The Effects of Fermented Vegetables on the Gut Microbiota for Prevention of Cardiovascular Disease

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This peer-reviewed article has been accepted for publication in Gut Microbiome but has not yet been copy-edited or typeset so may be subject to change during the production process. The article is considered published and may be cited using its DOI: 10.1017/gmb.2024.4
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https://doi.org/10.1017/gmb.2024.4 Published online by Cambridge University Press

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https://doi.org/10.1017/gmb.2024.4 Published online by Cambridge University Press

ABSTRACT

This study investigated the impact of regular consumption of fermented vegetables on inflammation and the composition of the gut microbiota in adults at increased risk for cardiovascular disease. Eighty-seven adults ages 35-64 were randomized into a Fermented Vegetable (FV) group, who consumed 100g fermented vegetables daily at least 5x/wk for eight weeks, or a Usual Diet (UD) group. Blood and stool samples were obtained before and after the intervention. Dependent samples *t*-tests and adjusted linear models were used for within and between group comparisons. Mean age and BMI of participants were 45 years and 30 kg/m^2, and 80% were female. Bloating or gas was the most common side effect reported (19.3% FV group *vs* 9.4% UD group). There were no changes in C-Reactive Protein, oxidized LDL-receptor 1, angiopoietin-like protein 4, trimethylamine oxide, and lipopolysaccharide binding protein or bacterial alpha diversity between groups. Our findings indicate that consuming 100g of fermented vegetables at least five days per week for eight weeks does not change inflammatory biomarkers or microbial alpha diversity as measured by Shannon index. It is possible that higher doses of fermented vegetables are necessary to elicit a significant response by gut bacteria.

SOCIAL MEDIA SUMMARY

New study finds that daily consumption of half a cup of fermented vegetables for two months is not sufficient to alter inflammation markers or the diversity of the gut bacteria in adults at risk for cardiovascular disease.

IMAGE FOR THUMBNAIL

Figure 1

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INTRODUCTION

There is a large body of evidence documenting the role of the gut microbiota in health and disease. It has been reported that individuals diagnosed with coronary artery disease (CAD) have decreased diversity of the gut bacteria and increased richness of certain types of bacteria (Toya *et al*., 2020). It has also been suggested that the gut microbiota may be fostering inflammation by increasing production of inflammatory molecules, therefore contributing to CVD (Tang *et al*., 2019). These findings suggest that the gut bacteria may play an important role in the development and progression of CVD. One recent case-control study found significantly decreased alpha diversity (p=0.002), richness (p=.001) and evenness (p=0.014) of gut bacteria in patients with advanced CAD (Toya *et al*., 2020). A cross-sectional study of 322 participants with large artery atherosclerotic stroke or transient ischemic attack (TIA), and 231 healthy controls found the control group had fewer opportunistic pathogens including *Enterobacter*, *Megasphaera*, *Oscillibacter*, and *Desulfovibrio*, and more abundant beneficial bacteria: *Bacteroides*, *Prevotella*, and *Faecalibacterium* (Yin *et al*., 2015). Researchers have also identified microbial strains associated with atherosclerotic cardiovascular disease (ACVD) (Jie *et al*., 2017). When comparing 218 patients with ACVD and 187 healthy controls, a higher abundance of *Roseburia instestinalis* (a butyrate producing bacteria) and *Faecalibacterium cf. prausnitzii* was found in the control group, and a greater abundance of *Enterobacteriaceae* and *Streptococcus spp* was noted in the participants with ACVD (Jie *et al*., 2017).

The gut microbiota plays a pivotal role in regulating inflammation through a multitude of mechanisms. These microorganisms are involved in maintaining the intestinal barrier integrity, primarily by promoting the production of mucin and tight junction proteins. This barrier prevents the translocation of pathogenic microbes and their products into systemic circulation, thereby

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averting immune responses. Additionally, the gut microbiota regulates the differentiation and function of immune cells, such as T cells, B cells, and macrophages, through direct interactions and metabolite production. Short-chain fatty acids (SCFAs), produced by certain gut bacteria during the fermentation of dietary fiber, exert anti-inflammatory effects by modulating immune cell responses and inhibiting pro-inflammatory cytokine production. Gut microbes can also metabolize dietary components into bioactive molecules that influence host immune and inflammatory responses. Dysbiosis or alterations in the composition of the gut microbiota can disrupt these regulatory mechanisms, contributing to chronic inflammation and various inflammatory diseases (Belkaid & Hand, 2014; Cani & Jordan, 2018). For example, a reduced or disrupted intestinal mucous layer thickness can increase leakage of lipopolysaccharide (LPS), a component of the cell walls of Gram-negative bacteria, into the circulatory system. LPS activates the innate immune system, eliciting a low-grade systemic inflammatory response (Yücel *et al*., 2017), which is a key factor in triggering the onset of cardiovascular diseases (Cani & Jordan, 2018). Trimethylamine oxide (TMAO) is another metabolite derived from microbial metabolism of animal-derived foods which has been associated with increased risk of cardiovascular disease (Krueger *et al*., 2021) Furthermore, gut microbiota metabolites have also been implicated in changes in the activity of angiopoietin-like protein 4 (ANGLPT4), which disrupt lipoprotein lipase activity and lead to lipid dysregulation, thus increasing the risk of cardiovascular disease (Zwartjes *et al*., 2021). Lastly, certain microbial taxa have been associated with low-grade inflammation assessed via high sensitivity C-reactive protein (hsCRP) and LPS levels (van den Munckhof *et al*., 2018). *Bifidobacterium* abundance has been associated with lower hsCRP and LPS levels and *Faecalibacterium prausnitzii* abundance has been associated with lower hsCRP

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levels. Conversely, lower abundance of *Ruminococcaceae* and *Ruminococcus* have been associated with higher levels of hsCRP (van den Munckhof *et al*., 2018).

Among the known risk factors for cardiovascular disease (CVD), diet is arguably the most significant, since it not only contributes to other risk factors, such as high blood pressure, obesity, inflammation, high blood lipids, and diabetes, but also decreases the risk for CVD through delivery of protective nutrients. Fermented vegetables contain live probiotic bacteria that are associated with health benefits, but only a few human studies have evaluated the effects of consumption of fermented vegetables on changes to the gut microbiota and possible impact on human health. There is very limited prior research investigating fermented vegetable consumption in the context of cardiovascular disease and the gut microbiota. The purpose of this study was to investigate the impact of regular consumption of fermented vegetables on markers of inflammation and the composition of the gut microbiota in adults at increased risk for cardiovascular disease. We hypothesized that regular consumption of fermented vegetables would improve both biomarkers of inflammation and the composition of the gut microbiota in adults at risk for cardiovascular disease.

METHODS

Study Design and Participants

This study was a randomized controlled clinical trial with two parallel groups, a fermented vegetable (FV) group and a usual diet (UD) group (clinicaltrials.gov ID: NCT04887662). Participants in the UD group followed their usual diet while participants in the FV group consumed 100 grams of fermented vegetables five days a week and otherwise followed their usual diet. Figure 1 shows the study design and Table 1 shows participant inclusion and

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exclusion criteria. The target population was men and women ages 35-64 who had at least one risk factor for cardiovascular disease. This study aimed to recruit between 90-100 participants with a goal of having 80 participants complete the study (a 20% dropout rate was assumed). The sample size was calculated using C-reactive protein (CRP) data from a recently completed pilot study.(A. E. Galena *et al*., 2022) The following parameters were used for the sample size calculation: mean CRP levels = 176 ng/ml, standard deviation = 100 ng/ml, power = 85%, and alpha = 0.05. Based on these parameters, the target sample size was 37 participants per group.

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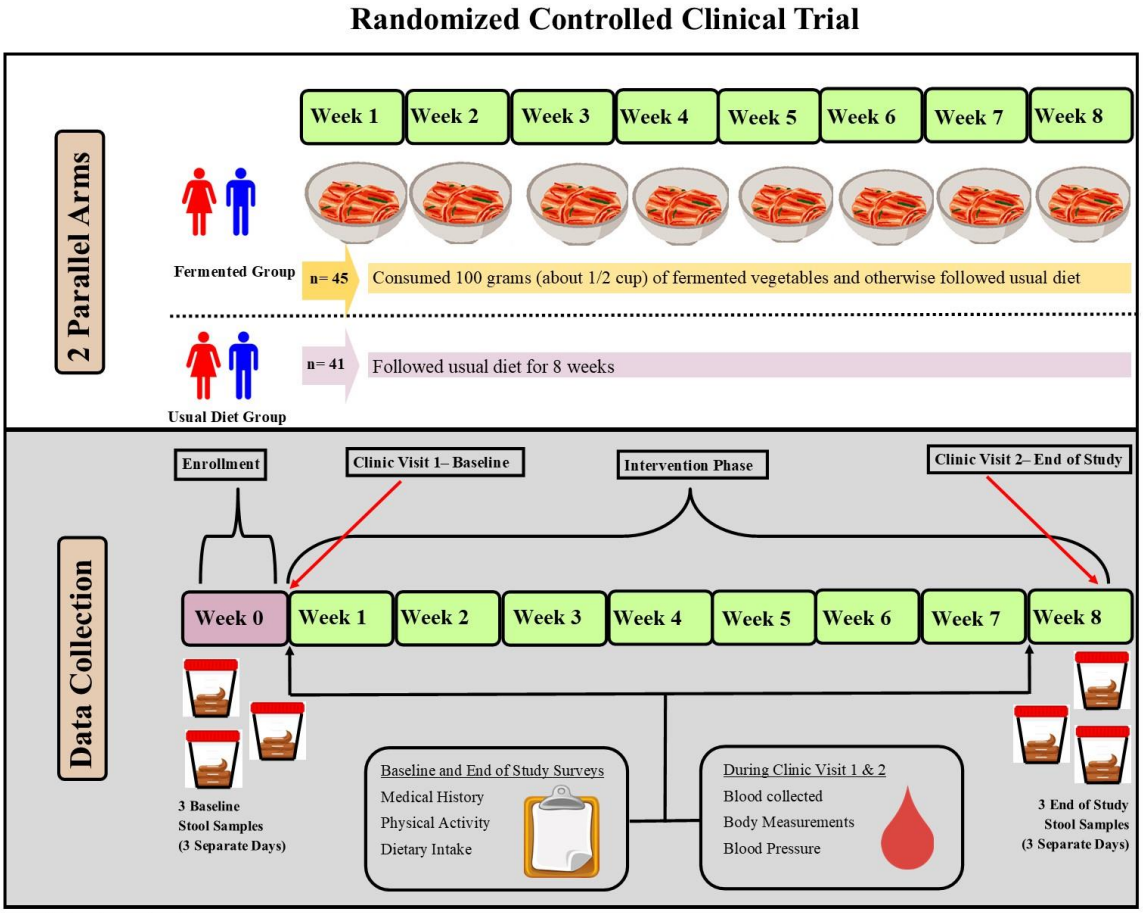

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138 Figure 1. Diagram of the Study Design. This randomized controlled trial consisted of two
139 parallel groups, the fermented vegetable group consumed 100 g of fermented vegetables once
140 daily for 8 weeks and the usual diet group consumed their usual diet for 8 weeks. Stool and
141 blood samples were collected prior to randomization and at the end of the 8-week period.
142 Participants also filled out questionnaires twice during the study.

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Table 1. Inclusion and Exclusion Criteria for Study Participants
Inclusion Criteria
• Age 35-64 • Participants must meet at least one of the following criteria: Overweight or obese (determined by BMI > 25), Family history of heart disease, controlled hypertension • Willing to consume one half cup of fermented vegetables 5 days a week for 8 weeks • No disabilities that may limit capacity to provide informed consent • Willing to visit the clinic twice during an 8-week period for body measurements and blood draws • A signed informed consent • Not on statin (Lipitor, Lesol, Crestor, Zocor, Altoprev) • Not on medication for diabetes (insulin, metformin, glipizide, glimepiride, glyburide) • Not on the following Monoamine oxidase inhibitors (Emsam, Marplan, Nardil, Parnate) • Not on Antirheumatic medications • Not on Biologics (Humira, Enbrel, Orencia, Kineret)
Exclusion Criteria
• Regular consumption of probiotics • Regular consumption of fermented vegetables (2 times per week or more) • Smoker • Previous or current diagnosis of cancer • Diagnosis of Diabetes Mellitus • Diagnosis of Crohn's disease • Diagnosis of colitis • Diagnosis of Rheumatoid arthritis • Diagnosis of Psoriasis • Taking antibiotics during the past three months

This project was approved by the University of North Florida (UNF) Institutional Review Board (IRB) and all participants provided informed consent prior to starting the study (UNF IRB Number: 1712254-1).

Treatment Groups

Participants in the FV group were instructed to consume 100 grams of fermented vegetables at least 5 out of 7 days each week for eight weeks. There were no restrictions on timing and the composition of meals consumed along with the fermented vegetables. However, participants

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were instructed by the study registered dietitian to not cook or heat the fermented vegetables and to reduce their sodium intake to account for the extra 600 mg of sodium consumed daily via the fermented vegetables. The UD group was asked to consume their current diet and not make any changes.

Throughout the study, fermented vegetables were delivered weekly by a local producer specialized in fermented krauts and kimchi. Eight varieties of kraut and kimchi were stocked in the study kitchen, and participants in the FV group selected their preferred varieties. Approximately 2.7 kg of fermented vegetables (about 27 100g servings) were provided to the participants randomized into the FV group at their first clinic visit. At the midpoint of the study these participants were contacted to plan for delivery or pickup of additional 2.7 kg of fermented vegetables.

Production of Fermented Vegetables

The fermented vegetables used in the study were made by a local producer through natural fermentation. The process starts by combining chopped cabbage with brine (2% sea salt) in a large wooden barrel as well as spices and condiments. Under anaerobic conditions, and at a temperature of approximately 23ºC, the bacteria that are naturally present in the cabbage start to grow while producing lactic acid, which lowers the pH of the environment and prevents growth of other bacteria that may be harmful to health. It takes approximately 4 to 5 days for the finished product to be ready for consumption, which is determined based on measurement of the salinity and pH of the product as well as on the taste, texture, and color. Once the fermented vegetables reach the desired sensory characteristics, it is stored at 4ºC for up to 6 months. All fermented vegetables provided were produced within one month of delivery to study participants. Our laboratory has previously analyzed the microbial composition of samples of

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fermented cabbage and the analysis indicates predominance of two major genera, namely *Lactobacillus* and *Leuconostoc*, which is consistent with previous reports (Park *et al*., 2021; Zabat *et al*., 2018). It is well established that once the pH of the fermented cabbage reaches 3.5, the presence of bacteria is restricted to the genera mentioned above (Zabat *et al*., 2018).

Study Procedures

Participants were recruited through many strategies: printed flyers in high visibility areas, a study website, social media, recruitment emails, and word of mouth. Interested participants were directed to the study website to fill out a screening questionnaire. Participants who met eligibility requirements were emailed a link to an orientation video and a consent form for their review. Upon consent to the study, participants scheduled an appointment time for the initial clinic visit. Participants attended a clinic visit twice during the study: at 0 weeks (initial clinic visit) and at 8 weeks (final clinic visit) where blood was drawn, and blood pressure and body composition were assessed. Additionally, participants completed online surveys to assess medical history, physical activity level, demographics, and prescription medication use. All participants were monitored through online weekly gastrointestinal symptom logs throughout the study period.

Data Collection

Surveys

The DHQ-3,(*Diet History Questionnaire III (DHQ III) | EGRP/DCCPS/NCI/NIH*, n.d.) 199, a 135-item food frequency questionnaire designed by the National Cancer Institute, was used to assess the participants' diet intake prior to the clinic visits. A 24-Hour Recall questionnaire was used to assess dietary intake at three timepoints: baseline (0 weeks), mid-study (4 weeks) and at end of study (8 weeks). Participants were also asked to complete eight weekly symptom logs to record their weekly vegetable intake and any symptoms they may have had related to

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gastrointestinal function including frequency of defecation, bloating or gas, abdominal pain, diarrhea/very loose stools, swelling in hands, legs or feet, and any other symptoms they chose to report.

Stool Collection

Participants received stool collection kits prior to their clinic visits and were instructed to collect three stool samples on three separate days on two timepoints (week 0 and week 8). Each stool collection kit contained three flushable stool collection sheets, three DNA/RNA Shield Fecal Collection tubes (Zymo Research, Irvine, CA) one biohazard bag, and detailed instructions for stool collection. Participants were asked to record the date and time of stool collection on each tube and store the tubes at room temperature until their clinic visit appointments. The time between collection of stool samples and the clinic visits ranged between one and six days.

Clinical Data

All biological samples were processed and stored at -70C until analysis. Blood was collected in two 8-mL tubes and left at room temperature for 30 minutes before centrifugation at 25C for 10 minutes at 1400 rpm. Serum was transferred to 1.5 mL cryogenic tubes in 1-mL aliquots. Stool samples were kept in their original collection tubes and stored at -70C until analysis. Study staff obtained participants' height, weight, and body composition at each clinic visit. A Detecto 439 Eye Level Beam Physician Scale 400ib x 4oz with Height Rod was used to measure height in centimeters. Weight and percent body fat were measured by multifrequency bioelectrical impedance (InBody 570, Cerritos, CA.) Blood pressure was measured by a digital blood pressure monitor (OMRON Model: HEM-907XL).

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Measurement of Biomarkers

Biomarkers related to cardiovascular disease and inflammation were selected based on other studies, particularly if there was a connection to the gut microbiome. Serum C-reactive protein (CRP) (RandD Systems, Minneapolis, MN - Cat#DCRP00), Angiopoetin-like protein 4 (ANGPTL4) (RandD Systems, Minneapolis, MN - Cat#DY3485), Oxidized low density lipoprotein receptor 1 (LOX-1) (RandD Systems, Minneapolis, MN – Cat#DY1798) and trimethylamine oxide (TMAO) (AFG Bioscience, Northbrook, IL - Cat#EK715704) were measured with commercial ELISA kits. Serum Lipopolysaccharide Binding Protein (LBP) was measured by a Pierce LAL chromogenic endotoxin quantitation kit (Cat#88282, ThermoFisher Scientific, Waltham, MA). All analyses were conducted in the laboratory of Dr. Arikawa at the University of North Florida.

16S rRNA Sequencing

DNA was extracted from the frozen stool samples with the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Germantown, MD, USA) per manufacturer's protocol. A NanoDrop One (Thermo Fisher Scientific, Madison, WI, USA) was used to measure DNA concentration and diluted to 10 ng/μL. Next-generation sequencing of the V4 region of the 16S rRNA gene was performed. Amplicon PCR was performed on the V4 region of 16S rRNA using the forward (5′-GTGCCAGCMGCCGCGGTAA-3′) and reverse (5′-GACTACHVGGGTWTCTAAT-3′) primers. PCR amplicons were barcoded and pooled in equal concentrations using the SequalPrep Normalization Plate Kit (Invitrogen, Carlsbad, CA, USA). qPCR was used to quantify and consolidate libraries using the Kappa Library Quantification Kit (Roche, Indianapolis, IN, USA), and the quality of the library will be determined by an Agilent 2100 Bioanalyzer (Agilent, Santa

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Clara, CA, USA). Positive and negative controls were sequenced for quality control. The ZymoBIOMICS™ Microbial Community Standard (Zymo Research, Irvine, CA, USA) were used to provide a commercial community DNA for a positive control, and DNA extraction and PCR amplification provided the negative controls. Sequencing was performed in a pair-end modality on the Illumina MiSeq 500 platform rendering 2 x 150 bp paired-end sequences (Illumina, San Diego, CA, USA)). Sequencing reads after quality control were denoised using Deblur integrated with QIIME2 (2022.02 released), alignment against a 16S reference database (SILVA v132), and clustering into amplicon sequence variants (ASVs) with 100% identity threshold. A total of 515 fecal samples were extracted for DNA and processed into QIIME2 pipeline. After filtering and denoising, 484 samples were retained for microbiome analysis.

Statistical Data Analysis

Descriptive statistics were obtained for each group (FV and UD) by calculating frequencies and percentages for categorical variables and means and standard deviation for continuous variables.

Comparisons were made between groups and within groups for all main and secondary outcomes. The independent variable of interest was consumption of fermented vegetables. Analysis of Covariance (ANCOVA) was used to compare the two groups with respect to blood biomarkers after controlling for sex, age at baseline, and BMI. All *p*-values lower than 0.05 were considered statistically significant. Pearson's correlation coefficients were calculated between all outcomes and alterations in the intestinal microbiota. IBM SPSS (Statistical Package for the Social Sciences) version 27 was used to perform statistical analysis.

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Alpha diversity (microbial diversity within each sample) and beta diversity (microbial diversity between samples) were calculated with q2-diversity plugin in QIIME2 on the ASV level. For alpha diversity, we used observed ASVs to measure microbial richness (number of species present), and the Shannon index to measure species richness and evenness (distribution). As for beta diversity, principal component analysis (PCoA) was used to discover the percent of variability and potential associations among the groups represented by the Bray-Curtis (measure of differences in taxa abundance between communities) and Jaccard index (taxa presence/absence). An analysis of similarity (ANOSIM) was used to assess significant clustering differences by comparing within-and between group similarity. Lastly, linear discriminant analysis effect size (LEfSe) was used to identify specific bacterial features that were enriched between conditions and diet patterns in each group or subgroup at the ASV level. The LEfSe analysis was performed using a web-based tool with 0.05 as the alpha value for the pairwise Wilcoxon test, and 2.00 as the threshold on the LDA score. All microbial analyses were conducted in the laboratory of Dr. Zhao in the Department of Animal Science at the University of Arkansas.

Results

Recruitment, screening and enrollment occurred between May and September 2021 and the study was completed by the end of November 2021. Figure 2 shows the participant flow through the study. Out of 426 potential participants who were screened prior to eligibility assessment, 87 participants were randomized into one of the two treatment groups and 86 completed the study. All study aims were assessed in at least 40 participants from each group. The most common reasons for being excluded from the study were not meeting either the age criteria or not presenting with a cardiovascular risk factor criterion.

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The demographic characteristics of the study population stratified by study group are shown in Table 2. The two groups were similar in baseline characteristics. Approximately 80% and 78% of participants in the UD group (*N*=33) and FV group (*N*=35) were female, respectively. The average age was 45 years for both study groups. Body Mass Index was 30.0 for both study groups. Most participants identified as white (81% and 85% in the UD and FV groups, respectively). There were no differences between groups in baseline nutrient intake (Table 2). The FV group showed a significant decrease in total calories and sodium intake at the end of the study compared with baseline, indicating that participants followed the instructions from the dietitian to reduce sodium consumption during the study.

Table 2. Dietary Variables Before and After the Study by Treatment Group. (*N*=42, usual diet and *N*=44, fermented vegetable).

	Mean (Standard Error)		
Variables	Before	After	*P*-value[a]
Calories (kcal)			
Usual Diet	1982 (104)	2025 (116)	0.992
Fermented Vegetable	2092 (106)	1807 (93.7)	**0.025**
P-value[b]	0.462	0.146	
Carbohydrates (g)			
Usual Diet	213.0 (15.0)	220.4 (14.9)	0.845
Fermented Vegetable	206.5 (12.3)	182.7 (11.0)	0.589
P-value[b]	0.191	0.148	
Protein (g)			
Usual Diet	80.1 (5.4)	81.5 (5.2)	0.478
Fermented Vegetable	96.2 (7.2)	80.0 (5.9)	0.177
P-value[b]	0.096	0.186	
Fat (g)			

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Usual Diet	87.4 (5.8)	90.2 (6.1)	0.885
Fermented Vegetable	92.9 (5.9)	81.3 (5.1)	0.184
P-value[b]	0.986	0.689	
Fiber (g)			
Usual Diet	19.1 (1.8)	21.6 (1.8)	0.376
Fermented Vegetable	20.2 (1.6)	18.3 (1.4)	0.271
P-value[b]	0.886	0.453	
Cholesterol (mg)			
Usual Diet	353.0 (41.6)	280.9 (28.7)	0.428
Fermented Vegetable	385.5 (57.0)	278.9 (32.8)	0.878
P-value[b]	0.939	0.497	
Sodium (g)			
Usual Diet	3.2 (0.2)	3.3 (0.2)	0.228
Fermented Vegetable	3.5 (0.2)	3.0 (0.2)	**0.048**
P-value[b]	0.449	0.977	
Alcohol (g)			
Usual Diet	7.8 (2.2)	5.4 (1.9)	0.601
Fermented Vegetable	9.8 (2.8)	7.4 (2.3)	0.165
P-value[b]	0.629	0.438	
Choline (g)			
Usual Diet	374.6 (32.1)	325.2 (22.8)	0.364
Fermented Vegetable	401.9 (40.8)	307.1 (27.0)	0.846
P-value[b]	0.938	0.732	

[a]*P*-value for within group comparisons using a dependent samples *t*-test.
[b]*P*-value for between group comparisons using linear models adjusted for total calorie intake.

There were no significant differences in either systolic or diastolic blood pressure among the two study groups at baseline and after the intervention (Table 3). Additionally, there were no significant differences in any of the measured biomarkers between or within study groups (Table 2).

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Table 3: Demographic and baseline characteristics of study participants.[a]

Characteristics	**Usual Diet (n=42)**	**Fermented Vegetable (n=44)**
Age at Baseline (y)	45.4 (7.6)	44.8 (7.0)
Sex		
Male	8 (19.5)	10 (22.2)
Female	33 (80.5)	35 (77.8)
Race		
White	33 (80.5)	39 (86.7)
Non-white	7 (17.1)	6 (13.3)
Do not wish to provide	1 (2.4)	-
Ethnicity		
Hispanic	4 (9.8)	2 (4.4)
Non-Hispanic	37 (90.2)	42 (93.3)
Percent Body Fat	36.7 (9.7)	35.9 (9.1)
BMI (Baseline)	30.0 (6.0)	30.0 (6.4)
Systolic BP (Baseline)	120.7 (2.3)	122.1 (2.2)
Diastolic BP (Baseline)	80.0 (1.5)	80.2 (1.4)

[a]Values are expressed as mean (standard deviation) for continuous variables and Frequency (%) for categorical variables.

The most common side effect reported was bloating or gas (9.4% in the UD group and 19.3% in the FV group) (Table 4). Average intake of fermented vegetables was 630 g per week. Average bowel movement frequency was 0.8 times per day in the UD group, and 1.9 times per day in the FV group.

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320 Table 4: Frequency (%) of reported side effects by treatment group.
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Side Effect	**Usual Diet (n=42)**	**Fermented Vegetable (n=44)**
Bloating or gas	9.4%	19.3%
Abdominal pain	2.7%	6.4%
Nausea	1.6%	4.1%
Diarrhea or loose stool	0.2%	1%
Swelling of hands, feet, arms or legs	0.8%	3.2%

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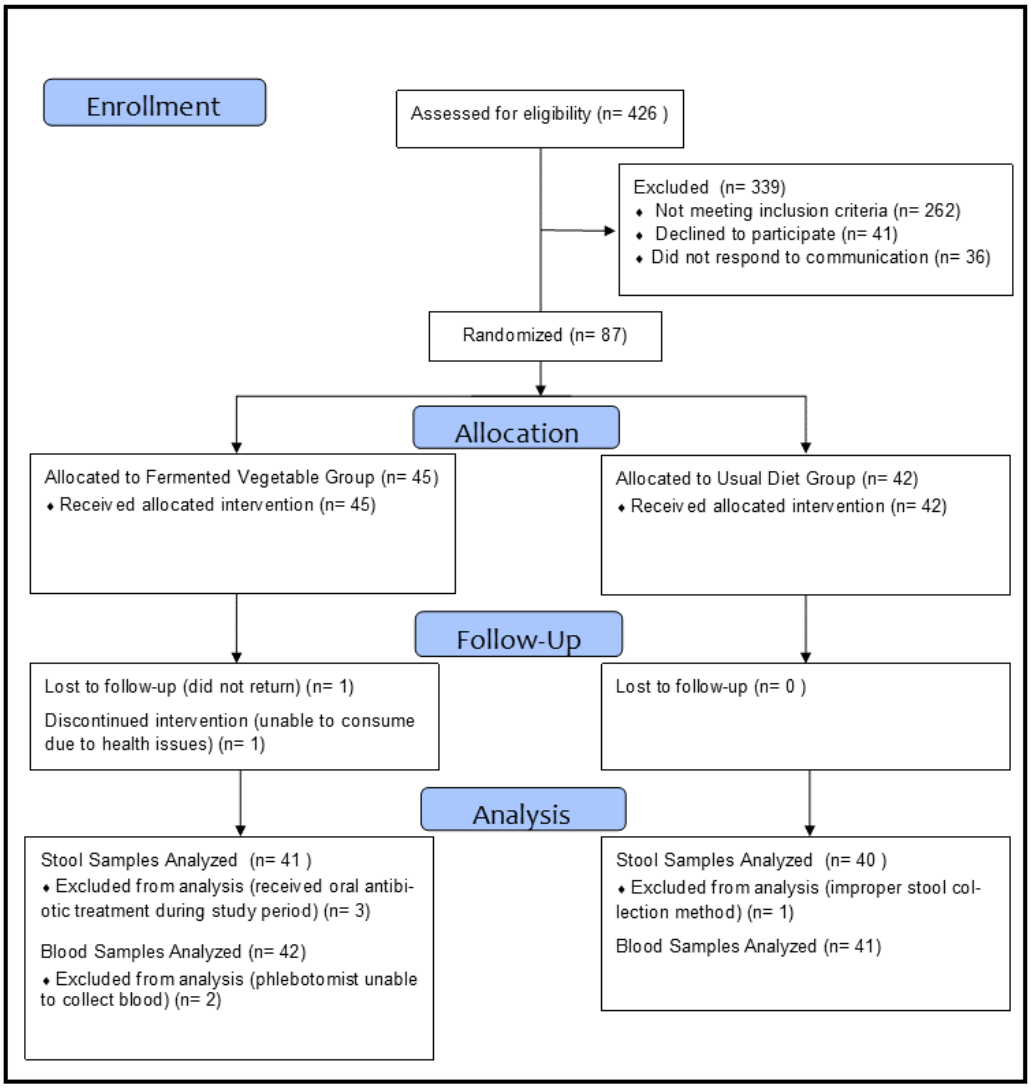

Figure 2. Flow of Participants

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326 Table 5: Changes in Inflammatory Biomarkers by Treatment Group[a].

Variables	Treatment Groups: Usual Diet	Treatment Groups: Fermented Vegetable	*P*-value[b]
TMAO (ng/mL)			
Week 0	479.8 (47.9)	360.3 (45.6)	0.425
Week 8	455.5 (43.9)	351.0 (41.8)	0.507
P-value[c]	0.059	0.661	
TMAO Change	-24.3 (11.3)	-9.3 (10.8)	0.339
LBP (ng/mL)			
Week 0	22.9 (1.2)	23.9 (1.2)	0.793
Week 8	24.5 (1.3)	22.5 (1.3)	0.651
P-value[c]	0.751	0.646	
LBP Change	0.6 (1.8)	-0.4 (1.7)	0.696
CRP (ng/mL)			
Week 0	7557.9 (2561.6)	5443.8 (2501.2)	0.882
Week 8	8053.9 (2646.1)	6086.6 (2583.8)	0.863
P-value[c]	0.848	0.456	
CRP Change	569.2 (894.6)	1008.5 (831.2)	0.72
ANGLPT4 (ng/mL)			
Week 0	136.2 (25.0)	120.2 (25.3)	0.868
Week 8	132.1 (23.3)	120.7 (23.6)	0.859
P-value[c]	0.426	0.991	
ANGLPT4 Change	0.05 (4.7)	-3.7 (4.6)	0.568
LOX-1 (pg/mL)			
Week 0	42.3 (13.4)	87.7 (35.2)	0.317
Week 8	40.6 (93.7)	80.9 (217.1)	0.36
P-value[c]	0.302	0.07	
OLR1 Change	-5.9 (27.5)	-8.3 (26.6)	0.949

327 Abbreviations: TMAO: trimethylamine oxide; LBP: Lipopolysaccharide binding protein; CRP:
328 C-reactive protein; ANGPTL4: Angiopoietin-like 4; LOX-1: oxidized LDL receptor 1.
329 [a]Values are expressed as mean (standard error)
330 UD group, *N*=40; FV group, *N*=44.
331 [b]*P*-values for between group comparisons, using Analysis of Covariance, adjusting for sex, age
332 and BMI.
333 [c]*P*-values for within group comparisons via dependent samples *t*-tests.
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335
336 *Changes in Inflammatory Biomarkers*

337 There were no significant changes in blood levels of TMAO, LBP, CRP, ANGPTL4 and LOX-1
338 in the FV group compared with the UD group following consumption of 100 grams of fermented
339 vegetables at least five days per week for eight weeks.

340

341 *Alpha and Beta Diversity*

342 Figure 3 portrays box plots of both Shannon Index and observed ASVs by treatment group and
343 study time point. No significant differences in alpha diversity were observed between or within
344 groups.

345 PCoA plots of Bray-Curtis and Jaccard indices are shown in Figure 4. Significant
346 dissimilarities in beta diversity were seen between the FV and UD groups at both timepoints
347 (P=0.004), however dissimilarities were not seen within each group over time. We also plotted
348 weighted and unweighted Unifrac distances and even though there was an increase in the
349 proportion of the PC axis explained by the treatments, there were no significant differences
350 between the two time points according to Analysis of Similarities (Supplemental Fig. 1).

351

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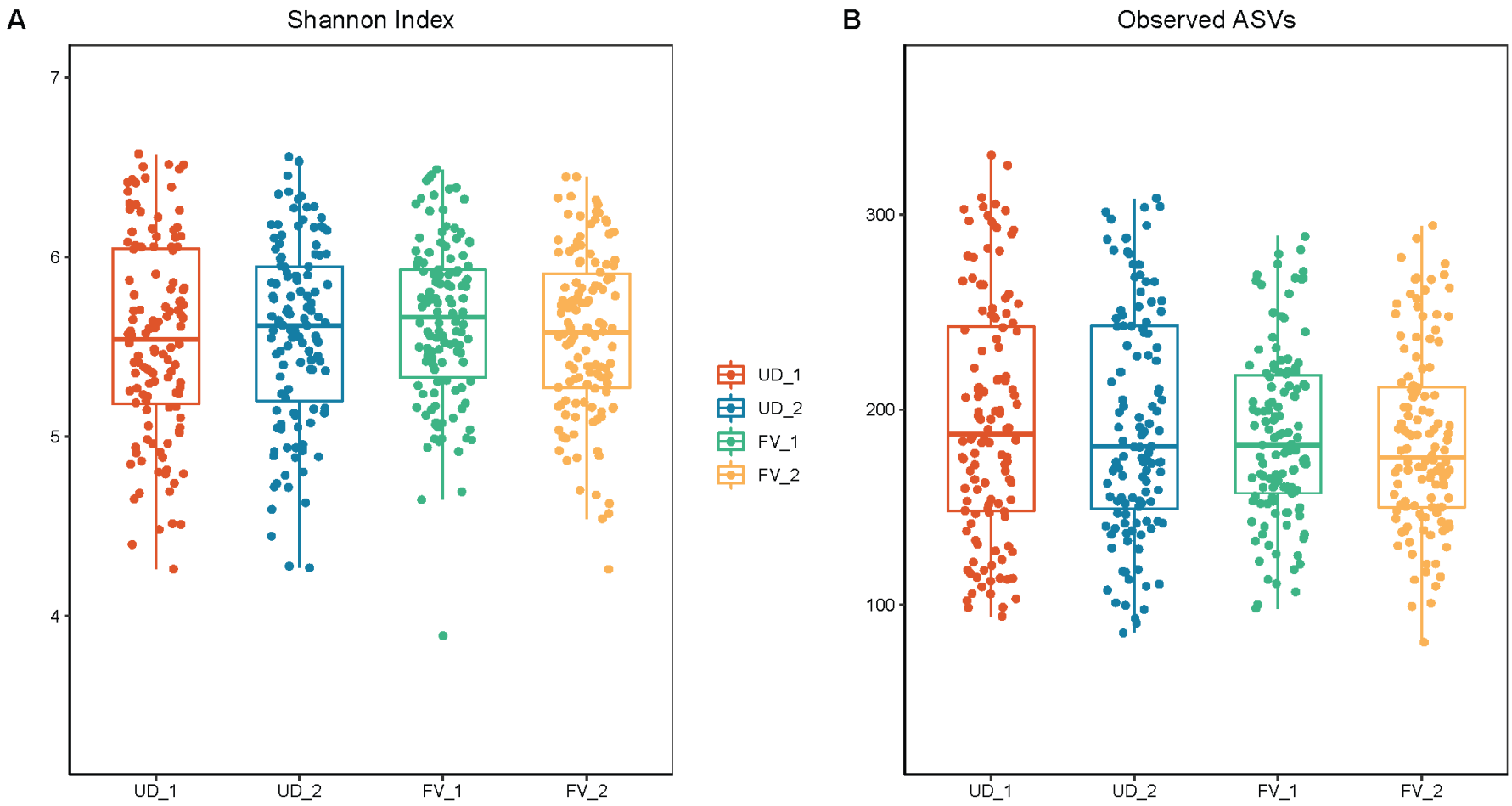

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353 **Fig 3.** Microbial diversity expressed as Shannon index and observed amplicon sequence variants (ASVs) by treatment group and time point.

354 Shannon index was calculated on the ASV level. UD_1 = usual diet group at time point 1, UD_2 = usual diet group at time point 2, FV_1=

355 fermented vegetable group at time point 1, FV_2= fermented vegetable group at time point 2.

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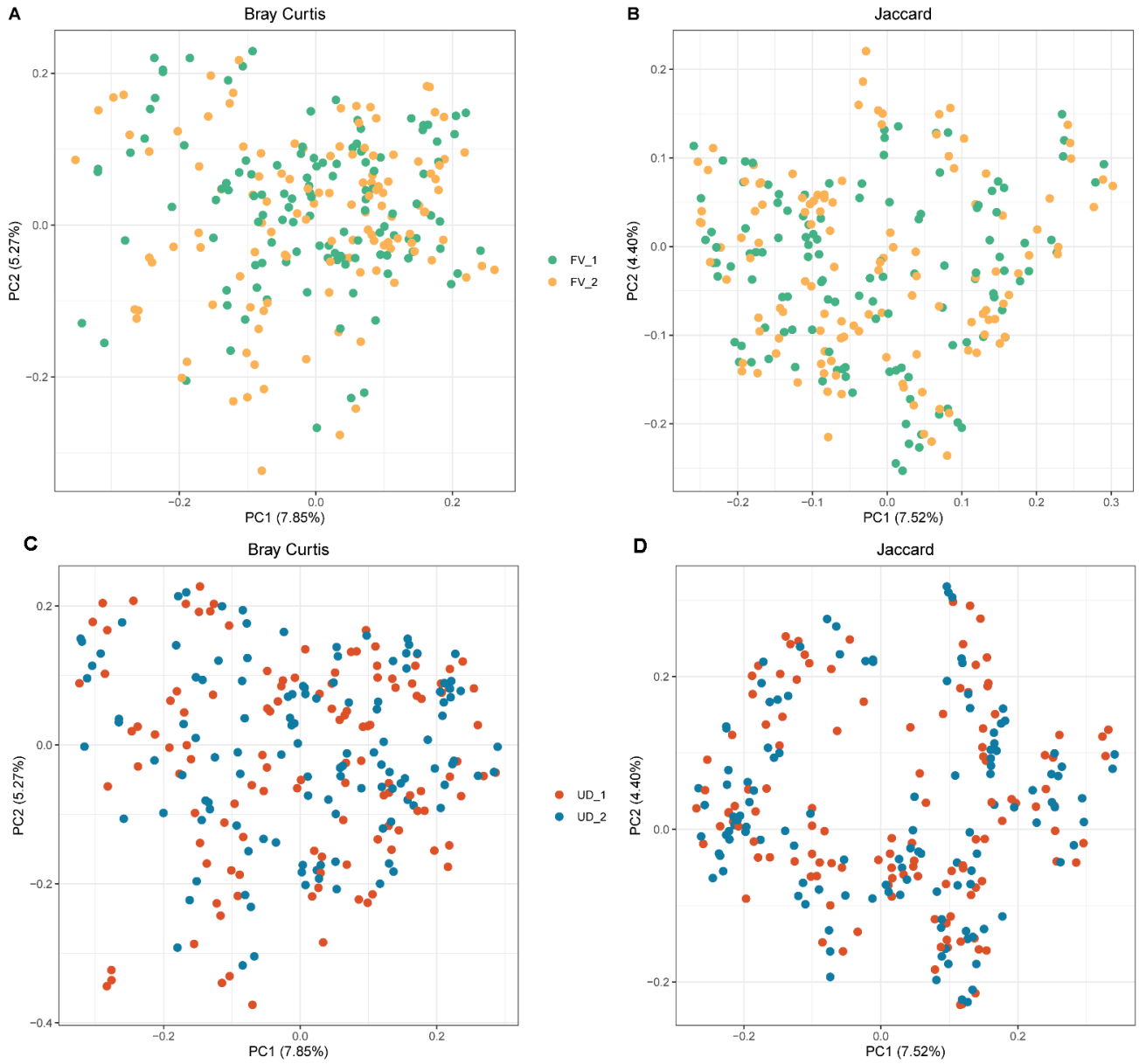

Fig 4. Microbial β-diversity plots expressed as Bray-Curtis and Jaccard distances by treatment group and study time point. The distances were calculated on the ASV level. UD_1 = usual diet group at time point 1, UD_2 = usual diet group at time point 2, FV_1= fermented vegetable group at time point 1, FV_2= fermented vegetable group at time point 2.

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Linear discriminant Analysis (LDA) Effect Size

Linear discriminant Analysis (LDA) Effect Size (LEfSe) was utilized to identify the bacterial taxa characterizing the differences between and within groups as shown in Figure 5. The FV group showed the most change in taxa between the two timepoints. Some notable changes were an increase in relative abundance of *Lactobacillaceeae Lactobacillus*, and *Lachnospiraceae Lachnoclostridium*. There was also a decrease in *Rumunococcaceae Faecalibacterium*, *Prevotella Prevotellacolorans*, *Actinomycetaceae Actinomyces*, *Peptostreptococcaceae Intestinobacter* and *Lachnospiraceae Roseburia* in the intervention group.

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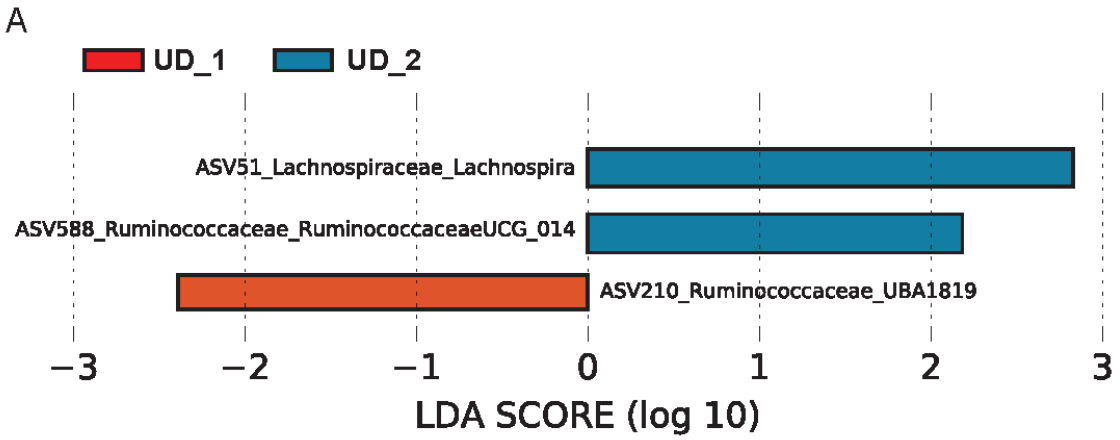

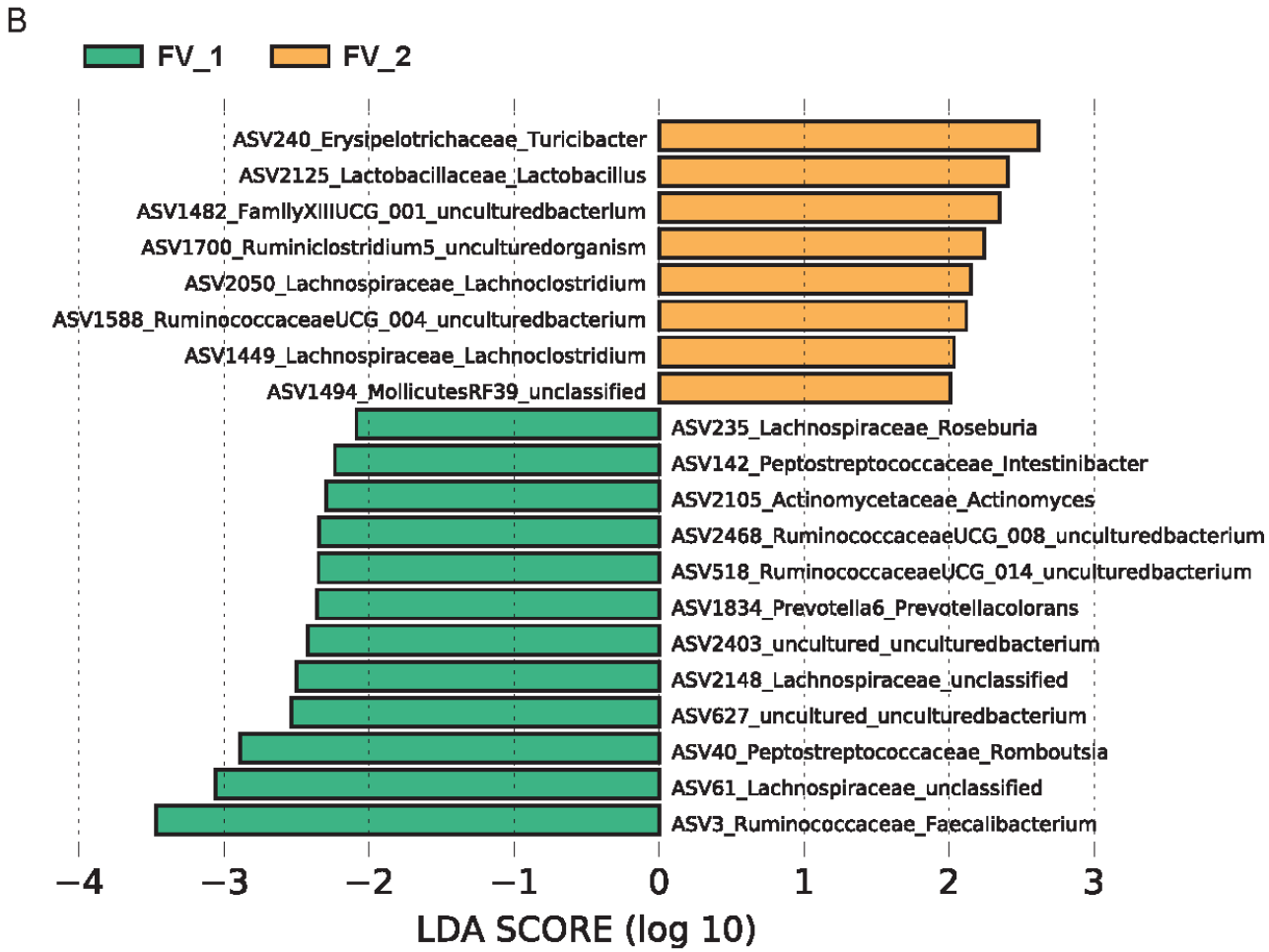

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370 **Fig 5.** Within Group Linear Discriminant Analysis Effect Size on ASV level. UD_1 = usual diet group
371 at time point 1, UD_2 = usual diet group at time point 2, FV_1= fermented vegetable group at time point
372 1, FV_2= fermented vegetable group at time point 2.

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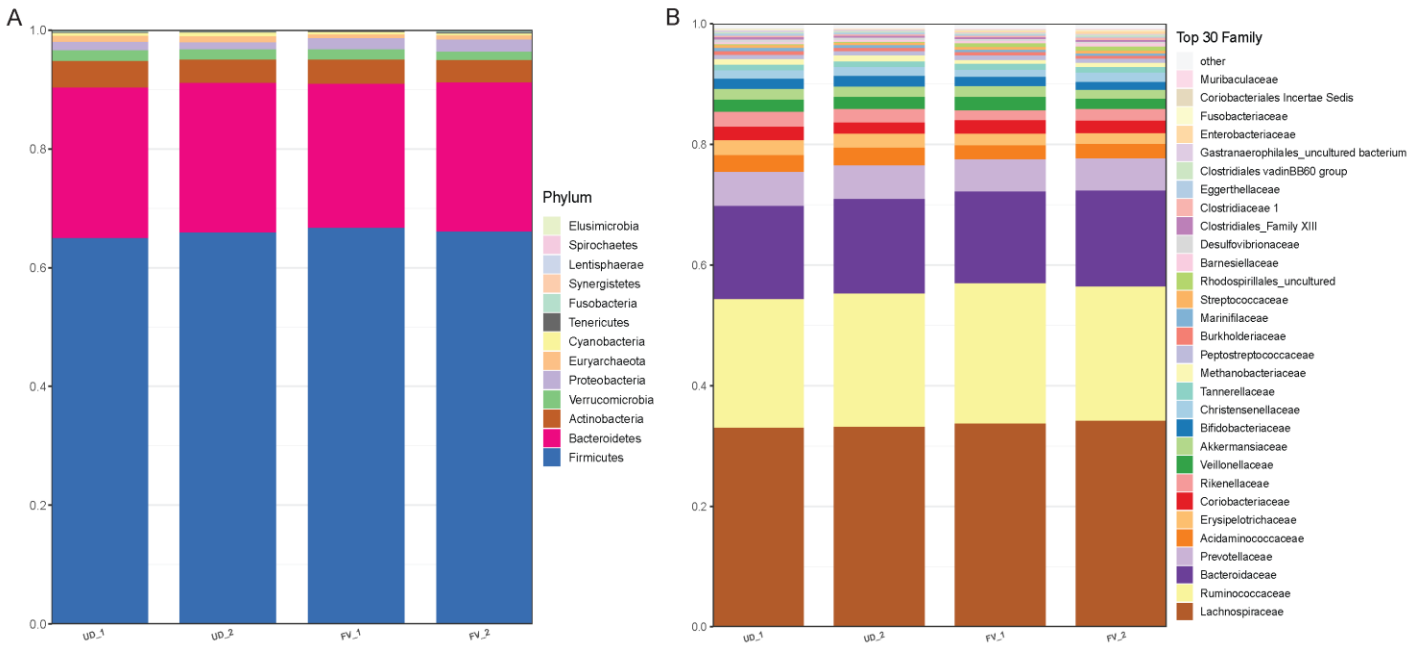
373 The top three predominant phyla in the stool samples of study participants were Firmicutes
374 Bacteroidetes, and Actinobacter. There were no significant differences within or between groups
375 in relative abundance of the top phyla or top 15 families shown in Figure 6.



376

377 **Fig 6.** Microbial composition at the phylum and family levels ranked by relative abundance per
378 treatment group and time point. UD_1 = usual diet group at time point 1, UD_2 = usual diet
379 group at time point 2, FV_1 = fermented vegetable group at time point 1, FV_2 = fermented
380 vegetable group at time point 2.

381

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382

383 **Discussion**

384 This randomized controlled trial explored the impact of regular consumption of
385 fermented vegetables on markers of inflammation and the composition of the gut microbiota in
386 adult men and woman at increased risk for cardiovascular disease.

387 *Effect on Inflammatory Markers*

388 There was no effect of fermented vegetables on serum biomarkers. Our results were
389 similar to a pilot study conducted by our laboratory in which 31 females consumed 100 grams of
390 fermented cucumbers or cabbage daily for six weeks (Galena *et al*., 2022). C-Reactive Protein
391 (CRP), Tumor Necrosis Factor (TNF) alpha, and Lipopolysaccharide Binding Protein (LBP)
392 were measured, and no significant changes were found between the fermented vegetable group
393 and the non-fermented vegetable group. In turn, Wastyk *et al* found decreased inflammatory
394 markers in response to a 10-week dietary intervention of fermented foods in healthy adults
395 (Wastyk *et al*., 2021). Circulating cytokines, chemokines, and inflammatory serum proteins were
396 measured in the serum and 19 of the 93 inflammatory markers decreased in the group consuming
397 an average of 6.3 servings of fermented foods per day. Another study investigated how total
398 antioxidant status (TAS) and serum lipids were influenced by kimchi consumption (Choi *et al*.,
399 2013). In this seven-day study, 100 participants living in South Korea were recruited and
400 assigned to either the low kimchi group (15 grams/day), or the high kimchi group (210
401 grams/day). TAS increased significantly in both groups ($p<.001$), and the greatest improvement
402 was in the higher dose kimchi group (7.5% increase in the high kimchi group and 5.1% increase
403 in the low kimchi group). Additionally, low density lipoprotein (LDL) and fasting blood glucose

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(FBG) were lower in the high kimchi group when compared with the low kimchi intake group. Fasting blood glucose (FBG) was also reduced in the high kimchi intake group (p=0.003) (Choi *et al*., 2013). Consumption of 210 g per day of kimchi for 12 weeks has also been shown to improve inflammatory markers and the composition of the microbiome in individuals suffering from irritable bowel syndrome, in a recent randomized controlled trial (Kim *et al*., 2022), indicating that fermented vegetable intake can be a viable treatment for patients affected by gut dysbiosis. The findings from these studies suggest that dose and duration of intake of fermented vegetables may be important to alter levels of inflammatory markers and the 100 g utilized in the present study may not have been sufficient to elicit changes in these markers.

To our knowledge, this was the first study to investigate the role of fermented vegetables on oxidized LDL receptor. Various dietary interventions have previously been shown to reduce oxidized LDL levels in different populations. Adherence to a 12-wk Mediterranean dietary pattern significantly decreased oxidized LDL by 11/3% in 71 healthy women (Lapointe *et al*., 2005). In addition, there is compelling evidence from in vitro studies to clinical trials reporting on the benefits of antioxidant supplementation to reduce LDL oxidation (Kiokias *et al*., 2018). While fermented vegetable intake did not change the levels of oxidized LDL receptor in the present study, it is possible that the dose provided was not sufficient to result in positive changes in lipid metabolism. Similarly, TMAO levels were not affected by consumption of fermented vegetables in this study. Indeed, the relationship between TMAO production, diet, and the gut microbiota is complex, and it has been hypothesized that TMAO may have different functions based on presence or absence of metabolic disease (Krueger *et al*., 2021). Previous studies have found that carnitine supplementation significantly increases TMAO levels in plasma (Samulak *et al*., 2019; Wu *et al*., 2019), while consumption of plant- based foods is associated with lower

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TMAO levels (Krueger *et al*., 2021). Wu *et al* (Wu *et al*., 2019) found that omnivores and vegetarians have distinct patterns of TMAO production and omnivores had 10 times higher odds of being high TMAO producers compared with vegetarians. In addition, it has been reported that high TMAO producers have higher Firmicutes to Bacteroidetes ratio compared with low producers (Wu *et al*., 2019). Li *et al* (Li *et al*., 2022) showed that the ability to produce TMAO following intake of animal-derived foods was dependent on a microbial profile which included species that predicted TMAO concentrations, such as *Eubacterium hallii*, *Eubacterium biforme*, *Roseburia hominis*, and *Alistipes shahii*. There was no evidence that the fermented vegetable consumption in the present study altered any of the TMAO-predicting species aforementioned, which may be one reason why there were no significant changes in plasma TMAO levels. We also examined red meat, choline and animal protein intake in the study participants (data not shown) and did not find any significant differences between or within groups. The current published evidence on the factors associated with TMAO production indicates that if consumption of fermented vegetables can modulate TMAO levels, it is likely to occur through more pronounced changes in intake of other dietary components, such as red meat and plant-based foods, and, most importantly, carefully considering the composition of the gut bacteria to focus on species that can predict TMAO production.

Effect on Gut Microbiota

Overall, we found slight changes in the gut microbiota profile in participants who consumed fermented vegetables, however there was no effect on alpha or beta diversity. Significant differences in beta diversity (Bray Curtis) were seen between the FV and UD groups at both timepoints (p=0.004), however there were no within group changes indicating that the dissimilarities were not related to the intervention. To further investigate whether specific

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bacterial taxa changed within each group during the 8-week intervention, Linear Discriminant Analysis (LDA) Effect Size (LEfSe) was utilized. Eight taxa increased and 12 decreased in the FV group, and two increased and one decreased in the UD group.

Lactobacillaceae Lactobacillus was enriched in the final stool samples of only the FV group. This may be related to the content of fermented vegetables, as *Lactobacillus* is commonly found in fermented foods like kimchi and sauerkraut. Interestingly, *Lactobacillaceae Lactobacillus* was not enriched in our pilot study where participants consumed the same amount of fermented vegetables.(A. E. Galena *et al*., 2022) The increase in *Lactobacillaceae Lactobacillus* in the present study may be related to the longer study period of 8 weeks, compared to six weeks in the pilot study. Many studies on probiotics have examined the effects of *Lactobacillus* with positive outcomes. (Azad *et al*., 2018; Heeney *et al*., 2018; Tomova *et al*., 2019) Individuals with lower abundance of *Lactobacillus* may suffer from constipation.(Wang and Yao, 2021) Interestingly, participants in the FV group in the present study reported more frequent bowel movements compared with the UD group. Enrichment of *Lactobacillus* is also positively associated with increased abundance of both beneficial short chain fatty acids (SCFAs) such as butyrate, and saturated long-chain fatty acid (SLCFAs) which are thought to enhance gastrointestinal motility.(Zhao *et al*., 2018) Conversely, our findings also indicated a lower abundance of the genus *Faecalibacterium* in the FV group at the end of the study, which are well known butyrate producers (Singh, et al., 2022). It is difficult to determine whether the overall production of short chain fatty acids was altered or not in the FV group without a metabolomic analysis of the stool samples. However, we speculate that production of SCFAs in the FV group may not have been significantly affected, given that the taxa enriched at both time points were capable of producing SCFAs.

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LEfSe analysis also revealed that there was an enrichment in bacterial taxa in the *Lachnospiraceae* family in the FV group at the end of eight weeks. The *Lachnospiraceae* family is part of the clostridial cluster XIVa of the phylum Firmicutes. The increase in taxa from the *Lachnospiraceae* family is supported by two other feeding studies. One study reported an increase in *Lachnospira* in participants consuming high fiber diets (Wastyk *et al*., 2021), and another study found an association between *Lachnospira* and high dietary fiber intake. (Lin *et al*., 2018) Bacterial taxa within the *Lachnospiraceae* family form a core part of the microbiome and are known short chain fatty acid producers. (Vacca *et al*., 2020) These bacteria colonize the gut during infancy and increase in abundance throughout adulthood. There are studies showing both positive and negative health effects related to *Lachnospiraceae* taxa (Vacca *et al*., 2020), but there are limited human studies linked to cardiovascular health. One of the few large cohort studies to explore the effect of fermented plants on the gut microbiome used participants enrolled in the American Gut Project (AGP).(Taylor *et al*., 2020) In this 4-week longitudinal study, 115 individuals in the AGP were enrolled to investigate if regular consumption of fermented plant foods had an impact on the diversity of the gut microbiome.(Taylor *et al*., 2020) No significant differences in alpha diversity were seen between the two groups, however researchers noted increased levels of conjugated linoleic acid (CLA) in the fermented plant food consumers. CLA has been purported to have many health benefits, including positive effects on atherosclerosis through improvement in the blood lipid profile.(Dilzer and Park, 2012; Koba and Yanagita, 2014) In a pilot study investigating the effect of consumption of 100g of fermented vegetables per day, there was an increase in *Faecalibacterium prausnitzii (P*=.022) and a trend towards a decrease in *Ruminococcus torques* (*P*=.074) in healthy female participants after six weeks of the intervention.(A. E. Galena *et al*., 2022) A study in South Korea investigated the effect of

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consumption of fermented kimchi on obesity.(Han *et al*., 2015) Study participants were assigned to either the fresh (n=12) or fermented (n=11) kimchi group for eight weeks, and participants consumed 300 grams of kimchi per day (100 grams per meal). Findings showed an increase in *Bacteroides* and *Prevotella* and a decrease in *Blautia* abundance in the fermented kimchi group. (Han *et al*., 2015)

Although our study did not show an effect on alpha diversity, a previous study with a higher dose and duration reported an increase in alpha diversity in healthy adults.(Wastyk *et al*., 2021) In this prospective study, fermented food intake steadily increased over the course of 10 weeks. Participants consumed on average 0.4 servings at baseline, and an average of 6.3 servings at week 10. A wide variety of fermented foods were consumed, which included fermented vegetables, yogurt, kombucha, and kefir. (Wastyk *et al*., 2021) Furthermore, a correlation was found between the number of servings of fermented food and an increase in diversity. (Wastyk *et al*., 2021). In the present study, there were significant differences in beta diversity between the two groups at both time points. It is possible that the high interindividual variability generally reported in the composition of the gut bacteria is responsible for these significant differences between the two groups. Dietary intake was assessed at both time points and there were no significant differences found in macronutrient intake and intake of other nutrients of interest in the context of cardiovascular disease, such as sodium, fiber, cholesterol, alcohol, and choline. Notwithstanding, it should also be noted that the method used to assess dietary intake was not sufficiently precise to account for variability in individual dietary intake.

Effects on Cardiovascular Risk Factors

Participants had at least one risk factor for cardiovascular disease (BMI>25, controlled hypertension, or a family history of heart disease). Over the course of the eight weeks, no

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519 significant change in weight, percent body fat, or blood pressure was observed. Previous studies
520 have shown an impact on risk factors for cardiovascular disease. Researchers in a kimchi feeding
521 study in South Korea studied 22 overweight and obese participants.(Kim *et al*., 2011)
522 Observations included a small reduction in BMI and body weight in participants who consumed
523 fermented kimchi. (Kim *et al*., 2011) In this crossover design study, participants were given both
524 fermented kimchi and fresh kimchi for two four-week periods with a two-week washout phase in
525 between. The amount of kimchi served was 300 grams per day (100 grams per meal) which was
526 three times more than the dose provided in our study. Participants had a small but significant
527 reduction in BMI, body weight, total cholesterol, fasting insulin and blood glucose levels
528 compared with baseline. (Kim *et al*., 2011). A different study investigating the effect of
529 consumption of fermented kimchi on obesity in South Korean women reported improvements in
530 waist-hip ratio, blood pressure, insulin and fasting blood glucose in the participants who
531 consumed 300 g of fermented vegetables per day. (Han *et al*., 2015) Participants were between
532 30 and 60 years, with a BMI >25. and participants consumed 300 grams of kimchi per day (100
533 grams per meal). Participants in both groups had decreases in body weight, body mass index, and
534 body fat, and the fermented kimchi group showed a significant decrease in the waist-hip ratio.
535 (Han *et al*., 2015) These findings indicate that higher doses of fermented vegetables (300 g per
536 day) in studies conducted in South Korea, where kimchi is commonly consumed, resulted in a
537 significant decrease in body weight. In our study, we used a lower dose of 100 g per day on at
538 least five days per week. While we considered increasing the dose to enhance the effect, most of
539 our participants were not accustomed to consuming fermented vegetables regularly, which posed
540 challenges to compliance to the treatment.
541 *Study Strengths and Limitations*

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One limitation of this study is that the results may not be generalizable to the whole population as the majority of study participants were female. In addition, over 80% of participants were white. Furthermore, we utilized volunteer sampling and even though study participants were recruited through many methods including flyers, social media, and mailing lists, it is possible that those interested in participating in the present study were more likely to follow a healthier lifestyle. When it comes to the study design and selection of the control group, it is possible that including 100 g of fresh cabbage as part of the control group treatment would have been a more appropriate comparison group. The fermented vegetables consumed during the study added up to 3 to 4 g of fiber each day, however, based on the nutrient intake comparisons between the two groups, we did not find any significant differences in fiber intake, which indicates that the addition of 100 of fermented vegetables to participants' diets did not result in an increase in fiber intake. In fact, it is more likely that participants in the fermented vegetable group substituted fermented vegetables for another vegetable that was part of their regular diet. Another limitation of this study is that measurement of microbial metabolites would likely have provided additional insight into the effect of consumption of fermented vegetables on microbial metabolism in the gut. Our laboratory has recently acquired funding to explore the role of fermented vegetables on microbial metabolites in stool samples, which will provide a more complete account of how fermented vegetables may modulate gut microbial metabolism. A unique aspect of the present study was the large amount of data collected: assessment of stool, body measurements and blood biomarkers combined with dietary surveys, gastrointestinal symptom collection surveys, and a physical activity assessment. Also, statistical power was strengthened by the stool collection protocol, which allowed for the analysis of three separate stools samples collected in three consecutive days at both time points for a total of six samples

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per participant. We also conducted a feasibility survey at the end of the study period to collect information on the participants' reactions to being in the fermented vegetable group. Most participants reported they had no difficulty consuming the fermented vegetables in the frequency and amount required. Many participants reported they would continue to consume fermented vegetables on at least a weekly basis after the study period, indicating that it is possible to incorporate fermented vegetables into the regular diet of Americans.

In conclusion, consuming 100 grams of fermented vegetables at least five days per week for eight weeks did not change the levels of inflammatory biomarkers or alpha diversity based on Shannon index measures. However, discriminant analysis showed a greater number of alterations in the fermented vegetable group compared with the usual diet group.

Future Directions

Understanding the symbiosis of the human diet and the microbial composition of the gut including metabolites is important for future research. In general, because there have been so few feeding studies, more of these types of studies are needed- particularly with a greater length of time, and a larger quantity and variety of fermented foods for a possible greater effect. Furthermore, future studies should consider the role fermented foods play in bowel health and regularity. In our study, participants in the FV group reported greater bowel movement frequency of 1.9 times per day, while participants in the UD group reported less than one bowel movement daily (0.8/day). Participants in the FV group reported positive changes in their bowel movements, particularly improved regularity, less malodorous stool, and a notable reduction in hard stools and constipation, although these data were not systematically captured during the present study. These results indicate a potential link between a reduction in constipation and fermented vegetable intake that should be further investigated. Another area that requires further

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588 investigation is identification of individuals who may or may not benefit the most from regular
589 consumption of fermented vegetables. Studies focusing on individuals with low or high levels of
590 inflammatory markers might also provide additional insight into commonalities and differences
591 in the microbiota, especially as it relates to protection against cardiovascular disease.
592
593 **ACKNOWLEDGEMENTS**
594 The authors would like to thank Valerie Morrison and Anthony Morrison for their support with
595 collection of biological specimens, and Paige Courtier, Larissa DePasqua, Sarah Masters and
596 Mary Tipton for their support with data collection.
597
598 **AUTHOR CONTRIBUTIONS**
599 Conceptualization, A.Y.A.; Methodology, A.Y.A., M.B., Formal Analysis, B.Z., A.Y.A.; Data
600 Curation, M.B., A.Y.A.; Visualization, B.Z., M.B., A.Y.A.; Writing – Original Draft, M.B. and
601 A.Y.A.; Writing – Review and Editing, A.Y.A., A.J.M., J.Z., B.Z.; Supervision, M.B., A.Y.A.,
602 J.Z.; Funding Acquisition, A.Y.A.

603 **FINANCIAL SUPPORT**
604 This study is financially supported in full by Jacksonville Florida's Scott R. MacKenzie
605 foundation, a private foundation established for the general purpose of funding initiatives to
606 advance cures for specific diseases through genome research.
607
608 **DISCLOSURE STATEMENT**
609 No potential conflict of interest was reported by the authors.

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RESEARCH TRANSPARENCY AND REPRODUCIBILITY

The data that support the findings of this study are openly available in Dryad at https://doi.org/10.5061/dryad.547d7wmfk

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. University of North Florida IRB # 1712254-1, April 28, 2021.

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