ELISA as we described.[52] We will also measure systematic inflammatory responses in both fasting and postprandial serum by ELISA (CRP, IL-6 and -8, TNFα, MPO) and by RTPCR analysis of peripheral blood mononuclear cells (PMBCs; isolated at blood collection) to assess the expression of genes involved in TLR4/NFκB signaling (TLR4, MyD88, p65, IL-6 and -8, TNFα, MPO, MCP-1).

***3. Endotoxin***. Serum endotoxin from the individual participant will be assessed on day 1 and day 14 using a high-sensitivity fluorometric assay (PyroGene rFC; Lonza) and endotoxin-free consumables as reported in our previous studies.[55] In our hands, inter- and intra-assay CV is 4.2-6.1%.

***4. Preparation of Whole Wheat Bread.*** To ensure uniformity in WB and WWB test products throughout this controlled study, both types of breads to be used will be prepared by co-Investigator Dr. Devin Peterson (Department of Food Science and Technology) and provided to the Bruno lab for clinical study. WWB and WB will be prepared in the Peterson lab in the Parker Food Science and Technology Building as previously reported by Cong et al.,[56] using the modified AACC straight-dough bread-making method.[57] The formula, is 200 grams of flour, included 10.6 g yeast (active dry), 12.0 g sucrose, 3.0 g salt (NaCl), 6.0 g shortening, and 130.0 g of water. Ingredients will be mixed (KitchenAid, Benton Harbor, MI) until a sponge dough is formed, approximately 2.5 min. The dough will be placed into a 5-3/4" x 3-1/4" x

2-1/4" (14.6 × 8.3 × 5.7 cm) loaf pan and fermented for 52 min at 30 °C and 85 % relative humidity, then proofed for 25 min, again for 33 min and punched between proofs. Finally, the dough will be baked at 215 °C (Doyon convection oven, Menominee, MI) for 17 min. The bread in this study will be made with limited ingredients to focus on the influence of endogenous chemistry in whole wheat flour on health activity of whole wheat bread.

***5. Dietary Control.*** To improve diet homogeneity and limit potential confounding effects of whole grain/wheat intakes on study outcomes, participants' basal diets will be rigorously controlled during each 2-wk intervention by a registered dietitian (PI Bruno) to provide 50-60% energy from carbohydrate, 2535% fat, and 15-25% protein consistent with the typical American dietary pattern. Participants will be required to consume bread-containing test meals at breakfast and lunch each day; these will be consumed at the study center 4 d/wk during each 2-wk study arm to establish rapport and ensure compliance to study procedures. The WB and WBB used in this study will be prepared in the pilot lab in the Department of Food Science and Technology under the auspice of Dr. Peterson (co-investigator of the project) to ensure compositional consistency.

Participants' basal diets, including bread-containing test meals, will be administered in a eucaloric manner by estimating energy requirements using the Harris-Benedict equation,[58] with appropriate adjustment for physical activity.[45] During each 2-wk study arm, participants will return any uneaten foods, which will be weighed to determine actual energy and nutrient intakes using NDSR dietary analysis software. All foods and beverages will be identical between study arms with the exception of WBB and WB. Participants will also complete a food diary to record the consumption of any non-prescribed foods. Non-fasting body weight will be measured 3 times/week throughout the study to monitor weight stability. If body mass fluctuates >1 kg, the participant's prescribed energy intake will be adjusted to prevent additional weight loss or gain. Thus, our approach will minimize participant burden while applying sufficient dietary rigor to test the hypothesis that WBB consumption, principally through its bioactive phytochemicals, improves glucose tolerance in a microbiota-mediated manner.

***6. Gut Permeability.*** On day 14 of each intervention, fasted participants will ingest a glucose solution (75 g; oral glucose tolerance test) containing 4-sugar probes [lactulose (5 g), mannitol (1 g), sucralose (1 g), and erythritol (1 g)] as described.[59, 60] Urinary sugars will be assessed by LC-MS as we established.[59] Sugar excretion (%), and excretion ratios of sugars from 0-5 h (lactulose/mannitol) and 6-24 h (sucralose/erythritol) will be calculated to reflect upper and lower gastrointestinal permeability, respectively.[61-64] This approach is based on lactulose being absorbed in the small intestine, but not the colon, whereas sucralose is absorbed in the colon.[65] Thus, these studies will define site-specific gut permeability, and the benefit of WWB treatment.

***7. Microbiota Composition.*** Microbiota composition and the predicted functional metagenome will be assessed from fecal samples provided on day 14 of each intervention arm as previously described.[66-70] Effects of WWB (vs WB) on total bacterial abundance will be determined by qPCR.[71] Microbiota will be characterized for diversity and community structure by sequencing 16S rRNA genes using MiSeq. Bacteria will be identified by comparing sequences representing each operation taxonomic unit (OTU) with RDP and Silva databases. [72, 73] α-Diversity (OTU richness, Shannon-Wiener diversity index, evenness) will be calculated using Qiime. Comparisons of diversity among samples (β-diversity) will be performed using the Bray-Curtis dissimilarity,[74] followed by multivariate analyses (e.g. principle coordinate analysis, permutational multivariate analysis of variance, analysis of similarity, partial least square discriminant analysis). The functional metagenome will be predicted using PICRUSt[75] and PanFP[76] based on reference genome databases and the KEGG Orthology classification scheme to obtain a functional gene abundance matrix.

***8. MS-Metabolomics & Short Chain Fatty Acids.*** We will perform rigorous metabolomics analysis of *serum samples on day 1 and day 14 and fecal samples provided at day 1 and day 14* from study participants use multiplex MS-based metabolomics with the following techniques: (1). Targeted LC-MS/MS metabolic profiling (TMP) methods using both regular and stable isotope-labeled chemical standards to achieve accurate, specific, and quantitative metabolite measurements of >200 metabolites from more than 25 metabolic pathways. [77] This method can provide accurate pathway-by-pathway measurement and validated metabolite identification for further biological system analysis, as demonstrated by our recent publications [78-80]. (2). We will also use a newly developed metabolomics approach, Globally Optimized Targeted (GOT)MS [81] to analyze broader coverage of metabolites. GOT-MS combines many of the advantages of targeted detection and global profiling in metabolomics analysis, including the capability to detect unknowns, broad metabolite coverage, and excellent quantitation. (3) We will also use untargeted metabolomics for exploratory studies: Briefly, a Thermo LC-Q-Exactive Hybrid MS system will be used for untargeted analysis. Compound Discoverer 3.1 software will be used with customized workflows for different types of samples. Compound identification will be achieved by multiple database searches (HMDB, m/z cloud, METLIN, mass bank, etc.) [82-84] and MS/MS fragmentation pattern comparisons to authentic standards. In addition, using our targeted LC-MS/MS-based panel of >50 frequently reported phytochemicals in whole wheat, such as representative alkylresorcinols, benzoxazinoids, phenolics (e.g., ferulic acid),[4, 5] we will quantify these phytochemicals and metabolites in serum and fecal samples following enzymatic hydrolysis with β-glucuronidase/sulfatase.[85] Analyte identification and quantification will be confirmed by comparing fragmentation patterns relative to purified standards or reference databases. In addition to defining the effect of WWB treatment on host metabolism, time-dependent serum measures of alkylresorcinol concentrations of 19:0, 21:0, and 23:0 and total alkylresorcinols in study participants who consume WWB will objectively establish *subject compliance* to the dietary intervention.[45, 86]

Separate LC-MS/MS procedures that are established by our research team, based on our published report,[87] and will be used to evaluate a panel of ten $C_2$-$C_6$ straight chain SCFAs (i.e. butyrate, acetate, propionate) and branched-chain SCFAs (e.g. isobutyric acid, isovaleric acid). Straight chain SCFAs are predominantly from bacterial fermentation of fibers whereas branched-chain SCFAs are derived mainly from bacteria metabolism of branched-chain amino acids. The former is often depleted with gut dysbiosis whereas the latter is often increased. In brief, fecal SCFAs are derivatized with 3-nitrophenylhydrazine to increase their stability prior to injecting on the LC-MS/MS system operated in negative ESI mode for quantitative analysis.

## D. Detailed Study Procedures

***1. Overview of Study Procedures.*** Potential participants who call the study center in an anonymous manner for more information will be given a brief description about the study and asked a few questions to determine their eligibility (*see Phone Script* attachment). If they meet the eligible criteria, they will be invited to the study center for a screening meeting. Prospective participants will be provided the Informed Consent form via email for review prior to an in-person discussion at the study center. During the in-person meeting, the Informed Consent (see *Informed Consent* attachment) will be explained and a hardcopy provided for them to review. If he/she chooses to participate in the study, they will then be asked to provide written consent. After receiving informed consent, the participants' height, weight, and blood pressure will be measured. Additionally, a small fasting blood sample will be collected for blood chemistry analysis. If they are not fasted at least 10 hours, they will be asked to come back in the fasted state at a time of mutual convenience. These blood results in combination with anthropometric parameters will determine the participant's eligibility. Eligible participants who agree to proceed with the study will then complete two 2-week intervention periods in a randomized order, each followed by a 3-h postprandial trial where a glucose tolerance test will be administered (Figure 1). We estimate that completion of all study procedures will take ~6-8 weeks per participant. Each study procedure will be discussed in detail below:

***2.*** ***Screening Meeting.*** Potential participants who have met the initial criteria of the study (based on the telephone interview) will be invited to the study center at a mutually convenient time. During this time, the participant and a member of the research team will meet in a private, quiet conference room or office. The individual will be provided the informed consent form, and its contents will be described to the potential participant. The participant will then review it, and if they choose to participate in the study, they will be asked to provide written consent. Although the participant will be asked to sign the informed consent, the participant will be told that they will not be asked to participate if their body measurements or plasma chemistries (see *Enrollment Criteria*) do not meet the study criteria. If the participant has provided consent, we will then measure the participant's height, weight, and blood pressure. Next, if the participant is fasted for at least 10 hours, we will ask if a trained individual can draw a small blood sample (10 mL; 2 tubes) so that we may measure blood chemistries (glucose, total and HDL-cholesterol, triglyceride). All samples will be coded to maintain participant anonymity. If the participant's anthropometrics or plasma chemistries do not meet the study criteria, they will be told that they do not meet the study criteria.

Potential participants who meet the criteria will be contacted within a few days after their screening meeting to provide them with their blood and body measurement results and inform them of their eligibility to participate in the study. Consistent with our CLIA exemption, blood results will be provided in a categorical manner (i.e. "normal", "marginally high", "high") rather than providing actual blood concentrations of lab values (see *Subjects Results Sheet*). Potential participants having any blood values outside of the "normal range" will be directed/encouraged to follow-up with their own physician. Those having body measurements and blood values within acceptable limits (see Inclusion/Exclusion criteria), will be invited to participate in the study. While only blood glucose and BMI will be used as the major inclusion/exclusion criteria, other blood chemistries and blood pressure measurements will also be assessed to characterize participants' general health status. Subjects will be included or excluded based on a best fit of the inclusion and exclusion criteria (an example of best fit would be if a potential subject says he/she exercises 4 h/wk, which is close to our exclusion criteria of 5 h/wk, they might still be included in the study if they meet all other inclusion and exclusion criteria more closely than other potential subjects). Participants will be read one phone script if they qualify and another phone script if they do not qualify (see *Participant Eligibility Phone Script* attachment). Potential participants who qualify for the study will be communicated a message as follows: "Congratulations! You have been selected for participation in our study based on your blood testing results. A study investigator will be telephoning you to invite you to the testing session. Would you like to know your blood chemistry results?" Subjects not selected for study will be told the following message: "You have not been chosen for our study, but thank you for your interest." This message will be followed by an explanation why they were not chosen, such as lab values outside of the range we are looking for: "Your blood testing data is…..we were looking for participants who had levels less than …..". "We can provide you a copy of your results if you would like…..how would you like us to provide them to you?". "You should also consider sharing these results with your physician."

If a participant is telephoned and is unavailable, a message will be left requesting a callback at a convenient time or that at a member of the study team will try calling again at a later time. No confidential or sensitive information will be shared with third parties or left on answering machines.

***3.*** ***Study timeline and activities occurring at each study visit.*** As indicated in **Figure 1**, each participant will complete a 2-arm (whole wheat bread; white bread), randomized, cross-over study. For this study, each participant will visit the study center a total of 13 times (screening visit plus 6 times per study arm), including:

- **Informed Consent & Screening Visit.** Potential participants will visit the study center to meet with a designated study team member. At this meeting, the informed consent will be reviewed with the potential participant and be allotted time to address any questions. If the participant agrees, the informed consent form will be signed for enrollment. If the participant is fasted, anthropometrics and blood pressure will be measured, and a fasting blood sample will be collected to determine whether circulating chemistries are within acceptable limits. If the participant is not fasted, these procedures will be scheduled at a time of mutual convenience. If the participant fulfills study inclusion criteria, the participant will proceed to complete Trial 1 procedures.

- **Trial 1 Procedures (2-week intervention to WWB or WB)** ○ *Day 1:* Participant will visit the study center for measures of anthropometrics and blood pressure, the collection of a fasting blood sample, and to pick-up daily meals for Days 13. All foods and beverages will be provided, and daily test meals will include WWB or WB. The participant will also provide a fecal sample that was self-collected within the preceding 24-hours.

    - *Day 3:* Participant will visit the study center briefly to pick-up meals that will be consumed on Days 4-6. Participant will return all uneaten foods and containers to the study team to determine actual food consumption during the preceding period.

    - *Day 7:* Participant will visit the study center briefly to pick-up meals that will be consumed on Days 7-10. Participant will return all uneaten foods and containers to the study team to determine actual food consumption during the preceding period. Measures of anthropometrics will be conducted and a urine sample will be collected to assess compliance to test meals.

    - *Day 10:* Participant will visit the study center briefly to pick-up meals that will be consumed on Days 11-14. Participant will return all uneaten foods and containers to the study team to determine actual food consumption during the preceding period. A stool collection kit will also be provided.

    - *Day 14:* Participant will visit the study center in the fasting state. The participant will provide a fecal sample that was self-collected during the preceding 24-hours. A urine sample will be collected to assess compliance to test meals. Participant will also return all uneaten foods and containers to the study team to determine actual food consumption during the preceding period. Anthropometrics and blood pressure will be assessed. Blood will be collected (0 min) and then an oral glucose tolerance test (75 g) and gut permeability test as described.[59] will be completed simultaneously. After emptying their bladder, the participant will ingest a 75 g glucose solution containing non-digestible sugars. Blood samples will be collected at 30-min intervals for 3-hours. Participant will be provided urine collection containers to self-collect urine for the subsequent 24-hours. During this 24-hour period, participants will also be provided controlled meals and beverages.

    - *Day 15:* Participant will return to the study center to provide collected urine. All procedures for Trial 1 are deemed complete.

- **Wash-out Period.**

Trial 1 and Trial 2 will be separated by at least two-weeks for men. However, the washout period for women may be extended to enable testing during the same phase (± 3 days) of the menstrual cycle.

- **Trial 2 Procedures (2-week intervention to WWB or WB).**

All procedures for Trial 2 will be identical to those of Trial 1 with the exception that the participant will receive the alternative test meals. If the participant was assigned WWB in Trial 1, WB will be assigned in Trial 2. If WB was assigned in Trial 1, then WWB will be provided in Trial 2.

- Study Completion

On Day 16 of Trial 2, the participant's involvement in the entire study is completed. A check will be provided to the participant in accordance with the payment schedule (up to $350) that is outlined in the consent form.

***4. Blood Collection and Sampling Handling.*** Once at baseline (day 0; 14 mL), and at each time point of the postprandial trial (20 mL * 7 timepoints over 3-h), a blood sample will be collected into evacuated blood collection tubes. Collecting a total of 154 mL or 0.65 cups once during each study arm is necessary to ensure an adequate volume of plasma and serum for each time point to accurately analyze levels of glucose, insulin, endotoxin, and pro-inflammatory genes. During blood collection, participants may feel an initial pain when inserting the needle, bruising around the insertion area, lightheadedness, or fainting, which are common when donating blood. However, we do not foresee any additional significant risks for collecting this amount of blood over 3 hours, other than the risks stated previously.

Throughout the span of the study (~8-12 weeks in duration depending on participant/investigator availability) which consists of four weeks total of controlled feeding, a blood collection at day 0 and 14 and then repeated following a ≥2-week washout period, and 1 screening day, we will be collecting a total of ~318 mL or ~1.34 cups) of blood. Urine will be collected in provided containers (VWR) containing 10% thymol to inhibit bacterial growth. We will then have the participants return their urine samples to the study center or coordinate with study personnel to meet at a public and mutual location to return their samples within 24-h after collection. Feces will be collected using a commercial commode specimen collection system (Fisher Scientific). Briefly, the collection kit consists of the necessary materials (e.g., gloves, waste bag) for participants to easily and hygienically collect their stool without contaminating the sample or themselves. Volumes of urine will be recorded and fecal mass and observations will be recorded based on the Bristol Stool Chart.[88]

During each blood collection, plasma will be obtained by centrifugation and then transferred to cryogenic storage tubes. Serum samples will be obtained by allowing the blood to clot, followed by centrifugation and transfer to cryogenic storage tubes. Tubes will be stored at -80° C until analysis can be completed. Analyses will include plasma glucose, insulin, triglyceride, HDL cholesterol, proinflammatory genes and/or proteins (e.g., TLR4, CD14, MD2, MyD88, p65, IL-1, IL-6, IL-8, TNF-α, MCP-1), and serum endotoxin. Urine will be stored at -80ºC until analysis can be completed. Analysis from urine will include sucralose, erythritol, lactulose, and mannitol. Fecal samples will be stored at -80ºC until analysis can be completed. Feces analysis will include microbiota composition, calprotectin, myeloperoxidase (MPO), and fecal metabolomics analysis. Remaining plasma, serum, urine, and fecal samples not used for these analyses will be archived for 5 years at -80 C in the event we decide to measure additional biomarkers. Appropriate notation has been made in the informed consent to alert participants that we will be archiving specimens and that they have the right to refuse our use of these specimens for future analyses. Lastly, approval from OSU IRB will be sought via a protocol amendment prior to the analysis of any additional biomarkers not specified herein.

**E. Privacy/Confidentiality**

For all data and records that are a part of this study, a number (i.e. code) will be assigned to each participant and will only be available to research personnel. Any records containing the names of participants will be stored in a locked filing cabinet or on a password protected computer in the PI's laboratory or office.

Research personnel under the supervision of the PI and the PI himself will be the only individuals that have access to this information. The names of participants will not be used for publication in any form. The records will be maintained until the data are published, up to a maximum of five years. All archived samples will be coded, but the key linking the code to each participant's identifiable information will have been destroyed. In addition, participants will be instructed that their participation in this study is voluntary and that they may withdraw at any time without prejudice. Data (biochemical values) obtained from this study will stored on a computer in the PI's laboratory. In addition, a backup of digital data will be stored on the PI's computer in his office. Both computers are password protected and both doors are locked when work areas are not in use.

**F. Internal Validity**

For all data and records that are a part of this study, a number (i.e. code) will be assigned to each participant. This will minimize measurement bias when performing analysis on dietary records, and biochemical markers because all samples/records will be coded. The codes will only be broken once data analysis has been completed and verified by the PI.

**G. Medical Safety Plan**

All aspects of the clinical study will be conducted in Dr. Bruno's clinical lab located in 1013 Evans Laboratory. Participants will be fasted for 10-12 hours prior to each study visit. We recognize that certain risks associated with fasting include: hypoglycemia, weakness, and fainting. This duration of fasting is consistent with guidelines set forth by the American Diabetes Association to minimize risk to the individual[62] when determining fasting blood glucose concentrations.

Risks related to hypoglycemia are anticipated to be low due to participants' already marginally elevated fasting blood glucose (study entry criteria = 100-126 mg/dL). They will also be ingesting a glucose beverage containing 75 g of glucose immediately following the fasting period. Throughout each visit to the study center, participants will be closely monitored by a member of the study team (PI, postdoctoral scholar, or graduate student). They will monitor the safety and well-being of participants for any signs and symptoms of hypoglycemia including: confusion, dizziness, irritability, weakness, headaches, and fainting.
Additionally, Dr. Bruno (PI; 1013 Evans Laboratory) has an academic office in close proximity to the study center and has significant experience coordinating clinical research studies involving overnight fasting, and other dietary- and carbohydrate-challenges, thereby supporting the competency of our research team in managing potential adverse events relating to fasting glucose and glycemic responses.

Consistent with our prior studies of similar design, and to ensure participant safety, all blood collection procedures throughout each study visit will occur while positioned on a hospital bed in the prone position or while seated in a phlebotomy chair. In the event that a participant was to become weak, dizzy, or faint, they would already be in a safe position to minimize risks associated with these symptoms. In the event that hypoglycemia-related symptoms occur, the study would be terminated to allow the participant to recover. We are prepared to provide pre-packaged beverages and snacks containing simple carbohydrates (e.g. Gatorade, fruit juice, apple sauce, crackers) that will allow for rapid restoration of blood glucose. These food items will be stored in a refrigerator, located in the adjacent metabolic research kitchen, that is dedicated for foods used in research studies. Participant status (e.g. attentiveness, skin color) will be monitored in our clinical laboratory to ensure recovery. The clinical area where participants undergo blood draws is also equipped with first-aid measures (e.g. smelling salt) and all study team members are trained to assist with basic first-aid application if needed.

Any adverse hypoglycemic response that might occur during this study will receive care commensurate to the symptoms. For example, if a participant were to faint, then smelling salts would be administered along with a carbohydrate-containing food upon regaining consciousness. The study PI would also be contacted.

Alternatively, should a more severe hypoglycemic response occur (e.g. contusion or laceration relating to fainting), the research team would immediately contact medical services (i.e. 911). For non-life-threatening emergencies, any OSU students participating in these studies would be directed to the Wilce Student Health Center. For other emergencies or those participants who are not OSU students, individuals would be directed to the Wexner Medical Center either by transporting them directly or requesting ambulance service. Regardless of the complexity of the adverse events, the research team would monitor the participant in the interim, provide palliative care as appropriate, and follow-up after any medical care has been provided.

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