Randomize placebo-controlled trial of a dietary prebiotic alters distal gut microbiome but not body composition, stress, or clinical biomarkers

**Nutrients-1/Frontiers in Nutrition-2**

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**Abstract**

**Introduction**

Western style diets are typically high in fats, meats, and processed food, and subsequently low in dietary fiber (GBD 2017 Diet Collaborators et al., 2019)(Grooms et al., 2013). Lack of dietary fiber has been proposed as a major factor in development of non-communicable inflammatory diseases, including diabetes, heart disease, and obesity (Kovatcheva-Datchary et al., 2015)(Zou et al., 2018)(Lie et al., 2018)(O’Keefe, 2019) (Grooms et al., 2013). Obesity affects one-third of the U.S. population, yet those entrusted to care for this population, physicians, are themselves suffering from obesity at similar levels with 23% obese and 40% overweight (Physicians Health Study 2007). Several factors impact this obesity trend, including not only poor dietary habits, but also high stress, lack of sleep and long work hours, which are typically behaviors obtained during residency training. In residency specifically, our preliminary data along with others show that there is a higher likelihood of having a higher BMI towards the end of residency as compared to the beginning (1). More important, is data showing that physicians who are of normal weight versus those who are overweight/obese, were more likely to provide obesity care to patients (2). Together, this evidence indicates a significant effect of obesity among physicians and residents that impacts not only their overall health, but their effectiveness as physicians in supporting a healthy patient population.

One method for reducing obesity and preventing weight gain, is through increased dietary fiber intake or prebiotics (REF). A beneficial effect of increased dietary fiber in generally healthy individuals is modification of the microbial community and function, which can regulate metabolism and appetite (REF). It has been established in multiple studies that there is an inverse association between consumption of fiber and body weight and body fat. (3–5) The gut microbiome, the collection microbes and their genes, is implicated as a contributing factor in chronic disease such as diabetes (T2DM) and obesity and can be influenced by the intake of fiber via food or supplementation. (3) These results are associated with changes in the microbiome, which can trigger an inflammatory response that is compounded by “Western” style diets that are low in fiber (6,7). Several promising studies, however, indicate that dietary fiber (prebiotics) can resolve or prevent these microbiome changes, as well as, obesity. A systematic review of prebiotic interventions demonstrated significant positive changes in appetite and postprandial glucose and insulin responses (8). The Western diet, however, may be deregulating this process. High-fat diets often lack dietary fiber (i.e., microbe accessible carbohydrates or “MACs”). Dietary fiber is one of the main sources of carbohydrates which the microbiome uses for fuel and in the production of short chain fatty acids (SCFAs). When dietary fiber is lacking (i.e., reduced MACs), the production of metabolites from fermentation of SCFAs is altered leading to changes in the bacterial community, and thus carbohydrate metabolism (9). Lack of dietary fiber leads to increases in bile-resistant Bacteroides, whereas plant-based diets increase Prevotella spp. that has enhanced capacity to ferment polysaccharides and produce SCFAs (10). Although both these bacteria belong to the phylum Bacteriodetes, the effect on metabolism is species specific and currently poorly understood (11). When dietary fiber as a fuel source is scarce, bacteria that use MACs as a source of energy (e.g., Bacteroidetes) will be reduced and replaced by those (e.g., *Akkermansia muciniphila*) that start degrading the mucin in the intestinal wall seeking an alternate source of carbohydrate as energy. Such activity reduces gut barrier function leading to chronic low-grade inflammation similar to what is seen in the obesogenic state (12). Specifically, in a long-term (52 weeks) weight loss intervention in human subjects, those who were most successful in keeping the weight off for at least 2 years consistently had baseline microbial communities enriched in Akkermansia spp. abundance that remained stable over that time (13). Furthermore, researchers were able to use as a biomarker the higher Firmicutes:Bacteroidetes ratios to differentiate those who were obese but metabolically healthy from those who only had metabolic syndrome (e.g., increased blood pressure, high blood sugar and waist circumference, and abnormal blood lipids) independent of obesity, suggesting that it may be possible to use the microbiome (e.g., Akkermansia ) to identify patients who are most responsive to weight loss interventions.

However, not all dietary fibers have the same effects, due to several factors, including solubility, fermentability, viscosity, degree of methyl esterification, and accessibility to the gut microbiota (REF). In a recent meta-analysis of intervention studies of soluble fiber supplementation, overall effects were significant for BMI, body fat, fasting glucose, and insulin (Thompson et al., 2017). Interestingly, in meta-regression analysis, these effects were independent of dose or duration, but the effect on HOMA-IR was specific to fermentable, non-viscous fibers. Specific types of dietary fiber, orange pectin but not citrus pectin, can block the inflammatory pathway through regulation of the Toll-like receptors (TLRs) in murine models of chemically-induced inflammation (Sahasrabudhe et al., 2018)(Ishisono et al., 2019). The specificity of these effects may be not only fiber-type specific, but also microbiome specific. In particular, inulin, which are polymers of fructose, and a subgroup polymer with degree of polymerization </=10, oligofructose (FOS), have had mixed success in randomized placebo-controlled trials of adults and children with obesity or diabetes (Reimer et al., 2017)(Smith et al., 2015)(Ho et al., 2019)(Fernandes et al., 2017)(Dehghan et al., 2014)(Lightowler et al., 2018)(Cani, 2018). In general, the majority of studies show a benefit glycemic control, body composition, weight control, and appetite. Additionally, these studies demonstrated changes in the microbiome, most consistently with increases in Bifidobacteria and production of short chain fatty acids (SCFA). ADD INFORMATION ON INULIN-ENRICHED FRUCTOOLIGOSACCHARIDES – USED IN THIS STUDY – FROM GREG]

The interaction between the gut and brain is also apparent with evidence demonstrating microbiota involved in mediating the stress response, specifically anxiety and depression (14–16). Multiple taxa, including Bifidobacterium and Lactobacillus, Mucispirullum and Desulfovibrionaceae, have been identified in upregulation of inflammation and mediating the stress response (17). This response is likely through the production of neurotransmitters or their inhibitors by taxa such as Bifidobacterium, Clostridium sporogenes, Ruminococcus gnavus, and Lactobacillus, which produce the neurotransmitter gamma-aminobutyric acid (GABA) (14,18). Interestingly, dietary tryptophan is a source of the hormone melatonin and the neurotransmitter serotonin, which is also produced in the gut through SCFAs (18). Supporting this link, germ free mice (GF) have heightened responses to stress that is blunted by colonization with Bifidobacterium spp. (19). Further, obese mice treated with Bifidobacterium spp. have a reduced stress response (20). Thus, there is a clear link between the gut microbiome, diet, stress, neurotransmitters, metabolites and obesity.

The purpose of this study was to determine if supplementation with the dietary fiber supplement, inulin-enriched fructooligosaccharide (Prebiotin), is effective at mitigating excessive weight gain among individuals during medical residency training, and its impact on the structure and composition of the gut microbiome, stress, and clinical biomarkers of metabolism.

**Materials and Methods**

This study utilized a single blind, placebo controlled, parallel design to assess the effect of dietary fiber on the gut microbiome, perceived stress, and anthropometric variables.

*Participants*

All participants in this study were resident physicians at the Family Health Center in Waco, TX. This population was selected based on pilot data indicating they are at a greater risk for weight gain and have higher levels of stress than the average population. Exclusion criteria for this study included: 1) Pregnancy 2) Currently on prescribed metformin or NSAIDS 3) A diagnosed gastro-intestinal disease (Ie: irritable bowel syndrome or Crohn’s disease) 4) Known allergy to the supplement, placebo, or provided meals 5) Antibiotic use within the last 3 months. Interested individuals were verbally screened using the indicated inclusion/exclusion criteria, those passing the screening were enrolled in the study. Informed consent was obtained prior to partaking in any study procedures.

*Dietary Fiber Intervention*

After obtaining informed consent, participants were randomly assigned to two groups – fiber or placebo. This study lasted for thirteen weeks, with baseline assessments being conducted during the first week and the intervention occurring during the subsequent twelve weeks. The fiber group received a p-inulin supplement (Prebiotin; Camp Hill, PA) while the placebo group received an isocaloric amount of maltodextrin placebo. Supplement/placebo was consumed every day for twelve weeks, with the dosage ramped such that 2g/day were consumed during week 1, 4g/day during week 2, 8g/day during week 3, 12g/day during week 4, and 16g/day during weeks 5 through 12. In addition to the supplement, participants in the fiber group consumed a high fiber meal replacement three days per week (BuffBake; Santa Ana, CA) while participants in the placebo group consumed a low fiber meal replacement three days per week (MyCookie; Frisco, TX). To enhance compliance, supplements and meal replacements were provided to participants one week at a time. Participants reported their degree of compliance at the end of each week.

*Stool collection and Microbiome Analysis*

To assess for changes in the gut microbiome, stool samples were obtained from participants at baseline and at the end of weeks 4, 8, and 12 of the intervention. Prior to each sampling timepoint, each participant was provided with a stool sample kit (OMNIgene gut, DNA genotek Inc, Canada) and instructions to take home with them. The stool sample was turned into the researchers the following week and stored at -80 degrees until sequencing was performed.

Samples underwent DNA extraction and sequencing at the Alkek Center for Metagenomics and Microbiome Research (Baylor College of Medicine, Houston, TX). Using manufacturer’s instructions, DNA was extracted from distal gut samples using the MoBio (Carlsbad, CA) PowerSoil extraction kit, and both negative and positive controls were included to control for contamination. Specifically, four controls were used: two for the DNA extraction phase and two for the PCR amplification phase. The negative control for extraction was a blank sample with DNA extraction kits added to an empty tube. The positive control for extraction was a stool sample; specifically, an aliquot of a homogenized sample from a non-study donor. The negative control for PCR amplification is pathogen-free PCR clean water sample run in the reaction as normal and with water added to make up the volume. The positive control for PCR was a preparation of *F. tularensis* DNA. This control was chosen due it being a pathogen only found in <1% of samples regularly sequenced at the core facility.

The 16S rRNA V4 region was amplified by PCR and sequenced on the MiSeq platform (Illumina) using the 2 × 250 bp paired-end protocol [42] which generated a total of X quality reads (average = X) with a max sequencing depth of X reads. The 16S rRNA gene sequence processing pipeline incorporates phylogenetic and alignment-based approaches to maximize data resolution [43]. Read pairs were demultiplexed based on the unique molecular barcodes, and reads were merged using USEARCH v7.0.1090 [44] allowing zero mismatches and a minimum overlap of 50 bases. Merged reads were trimmed at the first base with PHRED < Q20. A quality filter was applied to the resulting merged reads and reads with >0.05 expected errors were discarded. 16S rRNA gene sequences were clustered into Operational Taxonomic Units (OTUs) at a similarity cutoff value of 97% using the UPARSE algorithm. OTUs were mapped to an optimized version of the SILVA Database containing only the 16S V4 region to determine taxonomies [45,46]. Abundances were recovered by mapping the demultiplexed reads to the UPARSE OTUs. A rarefied OTU table from the output files generated in the previous two steps was used for downstream analyses of alpha diversity, beta diversity, and phylogenetic trends [47]. Data were rarefied to a depth of X reads/sample for normalization; given the small sample size of this pilot study, this depth enabled the retention of all quality samples for analysis. Alpha diversity was assessed by calculating observed OTUs (richness), Shannon diversity index, Inverse Simpson, and Fisher’s alpha (measures of richness and evenness). We applied the UniFrac distance metric, which assesses beta-diversity (between-sample diversity), with the weighted measure to assess differences in abundances of microbiota between groups and the unweighted UniFrac measure to assess for differences between groups of rarer taxa. [ADD METHODS FOR ANALYSIS/TRANSFORMATION OF DIETARY DATA]

*Diet Analysis*

Baseline dietary habits were measured using the Diet History Questionnaire version 3 (DHQIII). The DHQIII is a web-based tool that was completed during the baseline week of the study. To control for acute dietary changes to the gut microbiome, a 24-hour dietary recall was obtained for the day prior to the collection of each stool sample (at baseline, and the end of weeks 4, 8, and 12) using the Automated Self-Administered 24-hour Dietary Assessment Tool (ASA24). The Healthy Eating Index (HEI-2015) was calculated from the DHQIII data, and was used to measure dietary patterns the month prior to study entry. These data were used to score the historical diet quality of each study participant, which is critical to microbiome-diet studies that assess the effect of a dietary intervention on the microbiome response. The HEI is calculated based on 12 components which each have an individually assigned score to give a maximum score of 100. The components of the HEI include Food Pyramid Group Equivalents calculated for total fruit, whole fruits (excluding juice), total vegetables, dark green and orange vegetables, vegetables and legumes, total grains and whole grains, as well as, milk, meat and beans, oils, saturated fat and sodium. Those individuals having a score above 80 are considered to have a healthy diet, whereas those below 50 are considered to have a poor dietary intake, and do not meet 75% of the recommended dietary allowances.

*Stress Response and Analysis*

To assess for the relationship between the gut microbiome and stress, participants completed the Perceived Stress Scale (PSS; Cohen, Kamarck, & Mermelstein, 1993) during baseline and at the end of the twelve-week intervention period. The PSS is a 10 item self-report measure of stress within the last month, in terms of unpredictability, overload and uncontrollability. Each question is answered with a range of 0-4 with 0 equating to “never” and 4, “very often.” Possible scores range from 0 to 40 with higher scores indicating greater perceived stress

*Body Composition Analysis*

Anthropometric measures were obtained during the baseline week and at the end of the twelve-week intervention. Height was measured to the nearest 0.10 centimeter using a stadiometer. Weight was measured to the nearest 0.10 kilogram using a calibrated digital scale. Waist circumference was measured three times to the nearest 0.10 centimeter using a Gulik tape measure at an area just above the iliac crest per National Heart, Lung, and Blood guidelines. Percent body fat was measured using the InBody...

*Blood Analysis*

Blood draws were conducted with participants in the fasted state before and after 12 weeks of supplementation. Blood samples were collected via venipuncture at the most prominent vein in the antecubital space. Samples were allowed to clot on ice for 60min before centrifuging at 3500g for 15 min. After centrifugation, serum samples were aliquoted into microcentrifuge tubes and stored at -80°C for later analysis.

Serum levels of Peptide YY (PYY) were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) following the manufacturer’s instructions (Raybiotech Inc., Peachtree Corners, GA). High-sensitivity C-Reactive Protein (hsCRP) levels were measured via particle enhanced immunoturbidimetry (Roche, Basel, Switzerland). Insulin levels were assessed utilizing electrochemiluminescence immunoassay (ECLIA) (Roche, Basel, Switzerland). A basic metabolic panel and lipid panel were conducted using Roche COBAS automated methodology (Roche, Basel, Switzerland).

*Statistical Analysis*

*Gut Microbiome Characteristics.* The statistical analysis of gut microbiome proceeded in three steps. First, three measures of alpha diversity were used to assess richness (Observed OTUs) and evenness (Shannon and Inverse Simpson). We investigated how alpha diversity metrics varied among participants, overtime, and between intervention groups using linear mixed effects models (LMM) in R (R Core Team, 2020) using the lme4 package (Bates et al., 2015). Models included a random intercept for each participant to account for repeated measurement of alpha diversity over time. Intervention, time, sex, and race were added to the model sequentially and removed if they did not significantly add to the prediction of alpha diversity. Secondly, the effect of the intervention on beta diversity (between and within subjects). We examined beta diversity by intervention (between subjects) and by study week (within subjects) using three measures of dissimilarity, Bray-Curtis and Unweighted and Weighted UniFrac distance measures. We used Permutational multivariate analysis of variance (PERMANOVA) to test beta diversity differences between intervention groups and within subjects over time. Lastly, the effect of the intervention on microbiome abundance was investigated with generalized linear mixed effects models (GLMM) also using the lme4 package. Due to the small sample size, abundance data was only used at the phylum level and for analysis we only used a random intercept using a Poisson (log) link function. A negative binomial or zero-inflated variant link may be more appropriate; however, we attempted these links which resulted in a convergence issues due to the small sample size. Models were built sequentially, similar to how we built the linear mixed models for alpha diversity. The main outcome was the effect of the intervention and the interaction between the intervention and time on the abundance of each phylum. Significance was determined if the p-value for effect was less than 0.05 after controlling for multiple testing of across all phylum using the false discovery rate (FDR) adjustment (Benjamani & Hochberg, 1995).

*Anthropometric Characteristics and Clinical Measures.* Differences in physical characteristics of subjects (anthropometric and clinical measures) was examined using linear modeling. Age, race (dummy coded as hispanic or not), stress, and intervention assignment were regressed onto body mass index (BMI), lean body mass (LBM), visceral fat level (VFL), and weight, separately. The difference score was used as the outcome variable to assess the change from baseline to last measurement observed for each subject. Clinical measures were similarly analyzed using linear modeling to assess the impact of the intervention. Age, measurement occasion (pre vs. post), intervention assignment and occasion by intervention assignment interaction were regressed onto levels of glucose, BUN?, creatinine, sodium, potassium, chloride, carbon dioxide, calcium, cholesterol, triglycerides, HDL, LDL, LDL-HLD ratio, hsCRP, insulin, and PYY. Multiple comparisons were corrected using the FDR adjustment.

**[NOAH PLEASE ADD TO THIS SECTION]**

**Results**

This study recruited 11 physicians in residency training from a local family clinic, 5 males and 6 females. Subjects were randomly assigned to receive either the dietary fiber intervention supplement or placebo for 12 weeks. There were XXX significant differences between groups on physical characteristics or dietary history (FFQ) (Table 1). At baseline, each subject was asked to complete the FFQ, ASA24, and provide stool samples. Blood was also drawn at the first baseline visit and again at the end of the study. Repeated measures included ASA24 and stool samples at baseline and weeks 4, 8, and 12 (Table 2), though not all subjects completed each measure for the duration of the study.

*Effect of Intervention on Distal Gut Microbiome Diversity*

To assess the effects of the dietary fiber supplement on the composition of the gut microbiome we first analyzed the alpha diversity, within subject diversity. We used three measures of alpha diversity to assess richness (Observed OTUs) and evenness (Shannon and Inverse Simpson). Comparing intervention to the placebo group, there was a significant difference at baseline, with the intervention group having significantly higher alpha diversity (XXX). In unconditional models, there was no effect of the fiber intervention on any of the alpha diversity measures. In mixed effect models, adjusting for sex and ethnicity, there was no effect of the fiber intervention over time for any of the measures of alpha diversity. When evaluating the individual changes in diversity for each subject, the amount of inter-individual variability was large and was independent of intervention or baseline alpha diversity measures (Fig. 1). These results indicate that the intervention did not have a significant effect on the alpha diversity as compared to placebo.

Next, we assessed the effect of the intervention on beta diversity, between and within subject beta diversity. We examined beta diversity by intervention (between subjects) and by study week (within subjects) using three measures of dissimilarity, Bray-Curtis and Unweighted and Weighted UniFrac distance measures (Fig. 2). Using the Permutational multivariate analysis of variance (PERMANOVA) test we did not find any significant difference in beta diversity for any measure by study week (Fig. 2A). However, we did find a significant difference in beta diversity by intervention using the Bray-Curtis measure (R2 0.07, *P*=0.018), but the other two measures did not show significance (Fig. 2B). Analysis of the joint effect of intervention over time also did not show any significant difference in beta diversity (Fig. 2C). Thus, the intervention did not change the beta diversity of subjects over the study period.

We next evaluated the individualized response to the intervention at the phylogenetic level and compared it to the fluctuations in food groups and nutrients over the study period by week (Fig. 3). Given the small study size, we kept all 24-hour recall data, but identified outliers for analysis (Supplemental Fig. X) and used the FFQ (DHQIII) data to estimate dietary history and generate Healthy Eating Index Scores. Additionally, we note that not all individuals in the intervention group were 100% compliant with their fiber supplements, especially subjects 1007 and 1015 (Supplemental Fig. X). Looking at total kcal intake by study week, we note that for the majority of those individuals who reported more than one 24-hour recall, kcal intake was highly variable, likely due to the shift work and being ‘on-call’. Macronutrients, carbohydrates, proteins, and fats were more consistent across participants, but for almost all subjects, fat intake was excessive (AMDR 20-35% of kcals); >35% of kcal intake (range 30-60 g/1000 kcals). Individual level fiber intake was also highly variable between subjects, ranging between 5-25g/1000 kcals (Recommended Daily Intake 14g/1000 kcals) (Fig. X). The addition of the fiber supplement, for those who were compliant, lead to a 5-10g/1000 kcal increase in total dietary fiber intake.

Because individuals eat many foods episodically but are consumed with other common foods as pairs or meals, the data are zero-inflated, like microbiome data. For this reason, we chose to take a similar approach to that developed by Johnton et al. (2019); using the Food and Nutrient Database for Dietary Studies (FNDDS) to create a hierarchical tree of foods. Using this approach allows us to compare the food and nutrient data to the microbial abundance data using similar metrics of diversity (Microbiome - UniFrac, Lozupone and Knight, 2005; Food/Nutrients – Faith’s Phylogenetic Diversity, Faith 2009). Microbiome phylogenetic diversity, measured at the phylum level, was variable by subject, but most subjects were dominated by Bacteroides and Firmcutes with four subjects having higher carriage of Verrucomicrobia (Fig. 4). Dietary variability as assessed by food group by subject also showed variability by subject, with most dominated by fat intake. Comparing nutrient variability to food and microbiome variability, nutrient intake over time appeared more stable, and was dominated by sodium, lycopene (tomatoes), and potassium. Due to the small samples size we did not perform any microbiome-nutrient analyses.

To assess the effect of our intervention on taxonomic changes over time in phylum level abundance we used generalized linear mixed effects model. For subjects in the intervention (Group B), we found that Actinobacterio (), Euryarchaeota (), Firmicutes (), Lentisphaerae (), Tenericutes (), and Verrucumicribia () decreased significantly in relative abundance compared to control. Meanwhile, we found that Cyanobacteria () and Fusobacteria () increased significantly in relative abundance compared to control subjects (Group A) over the course of the study. We did not find evidence of significantly different changes in abundance between intervention groups in Bacteroidetes () and Proteobacteria (). These results are summarized in Figure XX where the predicted log abundance for each phyla is shown for intervention and control cases. Covariates (sex, ethnicity) did not change the conclusions of the effects on the intervention on the phylum level abundance of any taxa. Complete results of all models, including diagnostics, are available openly on our online supplemental website. [CHECK WITH NOAH ON THESE STATEMENTS!]

*Effect of Intervention on Anthropomorphic and Clinical Measures*

The main purpose of this intervention was to assess the ability of a dietary fiber supplement to mitigate excess weight gain among physicians in medical residency, as well as detrimental changes in blood lipids and glucose; a problem previously identified among this group (Supplemental Table SX). Overall, there was no significant effect of intervention on BMI or weight gain; however, there was no observation of excessive weight gain [what would our metric be for excessive???] in either group, intervention or placebo (Fig. 5A-B).

In multivariable models, adjusting for age, ethnicity, and stress survey scores, there was no effect of the intervention on any of the anthropomorphic measures, BMI, LBM, VFL, or weight (kg) (Table 3). Further, while there was no effect of the intervention on any of the metabolic markers after adjustment for multiple comparisons, prior to adjustment we did observe a significant increase of LDL cholesterol as an effect of the intervention (unadjusted *P=*0.046, adjusted *P*=0.474).

What about stress and other blood markers? PYY, etc?

**Discussion**

**Figure Legends**

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