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EFFECTS OF A FLAVONOID-RICH DIET ON GUT MICROBIOTA COMPOSITION AND PRODUCTION OF TRIMETHYLAMINE IN HUMAN SUBJECTS

by

Justin S. Bell

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ABSTRACT

The Effects of a Flavonoid-Rich Diet on Gut Microbiota Composition and Production of Trimethylamine in Human Subjects

by

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There has been substantial epidemiological evidence suggesting a protective effect of dietary flavonoids on the incidence of cardiovascular disease. The protective effects of a high flavonoid diet are thought to occur through effects on traditional risk factors such as blood pressure, lipid profile, and systemic inflammation. However, recent clinical studies have demonstrated no beneficial effect on these risk factors. An alternative risk factor may be the production of trimethylamine oxide (TMAO) by the gut microbiota from dietary choline. Plasma TMAO has been positively associated with an increased risk for cardiovascular events in human subjects.

To determine the effects of a high flavonoid diet on gut microbiota composition and plasma TMAO, 30 overweight or mildly obese participants were administered a dietary intervention for 6 weeks with either the high flavonoid diet or low flavonoid diet

followed by a 1-2 week washout and crossover to the alternate dietary intervention. Anthropometric data, plasma samples, and fecal samples were obtained from the participants at the end of each dietary intervention period. The plasma samples were analyzed for TMAO concentration and fecal samples were analyzed for microbiota composition.

The results demonstrated that there were no significant effects of a high flavonoid diet on traditional risk factors for cardiovascular disease. There were significant increases in percent abundance of the Bacteroidetes and Cyanobacteria phyla and significant decreases in the Actinobacteria and Firmicutes phyla. There were no significant differences in plasma trimethylamine oxide between the high flavonoid diet and low flavonoid. Changes in the class Deltaproteobacteria were most significantly correlated with increased plasma trimethylamine oxide concentrations.

(70 Pages)

PUBLIC ABSTRACT

The Effects of a Flavonoid-Rich Diet on Gut Microbiota Composition and Production of Trimethylamine in Human Subjects

Justin S. Bell

The prevalence of cardiovascular disease is a major public health concern worldwide. It has been theorized that diets rich in fruits and vegetables may be protective against the development of cardiovascular disease mainly through their high content of flavonoids. Flavonoids were thought to influence traditional risk factors of cardiovascular disease such as blood pressure, lipid profile, and systemic inflammation. Recent clinical studies have shown that this may not be the case. The production of trimethylamine oxide (TMAO) by the gut microbiota from dietary sources of choline has been associated with an increased risk of cardiovascular events. The objectives of this study were to determine the effects of a high flavonoid diet on gut microbiota composition and plasma trimethylamine oxide concentrations. Potential benefits of this research include the determination of a potential correlation between diet and markers of traditional and non-traditional risk factors for cardiovascular disease. Also, the effects that a high flavonoid diet has on the composition of the gut microbiota and plasma trimethylamine oxide concentrations may provide insight into possible dietary interventions to prevent cardiovascular disease.

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LITERATURE REVIEW

Introduction

Cardiovascular disease (CVD) is one of the leading causes of death both in the United States and in many other countries around the world [1-2] There are many factors that contribute to the development and progression of CVD including diet, genetic factors, and environmental factors [3]. What we eat can have beneficial or detrimental effects on overall health and wellbeing. The National Health and Nutrition Examination Survey (NHANES) showed that the majority of individuals in the United States scored an intermediate or poor Healthy Eating Index in 2010 [3]. Specific components of foods may exert beneficial effects on human health and the development of chronic diseases such as CVD. There has been substantial epidemiological evidence gathered to suggest that the habitual intake of fruits and vegetables may reduce the risk for CVD, mainly through mechanisms related to the abundance of flavonoids contained within these fruits and vegetables [1, 4]. Flavonoids (a class of polyphenols), and other phytonutrients that are commonly found in many fruits and vegetables, are defined as bioactive nonnutritive secondary plant metabolites [1-2]. Flavonoids can be divided into subclasses such as flavones, flavanols, flavonols, flavanones, isoflavones, and anthocyanadins [4].

The habitual intake of diets rich in flavonoids has been associated with a decreased risk for the development of CVD [5-7]. An observational study that was conducted within the PREDIMED study sought to determine the effects of polyphenol-rich diets and the incidence of CVD in study participants [5]. The results of this study showed that there were 273 confirmed cases of CVD in the 7172 study participants

during the follow up period of 4.3 years. The polyphenol consumption was calculated based upon the study participants' responses to a validated food frequency questionnaire (FFQ). Following multivariate adjustment there was a 46% reduction in CVD risk (based upon the first cardiovascular event, such as nonfatal myocardial infarction, stroke or death from cardiovascular related causes) between the highest quintile of polyphenol consumption and the lowest quintile of polyphenol consumption. Flavanols demonstrated the strongest inverse relationship for CVD risk in participants. This study suggests that polyphenols and/or flavonoids, may have beneficial effects on the prevention of CVD. Other studies have been conducted which have come to similar conclusions.

A study was conducted in conjunction with the Nurses' Health Study II to determine if there was an association between anthocyanins and other flavonoids and the risk for myocardial infarction [6]. The flavonoid intake was calculated through use of a validated FFQ which was administered every four years. This study found that during the 18 year follow-up period there were 405 cases of myocardial infarction reported, with an inverse association between high levels of anthocyanin intake and risk for myocardial infarction being observed. Following multivariate adjustment, it was determined that there was a 32% reduction in the risk for myocardial infarction between the highest quintile of anthocyanin consumption and the lowest quintile of anthocyanin consumption.

The effects of flavonoid-rich diets on risk factors for CVD continues to be of keen interest due to the fact that although many pharmacologic intervention strategies exist for combatting the risk factors associated with CVD, the control of these risk factors remains low [7]. There are many traditional risk factors and biomarkers that predict the risk for

the development of many chronic diseases. In regards to CVD, these risk factors include hypertension, lipid profile, and markers of systemic inflammation, such as C-reactive protein [2, 7]

Flavonoids and hypertension

Several studies have attempted to discern the effects of a polyphenolic-rich diet on the prevalence of hypertension in individuals at risk for the development of CVD. In conjunction with PREDIMED study, Medina-Remon et al. observed the effects of Mediterranean-based diets, which were high in polyphenols, on the systolic and diastolic blood pressure of study participants [8]. This study was carried out with 200 participants in a sub-study of the PREDIMED trial in which participants were randomly assigned to a low-fat control diet, a Mediterranean-based diet supplemented with extra virgin olive oil or a Mediterranean-based diet supplemented with nuts. Following one year of intervention there was a significant decrease in both systolic and diastolic blood pressure in the Mediterranean-based diets as compared to the control diet with no significant difference between the two different Mediterranean-based diets. These changes were also associated with a significant increase in the total polyphenol excretion and nitric oxide production. These results demonstrate that there is a potential inverse association between polyphenol consumption and risk for development of CVD as observed through a decrease in systolic and diastolic blood pressure.

A second study sought to determine the nutraceutical value of black cherries as it relates to antioxidant and antihypertensive properties [7]. This study hypothesized that

black cherries contained high levels of polyphenolic compounds that could potentially possess vasorelaxant and antihypertensive properties. This study utilized a rat aorta model to observe the effects of the extracted polyphenols from the black cherry fruits on the cardiovascular function. The results of this study indicated that black cherries do indeed contain high levels of total phenolics and flavonoids and these compounds were demonstrated to exhibit vasodilator and antihypertensive effects in hypertensive animals. The L-NAME (N-Nitro-L-arginine methyl ester hydrochloride)-induced hypertensive rats demonstrated a significant reduction in systolic blood pressure from 128±4 mmHg at baseline to 114±3 mmHg while the normotensive rats had no significant changes in systolic blood pressure following the supplementation with the black cherry polyphenol aqueous extract.

The effects that flavonoid consumption has on systolic and diastolic blood pressure can be observed from a clinical study conducted to determine the effects of black tea in 19 participants [9]. The participants were randomly assigned to consume black tea (129 mg flavonoids) or placebo twice a day for 8 days. This study was conducted as a randomized, double-blind, controlled cross-over study with a 13 day washout period between arms. This study demonstrated that black tea consumption resulted in significant decreases in both systolic and diastolic blood pressure (-3.2 mmHg, $p < 0.005$ and -2.6 mmHg, $p < 0.0001$; respectively). These studies indicate that the habitual consumption of flavonoids may decrease systolic and diastolic blood pressure and therefore reduce risk for the development of CVD.

Flavonoids and lipid profile

Lipid profiles have typically been a major biomarker for the risk of CVD with high levels of LDL, triglycerides, and total cholesterol, in conjunction with low levels of HDL, being associated with the development of CVD. Several epidemiological studies have shown an inverse relationship between polyphenol consumption and risk for CVD [3, 4, 6, 8]. Yubero et al. conducted a study to determine the effects of polyphenol-rich grape extract on LDL cholesterol levels [10]. This study was conducted as a randomized, double-blind, placebo-controlled clinical trial with 60 participants randomly assigned to either the grape extract group (700mg) or the placebo group for 56 days. The grape extract supplemented participants exhibited significantly lower total cholesterol (214±4 mg/dL versus 246±4 mg/dL) and LDL cholesterol (142±3 mg/dL versus 165±3 mg/dL) as compared to the placebo study participants.

A second study sought to determine the effects of an anthocyanin-rich intervention in type II diabetic study participants on dyslipidemia, oxidative stress and insulin sensitivity [11]. Fifty-eight study participants were given either 160 mg of purified anthocyanin or placebo for 24 weeks in a randomized, double-blind, placebo-controlled trial. The study demonstrated that the anthocyanin supplemented group had significantly decreased serum LDL levels (-7.9%), triglycerides (-23%) and significantly increased HDL (+19.4%) in comparison to the placebo supplemented group.

A final study was conducted to determine the effects of high-flavonoid supplementation via freeze-dried strawberries (FDS) [12]. This study was conducted as a randomized, dose-response controlled trial with 60 volunteers (55 women and 5 men)

who were assigned to consume one of the following four beverages for 12 weeks: low-dose FDS (25g/day), low-dose control, high-dose FDS (50g/day), or high-dose control. The control beverages were matched for calories and fiber content. The high-dose FDS supplemented group had significantly decreased LDL cholesterol concentrations at the conclusion of the study compared to baseline values (103±5 mg/dL vs. 130±7 mg/dL). No other supplementation groups showed significant changes in LDL cholesterol concentrations at the conclusion of the study. The results of these aforementioned studies suggest that flavonoids may reduce LDL cholesterol concentrations.

Flavonoids and systemic inflammation

Markers of systemic inflammation have long been implicated as a risk factor for the development of chronic diseases, particularly CVD. Flavonoids possess an antioxidant and anti-inflammatory capacity which could be beneficial in reducing systemic inflammation [13]. Moderate consumption of red wine has often been considered to be beneficial in reducing the risk for the development of CVD, possibly due to the high polyphenol content [14]. Consumption of red wine (30g/day) for 4 weeks decreased Interleukin (IL)-1α and plasma concentrations of C-reactive protein (CRP). CRP is a marker for systemic inflammation [15]. A cross-sectional study of 1997 females examined the associations between habitual intake of various flavonoid subclasses, insulin sensitivity and inflammatory biomarkers [16]. The intakes of flavonoid subclasses were calculated through use of a USDA FFQ. Increasing quintiles of anthocyanin intake was associated with significantly lower hs-CRP concentrations (Q5–Q1 = -0.3 mg/L).

Another study was conducted to observe the effects of supplementation with a high cocoa flavanol (HCF) beverage for 4 weeks on gut microbial populations and markers of systemic inflammation, such as CRP [17]. This study was conducted as a double-blind crossover trial in which twenty-two study participants were randomly assigned to HCF supplementation (494 mg cocoa flavanols/d) or low cocoa flavanol (LCF) supplementation (23 mg cocoa flavanols/d) for 4 weeks, followed by a 4 week washout period before the participants were crossed-over to the alternate treatment group. Supplementation with HCF beverage significantly increased the *Bifidobacteria* and *Lactobacillus* bacteria populations as compared to the LCF group. The changes in the microbial populations also corresponded to a significant decrease in plasma triacylglycerol and CRP concentrations, with the reduction in CRP being closely correlated with changes in *Lactobacilli* counts. This study demonstrates an emerging role that polyphenol consumption plays in altering the composition of the human gut microbiota, along with the potential to influence risk factors associated with the development and progression of CVD.

Traditional risk factors may not be responsible for cardio-protective effects of dietary flavonoids

Although many studies have demonstrated that increased dietary flavonoid consumption may exert positive effects on cardiovascular disease outcomes through effects on traditional risk factors, several recent studies have shown that this may not be the case [18-20]. A recent study was conducted to determine the effects of flavanones

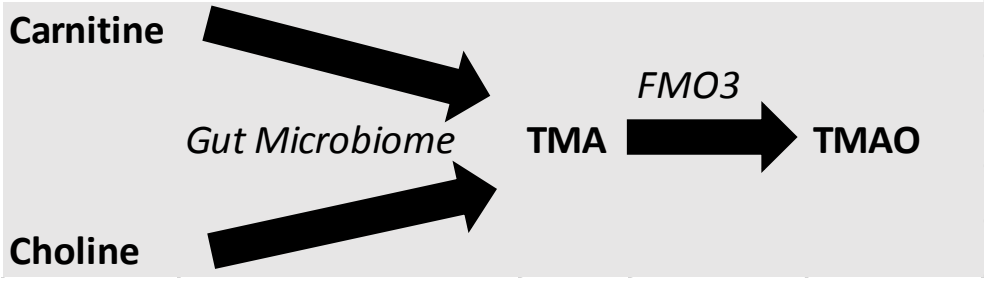
from grapefruit juice (GFJ) on vascular function in healthy postmenopausal women [18]. This study was conducted as a randomized, double-blind, controlled crossover trial with forty-eight women aged 50-65 years old. The participants were randomly assigned to consume 340 mL of GFJ/d or a matched control drink without flavanones for 6 months each with a 2 month washout between beverages. Consumption of the high flavanone-containing GFJ beverage did not affect traditional risk factors associated with CVD. There were no significant effects on systolic blood pressure, diastolic blood pressure, or plasma hs-CRP following the intervention period. A second study determined the effects of anthocyanin-rich (ACN-rich) beverage ingestion on oxidation and inflammation parameters in thirty healthy female participants [19]. This was a randomized crossover study in which the participants were randomly assigned to consume 330ml of either the placebo beverage (8.9 mg/l ACN), juice (983.7 mg/l ACN), or smoothie (840.9 mg/l ACN) daily for 14 days followed by a 4 day run-out and a 10 day washout before the start of the next arm of the intervention. Inflammation markers, such as hs-CRP, were not significantly affected by any of the study interventions. A final study determined the dose-response of fruit and vegetable intake (FV) on cardiovascular risk factors in adults at high CVD risk [20]. This study was a randomized controlled parallel group study in overweight adults with habitually low FV intake (<160g/day) and a high risk of developing CVD (estimated >20% over 10 years). Following a 4 week run-in period where FV consumption was restricted to less than 2 servings per day, the 92 participants were randomly assigned to one of three groups. These groups varied in portions of FV (2, 4, and 7 portions day; equivalent to 160g, 320g, and 560g respectively) to be consumed

daily for 12 weeks. Although there was good compliance with the intervention, there were no significant differences between the groups for blood pressure, plasma lipids, or hs-CRP. The conclusion that was drawn from this study is that there was no evidence for a dose-response effect of FV intake on traditional CVD risk factors in high CVD risk adults. The results of these studies suggest that increased flavonoid intake, through increased fruit and vegetable intake, may not affect the traditional risk factors of CVD in ways that can explain the epidemiological evidence of a favorable effect of increased fruit and vegetable intake on the development of CVD. If this is not the case, then the decreased risk for CVD observed with higher fruit and vegetable consumption must be occurring through a different, non-traditional risk factor. The emerging risk factor of trimethylamine oxide (TMAO), produced via the gut microbiota through the breakdown of choline and carnitine, may provide an explanation for the decreased risk of cardiovascular disease observed in those who consume diets high in flavonoids.

Flavonoids, gut microbiota, and TMAO production

Studies have suggested that elevated plasma TMAO have a strong positive association with CVD risk [21-24]. TMAO arises from dietary intake of choline and carnitine and subsequent metabolism by the gut microbiota through an intermediate, trimethylamine (TMA) [3, 21, 25-28, 34]. The intermediate, TMA, is then oxidized by flavin monooxygenase 3 (FMO3) resulting in the formation of TMAO (**Fig. 1**) [3, 21, 25, 27, 34].

Fig. 1 Conversion of L-Carnitine & Choline to trimethylamine oxide (TMAO)



The significance of TMAO production and the risk for CVD can be best demonstrated by two clinical studies. The first study involved a 3-year follow-up of over 4000 patients at the Cleveland Clinic in a study to determine the effects of fasting plasma levels of TMAO on the incidence of cardiovascular events (myocardial infarction, stroke, and death) [23]. This study showed a significant positive association between plasma TMAO and cardiovascular events. A study showed that elevated carnitine levels were significantly associated with cardiovascular events (myocardial infarction, stroke or death) over a 3 year follow-up. However, this relationship was only observed in individuals with concurrently high levels of TMAO [24]. The significance of the association between high circulating TMAO levels and CVD risk indicates that there may be new potential pathways and mechanisms for the prevention and onset of CVD [3, 21].

The gut microbiota has been implicated as the major producer of TMA through the metabolism of choline and carnitine [23]. A study demonstrating this relationship involved human subjects who were subjected to a phosphatidtylcholine-choline challenge in which the subjects were supplemented with a choline-rich diet containing approximately 750mg choline to be consumed within 10 minutes. Blood was then drawn from the subjects at 1, 2, 3, 4, 6 and 8 hours after baseline along with 24 hour urine

collection. These subjects exhibited significant increases in both urinary and plasma levels of TMAO following the initial visit. Following the first study period, the subjects were then supplemented in the same process as before but with the addition of an oral broad-spectrum antibiotic for 1 week. This resulted in an almost complete negation of detectable TMAO levels which indicates that the gut microbiota play a significant role in the production of TMAO.

It has been hypothesized that an alteration in the dietary substrate of individuals could lead to reductions in TMAO levels either by directly reducing the consumption precursors for TMAO production or through an alteration of gut microbiota which could potentially reduce the capacity of the gut microbiota to produce TMA and subsequently TMAO [3, 29]. Shifts in gut microbial communities have been shown to influence TMAO production in vegans/vegetarians compared to that of omnivores. Several different bacterial species have been implicated in the production of TMA from dietary sources including *Acinetobacter* spp.(Proteobacteria phylum), *Klebsiella pneumonia* (Proteobacteria phylum), *Escherichia coli* (Proteobacteria phylum) and *Proteus mirabilis* (Proteobacteria phylum) [21, 24]. There appears to be a selective advantage for bacterial populations that can utilize L-carnitine as a fuel source with subsequent production of TMA in those who regularly consume food products containing L-carnitine and choline [3, 27].

A recent study sought to determine which phyla and strains of bacteria present in the human gut microbiota were able to produce TMA from choline. This study utilized *in vitro* screening of seventy-nine bacterial isolates representing six phyla found in the

human intestinal tract [28]. Eight species of bacteria representing two phyla were able to produce TMA from choline. The two major phyla that contained species capable of producing TMA were Firmicutes and Proteobacteria. This study also demonstrated that germ-free mice that were colonized with these TMA producing species had significantly higher levels of plasma TMAO as compared to the germ-free mice colonized with non-TMA producing microbial species. Another study conducted in mice sought to determine if microbial transplantation of bacteria species found in high TMAO producing mice would confer changes to the gut microbiota and TMAO producing capacity in recipient mice [22]. Transplantation of microbial communities found in high TMAO-producing mice to low TMAO-producing recipient mice resulted in increases to TMAO production. The two major phyla that differed between the donor and recipient mice, and were therefore theorized to be implicated in the increased TMAO production, were Firmicutes and Actinobacteria. The phylum Bacteroidetes were present in large numbers in the recipient mice prior to transplantation and low numbers in donor mice, indicating that Bacteroidetes may be inversely associated with TMAO production. These aforementioned bacterial phyla, which have been implicated in TMAO production, have been shown to be affected by polyphenols/flavonoids [30].

Finally, a study conducted by Martinez-del Campo et al. sought to establish the link between a bacterial gene cluster (the choline utilization *cut* cluster) and anaerobic choline metabolism [34]. To determine the bacteria capable of choline metabolism, and subsequent formation of TMA, bacterial genomes were screened for two *cut* gene sequences. The key functional gene involved in choline utilization was choline TMA-

lyase (CutC). Both CutC and CutD are responsible for carrying out the initial cleavage of the C-N bond in choline, resulting in the formation of TMA and acetaldehyde. This study found that there were 459 CutC homologs which were distributed across four bacterial phyla including Proteobacteria, Firmicutes, Actinobacteria, and Fusobacteria. The majority of the CutC homologs were found within the Proteobacteria phyla, specifically Gammaproteobacteria and Deltaproteobacteria. The Firmicutes phylum was also found to be a major contributor, in particular the classes Clostridia and Bacilli. Less than 3% of the hits came from the Actinobacteria phylum (Coriobacteridae) and Fusobacteria (Fusobacteria). This study also found that CutC was absent from the Bacteroidetes phylum. These results demonstrate that members of the Proteobacteria and Firmicutes phyla, specifically Gammproteobacteria, Deltaproteobacteria, Clostridia, and Bacilli are likely the main choline utilizing bacteria in the human gut while members of the Actinobacteria and Fusobacteria phyla contribute to a lesser scale. These results also suggest that members of the Bacteroidetes phylum are not capable of metabolizing choline, and thus do not contribute to the production of TMA and the subsequent oxidation to TMAO.

Flavonoids and gut microbiota

The ability of the human gut microbiota to influence chronic disease development is beginning to be understood in a more in-depth manner [26]. The role that dietary polyphenols and flavonoids play in modulating gut microbiota has been shown in an animal study [13]. The bioavailability of polyphenols consumed in the diet depends

greatly on interactions with the gut microbiota and the conversion into bioactive compounds. The microbiota contained within the human gut has been shown to break down complex polyphenols into smaller phenolic acids which are then absorbed across the intestinal mucosa. A study attempting to discern the effects of high flavonoid containing apples on the gut microbial composition in a controlled feeding study in mice was conducted by supplementing the mice with one of the two experimental diets. The experimental diets of interest in this study were RG-F (Red Gala Flesh), RG-FP (Red Gala Flesh and Peel), MYB-F (Transformed Gala apples fused with MYB transcription factor which regulates biosynthetic genes involved in anthocyanin production) and MYB-FP (Transformed Gala apples, flesh and peel). The transgenic apples (MYB-F and MYB-FP) had increases in polyphenol content, specifically related to anthocyanins and flavanols. The controlled feeding of high flavonoid-containing apples in mice resulted in changes in both the overall population and numbers of individual species in the mice gut microbiota. There were significant increases in the *Bifidobacterium* spp. (Actinobacteria phylum) in mice fed the RG-F and RG-FP content diet as compared to the MYB-F, MYB-FP and control diets. These results coincided with decreases in the content of certain polyphenols in the MYB-F and MYB-FP diets compared to the RG-F and RG-FP, specifically phlorizidin-xyloside and quercetin-arabinoside, indicating that the increase in *Bifodbacterium* spp. observed in the RG-F and RG-FP groups could be correlated with differences in concentrations of these particular polyphenols. These findings suggest that diets high in specific polyphenol containing foods can alter specific gut microbiota species and potentially have implications for human health.

Several studies have demonstrated the ability of different polyphenol-containing foods to promote changes in the human gut microbiome [17, 31, 32]. To determine the effects of red wine polyphenol consumption on the composition of the human gut microbiome, Queipo-Ortuno et al. utilized a randomized, cross-over controlled intervention study consisting of 10 male participants [32]. The participants consumed de-alcoholized red wine (272 ml/d), red wine (272 ml/d) and gin (100 ml/d) for 20 days each. Fecal samples from the participants were obtained at the baseline of the study and at the conclusion of each 20 day intervention interval. Consumption of red wine polyphenols resulted in significantly increased numbers of *Enteroccocus* (Firmicutes phylum), *Bacteroides* (Bacteroidetes phylum) and *Bifidobacterium* (Actinobacteria phylum) species while also showing significant decreases in cardiovascular risk factors including systolic and diastolic blood pressure, total cholesterol, triglycerides and CRP concentrations [31-32]. The changes in cholesterol and CRP concentrations were correlated with changes in the *Bifidobacterium* population [32]. This study demonstrates that consumption of polyphenol-rich foods and beverages can promote changes in the human gut microbiota and subsequently risk for CVD development. A study conducted to observe the effects of tea phenolics on the growth of bacteria commonly found in the human digestive tract demonstrated that members of the Firmicutes phyla and Proteobacteria phyla had suppressed growth when exposed to green tea phenolics [30]. Another study examined the effects of Concord grape polyphenols on the gut microbiota and markers of metabolic syndrome in mice [33]. Consumption of Concord grape polyphenols changed the gut microbiota composition, specifically lowering the ratio of

Firmicutes to Bacteroidetes. The above studies are consistent in their findings that polyphenols decrease the abundance of Firmicutes and/or increase the abundance of Bacteroidetes. These results are of particular interest due to the fact that Firmicutes have been implicated in TMAO production while Bacteroidetes have not [22, 28].

TABLE 1 provides a summary of microbial communities that have been implicated in TMAO production and whether these bacterial phyla have been demonstrated to be affected by polyphenols:

TABLE 1 Summary of bacterial phyla, TMA production and effects of flavonoids

Phylum	TMA Production	Effect of Flavonoids
Actinobacteria	+[22]	↑[13, 32]
Bacteroidetes	- [22, 28]	↑[32, 33]
Firmicutes	+[22, 28]	↓13, 30, 33]
Proteobacteria	+[21, 24, 28]	↓[30]

+ indicates ability to produce TMA; - indicates no ability to produce TMA
↑ indicates increase in bacterial abundance; ↓ indicates decrease in bacterial abundance

The above interactions show that the main phyla of bacteria that have been implicated in TMAO production are bacteria belonging to Proteobacteria, Actinobacteria and Firmicutes while bacteria belonging to the Bacteroidetes phylum have not been implicated [21, 22, 24, 28]. Various different flavonoids have also been shown to affect the relative populations of these phyla in human and mouse studies [13, 30, 32, 33]. Based on these previously conducted studies, it appears that there is a potential link

between bacterial taxonomies associated with production of TMA and reductions in these implicated bacterial taxonomies through the consumption of flavonoids.

RESEARCH QUESTIONS AND HYPOTHESES

Research questions

1) How does the consumption of a flavonoid-rich diet affect the gut microbiota composition in study participants who were classified as overweight or mildly obese?
2) How does the consumption of a flavonoid-rich diet affect the plasma trimethylamine-oxide (TMAO) levels of study participants?
3) Does gut microbiota composition correlate with plasma TMAO levels?
4) Do changes in gut microbiota composition between diets correlate with changes in plasma TMAO levels?

Hypotheses

It is hypothesized that: 1) the consumption of a flavonoid-rich diet will promote a change in the composition of the gut microbiota in study participants; and 2) the consumption of a flavonoid-rich diet will also promote a decrease in plasma TMAO levels which will correlate with changes in gut microbiota population composition.

METHODS AND MATERIALS

The polyphenol study

This study was conducted in conjunction with the Polyphenol Study at Utah State University. A detailed description of the Polyphenol Study follows.

The Polyphenol Study was a randomized, double-blind, crossover study that was conducted at Utah State University by Dr. Michael Lefevre. This study was conducted to determine the effects of a dietary intervention involving a high and low flavonoid diet on intestinal bacteria composition, intestinal inflammation and blood measures of insulin resistance and systemic inflammation.

Participants

A total of 30 participants who were classified as overweight or mildly obese were selected from the Cache County, Utah regional population according to the following Inclusion Criteria and Exclusion Criteria.

Inclusion Criteria:

- Male or Female of any race or ethnicity between 18 to 70 years of age, inclusive
- Body mass index between 25-35 kg/m^2
- Free of chronic disease

- Willing to eat only the foods that are provided by the Center during the diet periods
- Willing to abstain from the consumption of alcohol for 48 hours prior to blood draw days
- Willing to abstain from the consumption of green tea, black tea, supplements containing green tea extracts, red wine, and supplements containing polyphenols

Exclusion Criteria:

- Age <18 or >70 years
- BMI <25 or >35 kg/m^2
- Presence of atherosclerotic disease, diabetes mellitus, renal, hepatic, endocrine, gastrointestinal or other systemic disease
- Uncontrolled hypertension defined as diastolic blood pressure >95 mm Hg or systolic blood pressure >160 mm Hg
- For women, pregnancy, breast feeding or postpartum < 6 months
- History of drug or alcohol abuse
- History of depression or mental illness requiring hospitalization within the last 12 months
- Use of antibiotics within the last 6 months
- Multiple food allergies or significant food preferences or restrictions that would interfere with diet adherence
- Chronic use of over-the-counter medication which would interfere with study endpoints including NSAIDS, laxatives and antacids

- Lifestyle or schedule incompatible with the study protocol
- Other medical, psychiatric, or behavioral conditions that in the view of the principal investigator may present a safety hazard to the participant or interfere with study participants or the ability to follow the intervention protocol

Diet description

There were two intervention diets that were designed to be identical in macronutrient content but different in flavonoid content (High Flavonoid Diet, HFD; Low Flavonoid Diet, LFD). The following tables provide a description of the diet characteristics (**TABLE 2**; **TABLE 3**).

TABLE 2 Composition of High (HFD) and Low (LFD) Flavonoid Diets

Nutrient	LFD	HFD
Protein, % kcal	17	17
Carbohydrate, % kcal	56	56
Fat, % kcal	30	30
SFA, % kcal	7	7
MUFA, % kcal	17	17
PUFA, % kcal	6	6
EPA + DHA, mg/d	125	125
Cholesterol, mg/d	250	250
Total Fiber, g/d	25	25
Soluble Fiber, g/d	4	4
Flavonoids + PAC*, mg/d	<100	>600

SFA, saturated fatty acid
MUFA, monounsaturated fatty acid
PUFA, polyunsaturated fatty acid
EPA, eicosapentaenoic acid
DHA, docosahexaenoic acid
PAC, proanthocyanidins

TABLE 3 Content of the flavonoid subclasses in the High (HFD) and Low (LFD) Flavonoid Diets

Flavonoid Subclass	**HFD**	**LFD**
Flavanones	34.9	0.8
Anthocyanins	184.4	0.9
Flavan-3-ols	69.6	3
Flavonols	28.6	4.6
Flavones	6.8	1.5
Poly Flavonoids	314.7	18.6
Total	639	29.4

Values are mean mg/day based on a 2200 kcal diet

The diets were fed for a 6 week period each with a 1-2 week washout between the diets. The diets were part of a controlled feeding program in which all meals, snacks, and caloric beverages consumed were prepared and provided by the Center for Human Nutrition Studies' Research Kitchen.

Blood collection and processing

Blood was collected in the morning prior to the breakfast meal and after a minimum 10 hour fast and 48 hour abstinence from alcohol. Blood was collected twice (on non-consecutive days) at the end of week 6 of each diet period.

Fecal collection and processing

During the last five days of each diet intervention period, participants were provided with five disposable coffee filter-like fecal collection devices which could be affixed to the toilet seat. Participants were provided collection tubes and "spoons" and

were asked to take five marble-sized fecal samples from each captured stool, deposit them in to the appropriate tubes and store the sample frozen until their next visit to the center.

DNA extraction

The fecal samples obtained from study participants at the conclusion of each dietary intervention period were analyzed using a QIAamp DNA Stool Mini Kit. Approximately 100mg of stool was used for each sample. The DNA extraction was performed following the process outlined by the QIAamp DNA Stool Mini Kit operating procedure.

Following the DNA extractions performed for each of the samples and in accordance with the outlined procedure, the isolated DNA was frozen for storage until later processing.

Polymerase Chain Reaction (PCR)

The PCR reaction was performed utilizing steps optimized for the Ion Torrent PGM platform. This platform works by amplifying the 16S rRNA gene sequences (obtained from extracted DNA via QIAamp DNA Stool Mini Kit) using the primers Probio_Uni and Probio_Rev which target the V3 of the 16S rRNA gene sequence [30]. The primer sequences are 5'-CCTACGGGRSGCAGCAG-3' and 5'-

ATTACCGCGGCTGCT-3’ for Probio-Uni and Probio_Rev, respectively [30]. Sixty forward primers were used, each containing a unique 8 bp “barcode”. Two pools were prepared and ran on two separate chips. After pooling, individual samples were able to be identified through their unique barcode primer sequences. A master mix was prepared according to a prescribed ratio (**TABLE 4**). The 0.2 mL tubes were labeled for each PCR reaction. The forward primer and 5µl of DNA for each sample were added to the PCR tubes. 42 µL of the master mix was added to each sample PCR tube containing DNA and forward primer. The samples were then put on the thermal cycler with a program optimized for Ion Torrent PCR (**TABLE 5**).

TABLE 4 Polymerase Chain Reaction (PCR) master mix composition

Reagent	**1 X Volume (µL)**
PCR Water	31.6
Buffer with 18 mM MgCl	5
Dimethyl sulfoxide (DMSO)	1
dNTP mix	1
Torrent Forward	3 (different primer/sample)
Torrent Reverse	3
Enzyme	0.4

TABLE 5 Ion Torrent PCR program description

Temperature (ºC)	Time	Cycles
95	5 minutes	1
94	30 seconds	
55	30 seconds	35
72	90 seconds	
72	10 minutes	1
4	Hold	

Following the PCR reactions, 1.5% agarose gel was prepared and gel electrophoresis was performed to determine that a PCR product exists for further purification and sequencing. Product size was approximately 280 bp and varied slightly between samples.

PCR product purification

The resultant PCR products were then subjected to a procedure designed to remove any existing impurities. A solution of 70% ethanol was prepared using PCR grade water and ethanol with 400 µL used for each sample. A 1X TE buffer was prepared with DNase/RNase free water. 50 µL of Agencourt AMPure beads were added to each well of a 96 well round bottom plate. PCR product was then added to the appropriate well and gently mixed. The well plate was then incubated at room temperature for 5 minutes. The well plate was then placed onto a 96 well magnet for 2 minutes. Once the beads were drawn to the center of the round bottom well, the clear fluid remaining was removed.

Two-hundred µL of 70% ethanol was added to each well followed by room temperature incubation for 30 seconds. The ethanol was then removed in the same process as described above. This process of ethanol washing and removal was repeated once more. The plate was then placed on the magnet for 5 minutes at room temperature to dry. The plate was removed from the magnet and 40 µL of TE buffer was added and mixed. The plate was placed on the magnet for 1 minute and the resulting eluent was transferred to corresponding tubes and stored at -20 ºC.

DNA concentration

A Quant-IT Picogreen dsDNA kit was used to assess the DNA concentration of each of the samples to determine the appropriate volume of each sample to be combined for grouping. A 1X TE buffer at a pH of 7.5 was prepared. The PicoGreen reagent (1:200 dilution) was prepared. 1.5 mL tubes were labeled for a serial dilution (**TABLE 6**). One-hundred µL of standard was added to each well of a black plate followed by the addition of 90 µL of 1X TE buffer to each sample well. 10 µL of sample DNA was added to each well containing the TE buffer. 100 µL of PicoGreen solution was added to each standard and sample well. The fluorescence was then read on a Tecan plate reader.

TABLE 6 Serial dilution preparation

Tube #	Volume of TE (µL)	Volume DNA stock and dilution series (µL)	Final concentration
A	480	20 from kit stock	4000 ng/mL
B	250	250 from tube A	2000 ng/mL
C	450	50 from tube B	200 ng/mL
D	450	50 from tube C	20 ng/mL
E	450	50 from tube D	2 ng/mL
F	500	0	Blank

DNA fragment sizing

DNA fragment sizing was carried out according to the procedure supplied by the Agilent Bioanalyzer.

Pooling samples

The barcoded PCR products were then combined to form a final concentration of 10^7 mol/µL with a duplicate created in case of sampling error or DNA lab sequencing error. The pooled samples were stored at -20 ºC until taken to the core lab for sequencing. The samples were split across two separate sequencing runs.

QIIME processing

The microbiota DNA sequences obtained were processed with Quantitative Insights into Microbial Ecology (QIIME) to determine the taxonomic compositions of the bacteria. The general workflow that was utilized via QIIME included the creation of a mapping file (in order to link the barcodes to each sample ID and metadata) and conversion of the Ion Torrent data from a FASTQ file to a FASTA (.fna) and QUAL (.qual) file. The data was then processed through the *split_libraries* function which resulted in demultiplexing and quality filtering of the data. The *split_libraries* workflow was performed at a minimum sequence length of 150 and a maximum sequence length of 300. The minimum quality score was set to 20. Next, the resultant *split_libraries* output for both run #1 and run #2 was concatenated using the *cat* command and a combined mapping file was created for use in the subsequent QIIME workflow scripts. The OTU's were picked through an open reference method utilizing the GreenGenes database as a reference at 97% similarity. Next, chimeric sequences were identified through the use of the ChimeraSlayer workflow with the GreenGenes database as a reference set. Following the identification of the chimeric sequences, the resultant data were filtered from the OTU table and subsequently an OTU table with the chimeric sequences removed was formed. A non-chimeric phylogeny tree was then created through the *make_phylogeny.py* workflow. Finally, a core diversity analysis was performed using the *core_diversity_analyses.py* workflow with the filtered OTU table and non-chimeric phylogeny tree as input data. The sampling depth was set to 33581 as indicated by the *biom_summarize_table* workflow.

The PICRUSt program utilizes an OTU tree and OTU table created through the QIIME pipeline to infer metabolic capabilities of the gut microbiome through bacterial abundances [35]. The results of the PICRUSt program provides an overview of the metagenome function for each sample. The first step in the PICRUSt pipeline was carried out by normalizing the OTU table through the script *normalize_by_copy_number.py* followed by the input of the resultant normalized OTU table into the Predict Function for Metagenomes script (*predict_metagenomes.py*). The resulting BIOM table was then used in the *categorize_by_function.py* script to collapse the predicted functions into higher Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

TMAO assay

The plasma samples of the participants were analyzed by the University of Utah's Metabolomics Core to determine the concentrations of TMAO present at the conclusion of each diet intervention period. One-hundred µL of plasma was aliquoted into microtubes (two samples per participant/per diet period) and stored at -80 ˚C until they were delivered to the Metabolomics Core. The plasma samples were analyzed utilizing a TMAO assay Standard Operating Procedure developed at the University of Utah. The following describes the SOP used in the analysis:

Frozen plasma samples were thawed in 4 ˚C refrigerator. Average sample volume needed is ~30 µL. Next, Proteins were precipitated by adding 120 µL methanol containing internal standard (1 µg/mL ^{13}C, ^{15}N-labeled amino acid mix) that was stored

in -20 ˚C freezer. Precipitation was pelleted down by centrifugation at 20,000 x g for 10 minutes in a refrigerated centrifuge. Obtained supernatant was removed and 20 µL were directly injected into LC-MS. LC-MS used for this study is an Agilent 1290 LC system coupled to an Agilent 6550 iFunnel Q-TOF mass spectrometer. Column used in the study was a SeQuant ZIC-cHILIC column. LC conditions used are: initial condition – 10% aqueous phase (10mM ammonium formate, pH 3.2), 90% organic phase (acetonitrile); aqueous phase was ramped up to 60% in 10 minutes; aqueous phase was then held at 60% for 0.5 minutes then ramped down to initial condition and held for 10 minutes for column re-equilibrium. Q-TOF mass spectrometer parameters were optimized for TMAO detection using MDDE program and used parameter settings are: detection mode positive; Drying Gas Temp 200 ˚C; Drying Gas Flow 15.1 L/min; Neubulizer pressure 37 psig; sheath gas temp 400 ˚C; sheath gas flow 11.9 L/min; VCap 3000 V; Nozzle Voltage 0 V.

Under the experimental conditions TMAO was eluted at ~ 7.0 min (+/- 0.2 minutes) at two ionized forms: $[M+H]^+$ (m/z 76.0757) and $[2M+H]^+$ (m/z 151.1441). The isotope-labeled amino acid proline (retention time ~6.6 min, $[M+H]^+$ (m/z 122.0844)) has similar retention time and was used as internal standard for analysis. The obtained mass spectra were analyzed using Agilent Mass Hunter Qualitative Analysis software. The area under TMAO curve (AUC) and internal standard (isotope labeled proline) were calculated and used for quantification.

For calibration curve construction, 30 µL water were spiked with 0, 1, 5, 10, 50, 100 ng TMAO, respectively. These standard samples were treated in the same way as

plasma samples, i.e. addition of 120 µL methanol containing internal standard, centrifugation then injection into LC-MS. Calibration curve was plotted with TMAO AUC/proline AUC ratio vs. TMAO amount (in ng).

Statistical analysis

Statistical analysis was conducted with SAS software (version 9.4). The samples for each participant were averaged for both the HFD and LFD prior to conducting statistical significance test. A paired t-test was used in conjunction with a Wilcoxon Signed Rank Test at the significance of $\alpha=0.05$ to determine if there were significant differences between the HFD and the LFD in the percent abundance of the gut microbiome at all taxonomic levels. A paired t-test and Wilcoxon Signed Rank Test were conducted at the significance of $\alpha=0.05$ to determine if there was a significant difference in plasma TMAO concentrations between the HFD and LFD. To determine the bacterial taxa that were significantly correlated with plasma TMAO concentrations, a Spearman non-parametric correlation test was conducted at the significance of $\alpha=0.05$. Linear regression analysis was conducted to correlate changes in taxa with changes in TMAO. The minimum detectable difference in TMAO was calculated through post-hoc power with parameters set to n=29 (sample size), $\alpha=0.05$, power 80%, and the variance for the difference between the HFD and LFD.

RESULTS

Participant characteristics

There were a total of 30 participants enrolled for the study with 29 participants completing the entire study (**TABLE 7**). There were 16 males and 13 females included in the study population. The average age of the study participants was 38.9 years with an average BMI of 29.48.

TABLE 7 Participant characteristics (age; weight; BMI; systolic blood pressure (SBP); diastolic blood pressure (DBP); blood glucose)

	Men	**Women**	**Combined**
n	16	13	29
Age (years)	35.9 (22.3-66.1)	42.5 (23.2-66.7)	38.9
Weight (kg)	94.9 (77.4-115.8)	77.9 (66.4-95.0)	87.3
BMI (kg/m^2)	29.4 (24.9-34.8)	29.6 (26.4-34.2)	29.5
SBP (mmHg)	121 (104-141)	108 (93-125)	116
DBP (mmHg)	76 (65-88)	74 (58-91)	76
Blood Glucose (mmol/L)	87.7 (77.5-100.8)	87.8 (77.3-107.5)	87.7

Diet intake

There were no significant differences between the HFD and LFD for calories consumed. Participants on the HFD consumed 2891±103 kcal/day while on the LFD they consumed 2907±113 kcal/day. The flavonoid consumption was significantly higher for the HFD in comparison to the LFD (983±33.8 vs. 30±6.9 mg/day; $p<0.0001$).

Traditional risk factors

Traditional risk factors for CVD, including blood pressure, plasma lipids, and CRP were not significantly affected by the HFD (**TABLE 8**).

TABLE 8 Effect of dietary flavonoids on traditional risk factors for CVD

Risk Factor	**HFD**	**LFD**	**HFD-LFD**	**p**
Weight Change (kg)	-0.80 ± 0.2	1.1 ± 0.2		ns
Systolic blood pressure (mmHg)	114.1 ± 2.4	115.0 ± 2.2	-1.03 ± 1.06	ns
Diastolic blood pressure (mmHg)	75.4 ± 1.5	77.0 ± 1.6	-1.72 ± 0.96	ns
LDL (mg/dL)*	100 ± 5	102 ± 4	-2.41 ± 2.21	ns
HDL (mg/dL)*	40.7 ± 2	40.8 ± 1.8	-0.03 ± 0.62	ns
TG (mg/dL)*	133 ± 11	131 ± 10	2.31 ± 5.22	ns
hs-CRP (mg/L)*	2.41 ± 0.62	1.65 ± 0.30	0.76 ± 0.46	ns

n=29 (High Flavonoid Diet, HFD; Low Flavonoid Diet, LFD)
Paired t-test (α=0.05)
Mean ± SEM
*Statistical analysis conducted on ln transformed data

Microbiota analysis

The results of the microbiota analysis revealed that there were significant changes in the gut microbiota between the HFD and LFD across 43 taxonomic levels (TABLE 9). The HFD resulted in a decrease in the abundance of the phyla Actinobacteria and Firmicutes and an increase in the abundance of the phylum Bacteroidetes, as compared to the LFD. There were no significant differences in the phyla Proteobacteria, Tenericutes,

and Verrucomicrobia. Taxa implicated in TMAO production are highlighted in yellow (**TABLE 9**, **Fig. 2**).

Alpha diversity was analyzed through use of chao1 and PD_whole_tree measurements. Chao1 estimates the estimates total species richness whereas PD_whole_tree measures the minimum total length of all the phylogenetic branches required to span a set of taxa on a phylogenetic tree [36-37]. The results of the Alpha Diversity analysis for the HFD and LFD showed that there were no significant differences in richness (chao1) or Branch length-based diversity (PD_whole_tree) (**Fig. 3**).

Beta diversity utilized Principal Coordinate Analysis (PCoA) that was conducted with the weighted and unweighted Unifrac distance for the HFD-LFD. Unifrac is a measure of dissimilarity in community composition between two groups (HFD; LFD) [37]. Unweighted Unifrac distance is used to compare microbial communities based on the presence or absence of members. Weighted Unifrac distance takes into account the relative abundance of each taxa. The results of the unweighted Unifrac distance showed that there was no significant shift in coordinates PC1 or PC3 (PC1= -0.011±0.006, α=0.065; PC3= 0.004±0.006, α=0.534). There was a significant shift in coordinate PC2 (PC2= -0.02±0.009, α=0.040). The results of weighted Unifrac distance showed that there was no significant shift in coordinates PC2 or PC3 (PC2= -0.008±0.011, α=0.486; PC3= 0.012±0.011, α=0.369). There was a significant shift in coordinate PC1 (PC1=0.09±0.04, α=0.025). Figures 4 and 5 provide a graphical summary of the Beta Diversity analysis.

TABLE 9 Effect of High Flavonoid Diet on gut microbiota percent abundance

Taxa	HFD	LFD	HFD-LFD	P-Value	P-Value*
p_Actinobacteria	0.533 ± 0.099	1.430 ± 0.434	-0.897 ± 0.407	<0.0001	0.007
c_Actinobacteria	0.220 ± 0.056	0.842 ± 0.412	-0.623 ± 0.398	<0.0001	0.007
o_Bifidobacteriales	0.220 ± 0.056	0.842 ± 0.412	-0.623 ± 0.398	<0.0001	0.007
f_Bifidobacteriaceae	0.220 ± 0.056	0.842 ± 0.412	-0.623 ± 0.398	<0.0001	0.007
g_Bifidobacterium	0.220 ± 0.056	0.842 ± 0.412	-0.623 ± 0.398	<0.0001	0.007
s_adolescentis	0.063 ± 0.021	0.372 ± 0.218	-0.310 ± 0.205	0.0005	0.0142
s_bifidum	0.005 ± 0.004	0.016 ± 0.014	-0.011 ± 0.011		
s_longum	0.056 ± 0.021	0.105 ± 0.024	-0.049 ± 0.019	0.0019	0.039
c_Coriobacteriia	0.313 ± 0.069	0.588 ± 0.137	-0.275 ± 0.094	0.0001	0.007
o_Coriobacteriales	0.313 ± 0.069	0.588 ± 0.137	-0.275 ± 0.094	0.0001	0.007
f_Coriobacteriaceae	0.313 ± 0.069	0.588 ± 0.137	-0.275 ± 0.094	0.0001	0.007
g_Collinsella	0.225 ± 0.051	0.487 ± 0.120	-0.262 ± 0.081	<0.0001	0.007
s_aerofaciens	0.218 ± 0.049	0.465 ± 0.116	-0.262 ± 0.078	<0.0001	0.007
s_stercoris	0.004 ± 0.003	0.013 ± 0.009	-0.009 ± 0.007		
g_Eggerthella	0.004 ± 0.001	0.010 ± 0.005	-0.006 ± 0.004		
s_lenta	0.004 ± 0.001	0.010 ± 0.005	-0.006 ± 0.004		
p_Bacteroidetes	51.516 ± 3.093	44.617 ± 3.075	6.899 ± 2.469	0.0073	0.069
c_Bacteroidia	51.516 ± 3.093	44.617 ± 3.075	6.899 ± 2.469	0.0073	0.069
o_Bacteroidales	51.516 ± 3.093	44.617 ± 3.075	6.899 ± 2.469	0.0073	0.069
f_[Barnesiellaceae]	0.520 ± 0.133	0.792 ± 0.234	-0.272 ± 0.134		
f_[Odoribacteraceae]	0.273 ± 0.049	0.259 ± 0.042	0.014 ± 0.065		
g_Butyricimonas	0.123 ± 0.032	0.100 ± 0.024	0.023 ± 0.023		
g_Odoribacter	0.150 ± 0.029	0.159 ± 0.028	-0.008 ± 0.013		
f_[Paraprevotellaceae]	0.527 ± 0.138	0.419 ± 0.107	0.109 ± 0.080		
g_[Prevotella]	0.057 ± 0.053	0.036 ± 0.027	0.022 ± 0.030		
g_Paraprevotella	0.386 ± 0.121	0.266 ± 0.075	0.120 ± 0.082		
f_Bacteroidaceae	29.668 ± 3.669	25.894 ± 3.455	3.773 ± 1.809		
g_Bacteroides	29.651 ± 3.669	25.885 ± 3.455	3.773 ± 1.809		
s_caccae	0.881 ± 0.245	1.118 ± 0.300	-0.240 ± 0.125		
s_coprophilus	0.074 ± 0.074	0.061 ± 0.061	0.013 ± 0.013		
s_eggerthii	0.326 ± 0.227	0.570 ± 0.378	-0.244 ± 0.441		
s_fragilis	0.106 ± 0.050	0.885 ± 0.567	-0.778 ± 0.532		
s_ovatus	2.831 ± 0.714	3.093 ± 0.830	-0.262 ± 0.259		
s_plebeius	0.448 ± 0.379	0.276 ± 0.210	0.172 ± 0.177		
s_uniformis	3.596 ± 0.847	2.651 ± 0.600	0.944 ± 0.447		
f_Porphyromonadaceae	3.029 ± 0.517	1.919 ± 0.910	1.110 ± 0.659	0.0005	0.0142
g_Parabacteroides	3.029 ± 0.517	1.919 ± 0.910	1.110 ± 0.659	0.0005	0.0142
s_distasonis	2.104 ± 0.461	1.318 ± 0.828	0.786 ± 0.601	0.0002	0.009
f_Prevotellaceae	8.661 ± 3.019	8.662 ± 3.009	-0.001 ± 2.407		
g_Prevotella	8.661 ± 3.019	8.662 ± 3.009	-0.001 ± 2.407		
s_copri	8.341 ± 2.996	8.386 ± 3.005	-0.045 ± 2.381		
s_stercorea	0.070 ± 0.050	0.044 ± 0.031	0.026 ± 0.025		
f_Rikenellaceae	6.967 ± 1.061	4.960 ± 0.682	2.007 ± 0.938	0.0079	0.07
f_S24-7	0.071 ± 0.053	0.178 ± 0.125	-0.107 ± 0.074		
p_Cyanobacteria	0.208 ± 0.075	0.199 ± 0.167	0.009 ± 0.158	0.0003	0.011
c_4C0d-2	0.068 ± 0.037	0.176 ± 0.168	-0.109 ± 0.141		
o_YS2	0.068 ± 0.037	0.176 ± 0.168	-0.109 ± 0.141		
c_Chloroplast	0.141 ± 0.068	0.023 ± 0.006	0.118 ± 0.065	<0.0001	0.007
o_Streptophyta	0.141 ± 0.068	0.023 ± 0.006	0.118 ± 0.065	<0.0001	0.007

Significance determined through Wilcoxon Signed-Rank Test.
Data are Mean ± SEM.
n=29 (High Flavonoid Diet, HFD; Low Flavonoid Diet, LFD)
P-Value*=Adj. P-Value for False Discovery Rate

TABLE 9 Cont. Effect of High Flavonoid Diet on gut microbiota percent abundance

Taxa	HFD	LFD	HFD-LFD	P-Value	P-Value*
p_Firmicutes	43.094 ± 2.737	49.714 ± 2.778	-6.620 ± 2.500	0.0073	0.069
c_Bacilli	0.443 ± 0.062	1.232 ± 0.448	-0.790 ± 0.440	0.0010	0.023
o_Lactobacillales	0.423 ± 0.061	1.164 ± 0.443	-0.740 ± 0.430	0.0023	0.032
f_Streptococcaceae	0.423 ± 0.061	1.164 ± 0.443	-0.740 ± 0.430	0.0023	0.032
g_Lactococcus	0.007 ± 0.003	0.005 ± 0.002	0.002 ± 0.003		
g_Streptococcus	0.416 ± 0.060	1.158 ± 0.443	-0.740 ± 0.430	0.0021	0.033
o_Turicibacterales	0.020 ± 0.005	0.068 ± 0.039	-0.049 ± 0.038		
c_Clostridia	41.301 ± 2.479	47.215 ± 2.470	-5.860 ± 2.220	0.0049	0.054
o_Clostridailes	41.301 ± 2.479	47.215 ± 2.470	-5.860 ± 2.220	0.0049	0.054
f_[Mogibacteriaceae]	0.063 ± 0.011	0.061 ± 0.016	0.002 ± 0.010		
f_Christensenellaceae	0.236 ± 0.119	0.180 ± 0.071	0.056 ± 0.109		
f_Clostridiaceae	0.793 ± 0.090	0.949 ± 0.166	-0.156 ± 0.161		
g_Clostridium	0.514 ± 0.079	0.411 ± 0.086	0.100 ± 0.080	0.0197	0.147
g_SMB53	0.190 ± 0.035	0.382 ± 0.095	-0.193 ± 0.092		
f_Lachnospiraceae	21.166 ± 1.329	24.579 ± 1.857	-3.410 ± 1.530	0.0197	0.147
g_[Ruminococcus]	0.410 ± 0.055	0.985 ± 0.295	-0.570 ± 0.250	<0.0001	0.007
g_Anaerostipes	0.205 ± 0.040	0.385 ± 0.147	-0.179 ± 0.130		
g_Blautia	2.401 ± 0.311	3.818 ± 0.637	-1.420 ± 0.500	0.0001	0.007
s_producta	0.364 ± 0.059	0.553 ± 0.103	-0.190 ± 0.070	0.0053	0.054
g_Coprococcus	0.012 ± 0.004	0.014 ± 0.003	-0.001 ± 0.003		
s_eutactus	0.005 ± 0.001	0.007 ± 0.002	-0.002 ± 0.002		
g_Dorea	0.145 ± 0.049	0.182 ± 0.058	-0.040 ± 0.020	0.0040	0.047
s_formicigenerans	0.130 ± 0.046	0.160 ± 0.050	-0.030 ± 0.014	0.0032	0.041
g_Lachnobacterium	0.029 ± 0.005	0.043 ± 0.008	-0.014 ± 0.007		
g_Lachnospira	1.650 ± 0.315	0.983 ± 0.112	0.667 ± 0.257	0.0386	0.274
g_Pseudobutyrivibrio	2.389 ± 0.467	2.774 ± 0.605	-0.384 ± 0.343		
g_Roseburia	1.514 ± 0.204	2.275 ± 0.331	-0.761 ± 0.334	0.0500	0.338
g_Shuttleworthia	0.017 ± 0.004	0.017 ± 0.004	0.000 ± 0.002		
f_Peptococcaceae	0.019 ± 0.006	0.011 ± 0.004	0.009 ± 0.005	0.0181	0.151
g_rc4-4	0.012 ± 0.006	0.003 ± 0.002	0.010 ± 0.004	0.0020	0.036
f_Ruminococcaceae	12.313 ± 1.248	12.916 ± 1.271	-0.604 ± 0.975		
g_Anaerotruncus	0.010 ± 0.006	0.020 ± 0.012	-0.011 ± 0.006		
g_Faecalibacterium	2.410 ± 0.237	2.378 ± 0.223	0.032 ± 0.225		
s_prausnitzii	2.359 ± 0.232	2.342 ± 0.218	0.016 ± 0.222		
g_Oscillospira	3.868 ± 0.402	4.847 ± 0.680	-0.979 ± 0.456	0.0184	0.145
g_Ruminococcus	3.496 ± 0.959	3.256 ± 0.812	0.240 ± 0.567		
s_bromii	0.662 ± 0.162	0.564 ± 0.144	0.099 ± 0.143		
s_callidus	0.082 ± 0.030	0.068 ± 0.029	0.013 ± 0.040		
s_flavefaciens	0.017 ± 0.011	0.020 ± 0.015	-0.004 ± 0.005		
f_Veillonellaceae	3.943 ± 0.502	4.815 ± 0.647	-0.873 ± 0.464		
g_Acidaminococcus	0.106 ± 0.084	0.121 ± 0.069	-0.015 ± 0.047		
g_Dialister	2.026 ± 0.530	2.446 ± 0.609	-0.420 ± 0.423		
g_Megamonas	0.102 ± 0.082	0.084 ± 0.056	0.018 ± 0.046		
g_Megasphaera	0.028 ± 0.024	0.122 ± 0.089	-0.095 ± 0.077		
g_Phascolarctobacterium	1.616 ± 0.412	1.908 ± 0.559	-0.292 ± 0.220		
g_Succiniclasticum	0.023 ± 0.023	0.002 ± 0.002	0.021 ± 0.021		
g_Veillonella	0.042 ± 0.008	0.132 ± 0.037	-0.089 ± 0.033	0.0003	0.011
o_SHA-98	0.009 ± 0.003	0.004 ± 0.002	0.005 ± 0.003		
c_Erysipelotrichi	1.285 ± 0.648	1.209 ± 0.360	0.076 ± 0.346		
o_Erysipelotrichales	1.285 ± 0.648	1.209 ± 0.360	0.076 ± 0.346		
f_Erysipelotrichaceae	1.285 ± 0.648	1.209 ± 0.360	0.076 ± 0.346		
g_[Eubacterium]	0.647 ± 0.558	0.385 ± 0.275	0.262 ± 0.286		
s_biforme	0.628 ± 0.559	0.332 ± 0.275	0.296 ± 0.284		
s_dolichum	0.013 ± 0.004	0.049 ± 0.026	-0.036 ± 0.022	0.0020	0.036
g_Catenibacterium	0.036 ± 0.019	0.141 ± 0.100	-0.105 ± 0.083		
g_Holdemania	0.014 ± 0.003	0.019 ± 0.006	-0.005 ± 0.005		

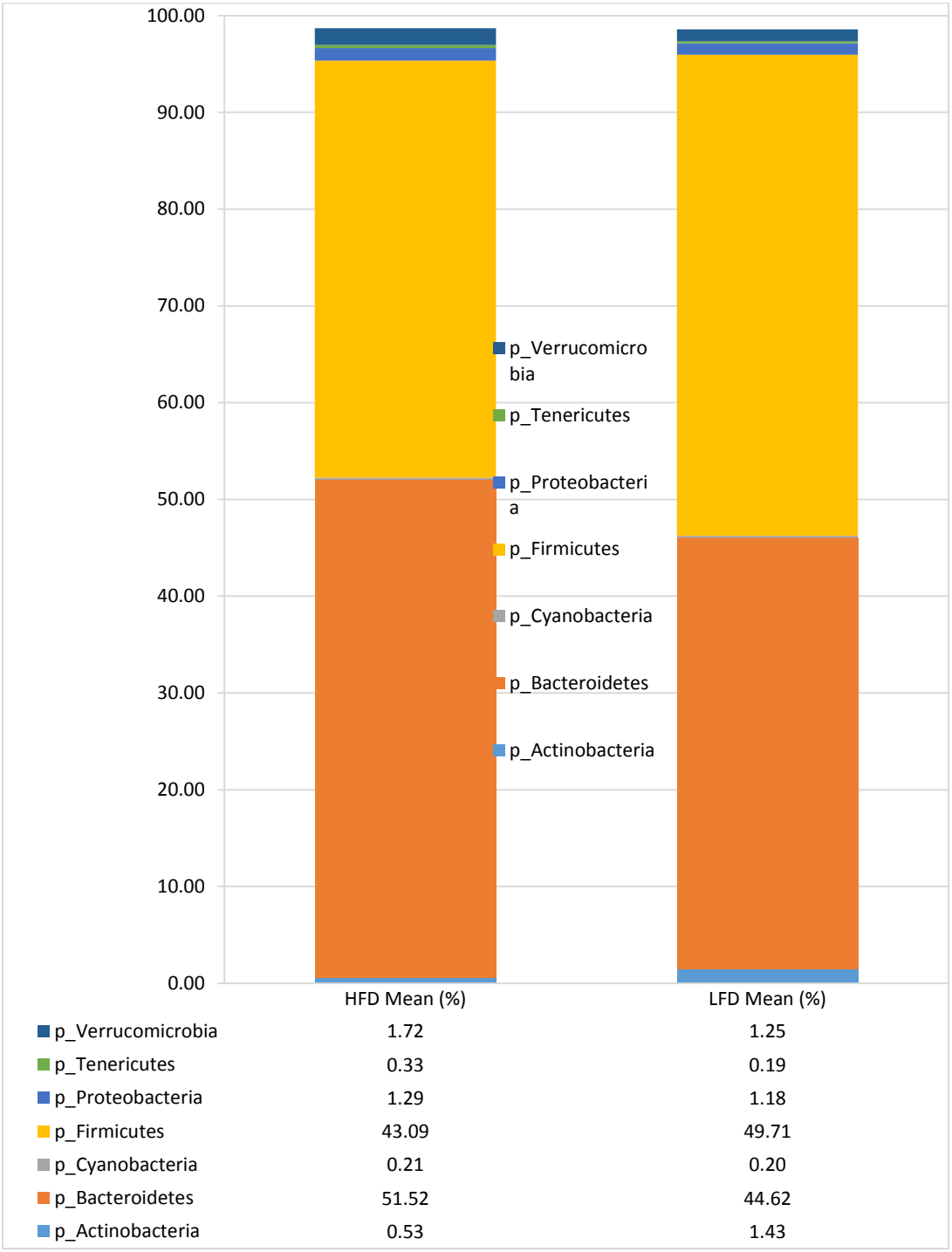
Significance determined through Wilcoxon Signed-Rank Test.
Data are Mean ± SEM.
n=29 (HFD; LFD)
P-Value*=Adj. P-Value for False Discovery Rate

TABLE 9 Cont. Effect of High Flavonoid Diet on gut microbiota percent abundance

Taxa	HFD	LFD	HFD-LFD	P-Value	P-Value*
p_Proteobacteria	1.289 ± 0.441	1.181 ± 0.267	0.108 ± 0.224		
c_Alphaproteobacteria	0.183 ± 0.052	0.157 ± 0.059	0.026 ± 0.040		
c_Betaproteobacteria	0.429 ± 0.076	0.504 ± 0.097	-0.074 ± 0.087		
o_Burkholderiales	0.429 ± 0.076	0.504 ± 0.097	-0.074 ± 0.087		
f_Alcaligenaceae	0.429 ± 0.076	0.504 ± 0.097	-0.074 ± 0.087		
g_Sutterella	0.429 ± 0.076	0.504 ± 0.097	-0.074 ± 0.087		
c_Deltaproteobacteria	0.178 ± 0.067	0.125 ± 0.035	0.053 ± 0.050		
o_Desulfovibrionales	0.178 ± 0.067	0.125 ± 0.035	0.053 ± 0.050		
f_Desulfovibrionaceae	0.178 ± 0.067	0.125 ± 0.035	0.053 ± 0.050		
g_Bilophila	0.016 ± 0.003	0.020 ± 0.006	-0.005 ± 0.006		
g_Desulfovibrio	0.163 ± 0.066	0.105 ± 0.035	0.058 ± 0.052		
c_Gammaproteobacteria	0.498 ± 0.426	0.395 ± 0.255	0.103 ± 0.182		
o_Enterobacteriales	0.484 ± 0.426	0.347 ± 0.255	0.137 ± 0.180		
f_Enterobacteriaceae	0.484 ± 0.426	0.347 ± 0.255	0.137 ± 0.180		
o_Pasteurellales	0.014 ± 0.005	0.048 ± 0.017	-0.034 ± 0.015		
f_Pasteurellaceae	0.014 ± 0.005	0.048 ± 0.017	-0.034 ± 0.015		
g_Haemophilus	0.014 ± 0.005	0.048 ± 0.017	-0.034 ± 0.015		
s_parainfluenzae	0.014 ± 0.005	0.048 ± 0.017	-0.034 ± 0.015		
p_Tenericutes	0.334 ± 0.206	0.192 ± 0.106	0.141 ± 0.120		
c_Mollicutes	0.318 ± 0.205	0.185 ± 0.105	0.134 ± 0.120		
o_Anaeroplasmatales	0.068 ± 0.068	0.070 ± 0.070	-0.003 ± 0.003		
f_Anaeroplasmataceae	0.068 ± 0.068	0.070 ± 0.070	-0.003 ± 0.003		
g_Anaeroplasmataceae_unk	0.068 ± 0.068	0.070 ± 0.070	-0.003 ± 0.003		
o_RF39	0.250 ± 0.196	0.114 ± 0.079	-0.003 ± 0.003		
c_RF3	0.016 ± 0.007	0.008 ± 0.004	0.136 ± 0.119		
o_ML615J-28	0.016 ± 0.007	0.008 ± 0.004	0.136 ± 0.119		
p_Verrucomicrobia	1.720 ± 0.391	1.249 ± 0.387	0.407 ± 0.301		
c_Opitutae	0.014 ± 0.009	0.006 ± 0.003	0.009 ± 0.007		
c_Verrucomicrobiae	1.706 ± 0.391	1.244 ± 0.387	0.462 ± 0.299		
o_Verrucomicrobiales	1.706 ± 0.391	1.244 ± 0.387	0.462 ± 0.299		
f_Verrucomicrobiaceae	1.706 ± 0.391	1.244 ± 0.387	0.462 ± 0.299		
g_Akkermansia	1.706 ± 0.391	1.244 ± 0.387	0.462 ± 0.299		
s_muciniphila	1.706 ± 0.391	1.244 ± 0.387	0.462 ± 0.299		

Significance determined through Wilcoxon Signed-Rank Test.
Data are Mean±SEM.
n=29 (HFD; LFD)
P-Value*=Adj. P-Value for False Discovery Rate

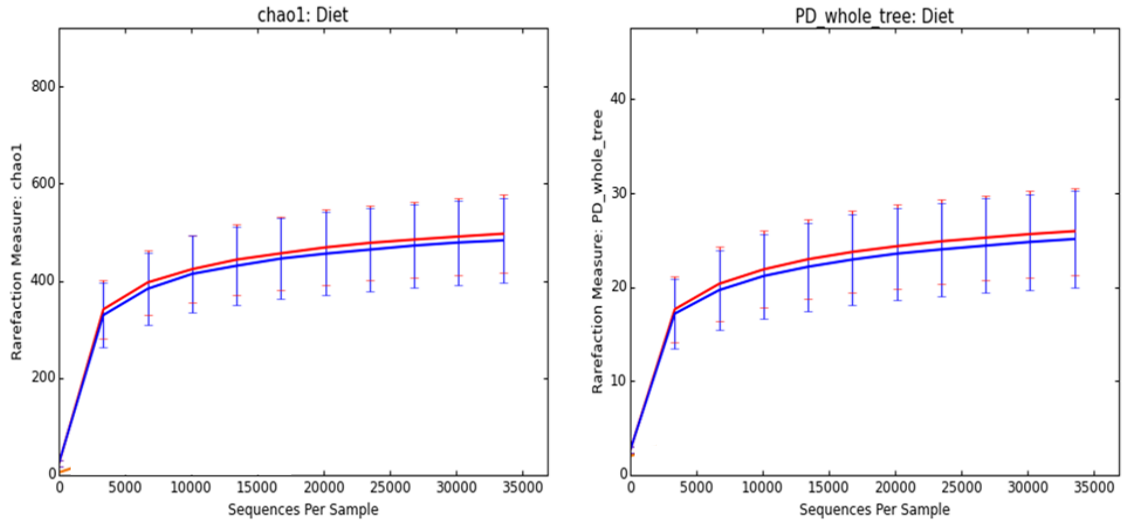
Fig. 2 Phyla percent abundance by diet



	HFD Mean (%)	LFD Mean (%)
p_Verrucomicrobia	1.72	1.25
p_Tenericutes	0.33	0.19
p_Proteobacteria	1.29	1.18
p_Firmicutes	43.09	49.71
p_Cyanobacteria	0.21	0.20
p_Bacteroidetes	51.52	44.62
p_Actinobacteria	0.53	1.43

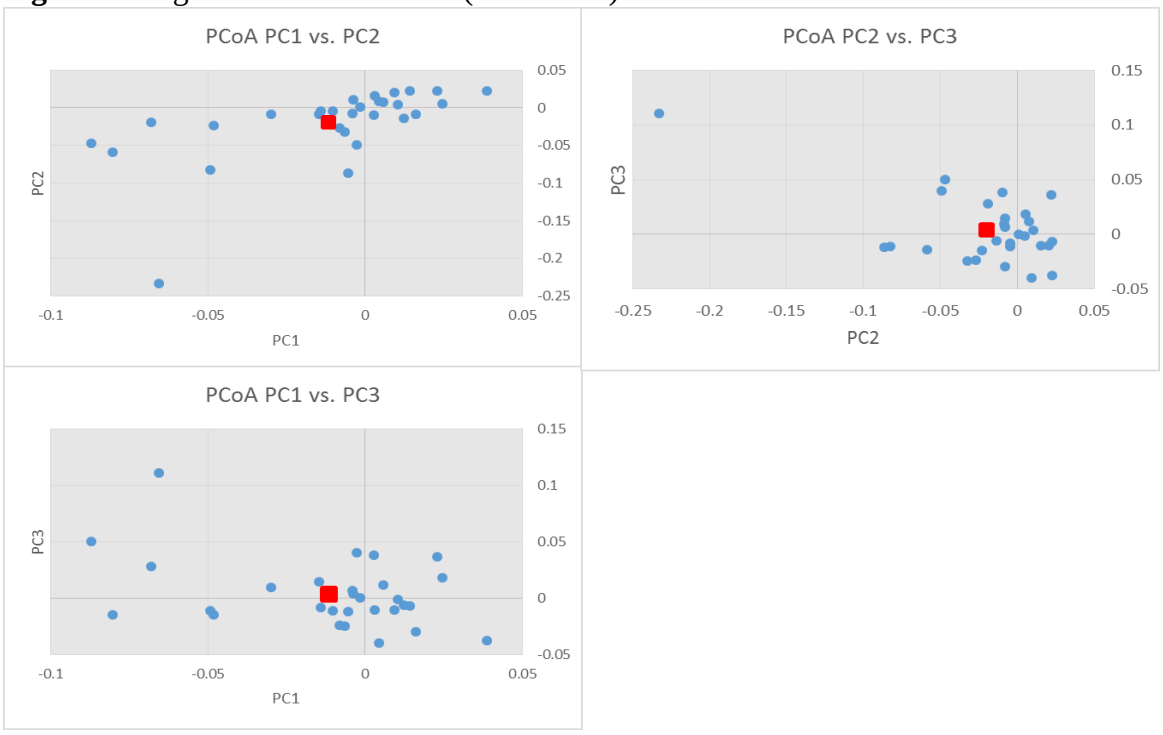
Values are mean percent abundance for all participants (n=29)

Fig 3. Alpha diversity measures (HFD; LFD)



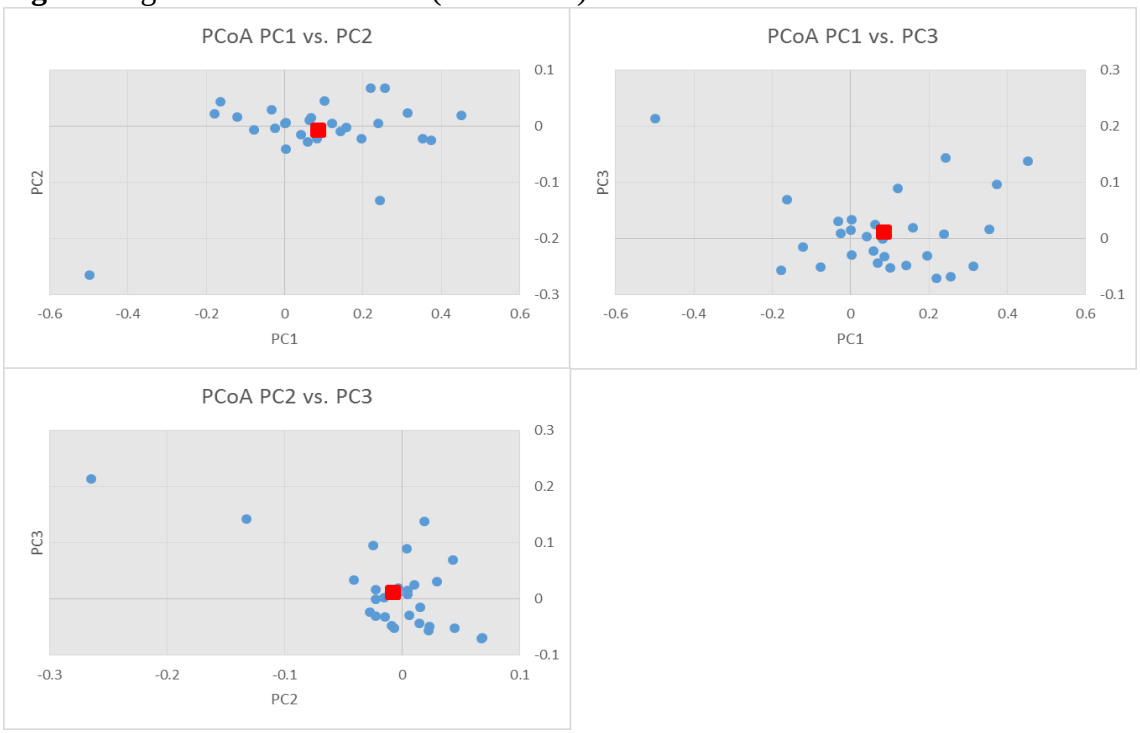
Error bars correspond to 95% confidence intervals
Blue=LFD (Low Flavonoid Diet)
Red=HFD (High Flavonoid Diet)

Fig. 4 Unweighted unifrac distance (HFD-LFD)



PCoA is Principal Coordinate Analysis; PC is Principal Coordinate; Blue dots represent the unweighted Unifrac Distance for the High Flavonoid Diet (HFD) minus the Low Flavonoid Diet (LFD); Red square corresponds to the mean change for all participants

Fig. 5 Weighted unifrac distance (HFD-LFD)



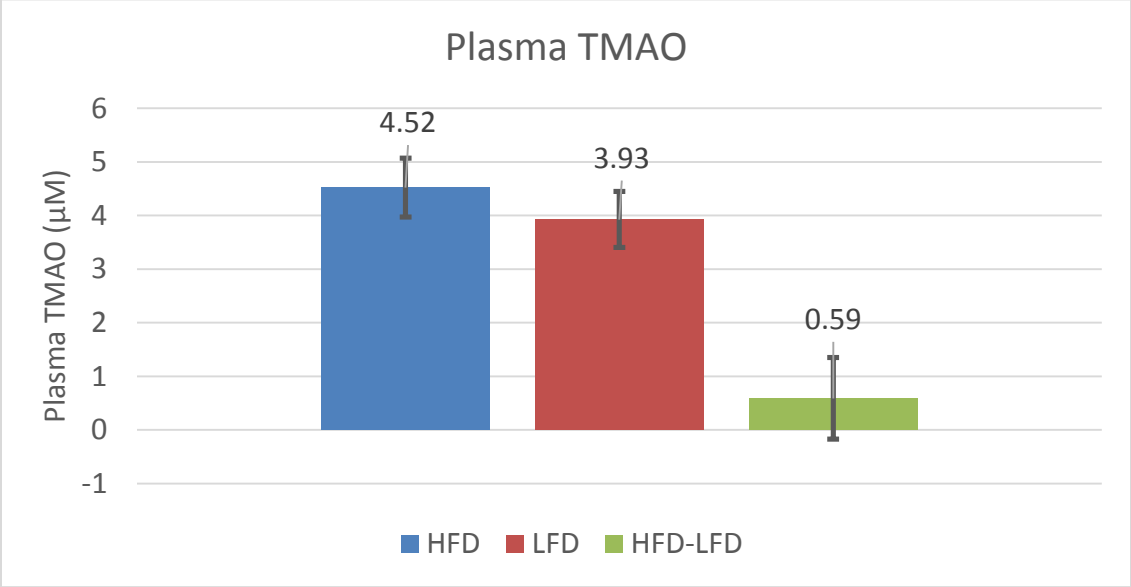
PCoA is Principal Coordinate Analysis; PC is Principal Coordinate; Blue dots represent the weighted Unifrac Distance for the High Flavonoid Diet (HFD) minus the Low Flavonoid Diet (LFD); Red square corresponds to the mean for all participants

TMAO analysis

There were no significant differences in plasma TMAO concentrations between the HFD and LFD (**Fig. 6**). The HFD resulted in an average plasma TMAO concentration of 4.52 ± 0.55 µM while the LFD resulted in an average of 3.93 ± 0.52 µM. The mean difference was 0.59 ± 0.76 (p=0.074).

Post-hoc power calculations showed that the minimum detectable difference in plasma TMAO was 0.394 µM given the sample size (n=29), alpha (α=0.05), power (80%), and variance for the difference between the HFD and LFD.

Fig. 6 Plasma TMAO concentrations (HFD; LFD; HFD-LFD)

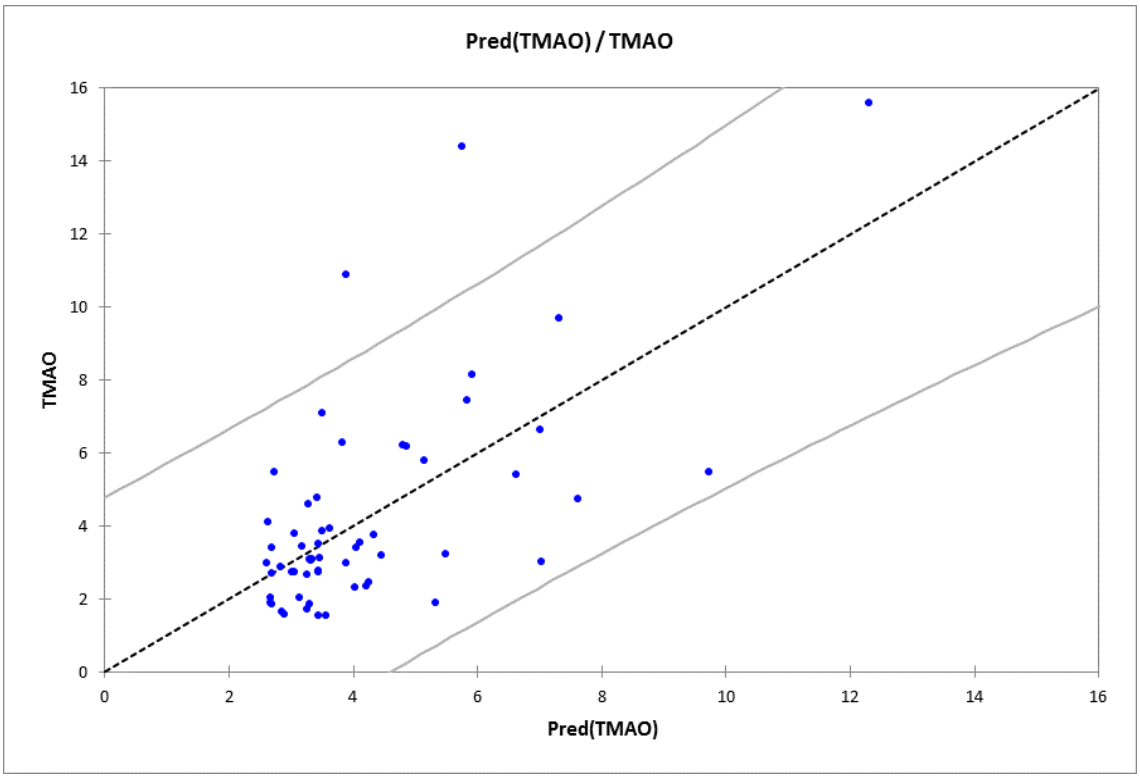


Significance determined through Wilcoxon Signed-Rank Test.
Data are Mean ± SEM.
n=29
HFD; High Flavonoid Diet
LFD; Low Flavonoid Diet

TMAO and gut microbiota analysis

Through linear regression modeling of TMAO and gut microbiota composition for both the HFD and LFD it was determined that the plasma TMAO concentrations were best predicted by the class Deltaproteobacteria, the genus *Pseudobutyrivibrio*, and the family Christensenellaceae. The percentage of variance in the plasma TMAO concentrations that was explained by the model was 37.7% (**Fig. 7**). The equation for the model of TMAO concentration as predicted by bacterial taxonomies showed that increases in abundance of f_Christenellaceae, g_Pseudobutyrivibrio, and c_Deltaproteobacteria were associated with increases in plasma TMAO concentrations (**TABLE 10**).

Fig. 7 Predicted plasma TMAO concentrations vs. observed plasma TMAO concentrations



Blue dots are observed TMAO vs. predicted TMAO for study participants (n=29).
Dashed line represents the fitted linear regression model.
Solid gray lines represent 95% confidence interval.
Values are µM.

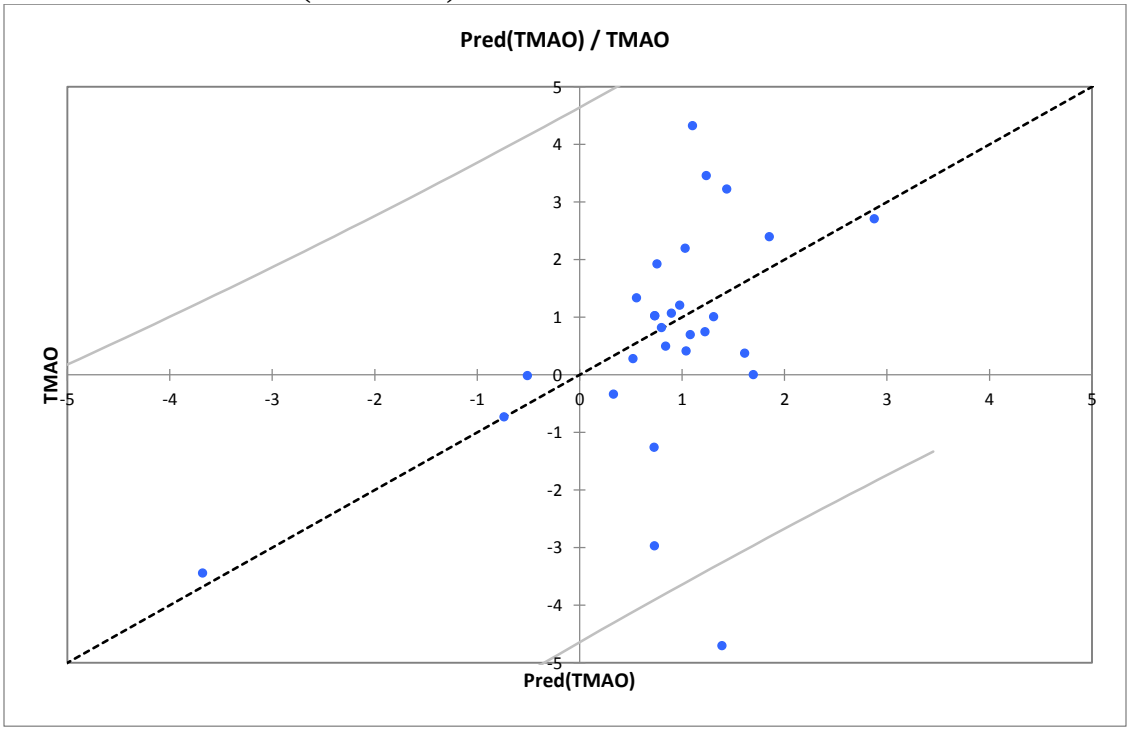
TABLE 10 Model parameters (TMAO and gut microbiota; HFD & LFD)

Source	Value	Standard error	t	Pr > |t|	Lower bound (95%)	Upper bound (95%)
Intercept	2.566	0.476	5.388	<0.0001	1.611	3.521
f_Christensenellaceae	2.524	0.601	4.201	0.000	1.320	3.729
g_Pseudobutyrivibrio	0.248	0.109	2.274	0.027	0.029	0.467
c_Deltaproteobacteria	3.237	1.153	2.808	0.007	0.926	5.547

HFD, High Flavonoid Diet
LFD, Low Flavonoid Diet

Through linear regression modeling of changes in TMAO concentration (HFD-LFD) and changes in gut microbiota composition (HFD-LFD) it was determined that changes in c_Bacilli, c_Erysipelotrichi, and f_Streptococcaceae were most significantly correlated with changes in plasma TMAO concentrations. The percentage of variance in changes in TMAO concentrations explained by the model was 29.5% (**Fig. 8**). The equation for the model showed that increases in the abundances of c_Bacilli were associated with increases in plasma TMAO while increases in c_Erysipelotrichi and f_Streptococcaceae were associated with decreases in plasma TMAO (**TABLE 11**).

Fig. 8 Predicted change in plasma TMAO concentrations vs. observed change in plasma TMAO concentrations (HFD-LFD)



Blue dots are observed TMAO vs. predicted TMAO for study participants (n=29).
Dashed line represents the fitted linear regression model.
Solid gray lines represent 95% confidence interval.
Values are µM.

TABLE 11 Model parameters (TMAO and gut microbiota; HFD-LFD)

Source	Value	Standard error	t	Pr > |t|	Lower bound (95%)	Upper bound (95%)
Intercept	1.025	0.444	2.309	0.029	0.111	1.939
c_Bacilli	4.490	1.854	2.421	0.023	0.663	8.317
c_Erysipelotrichi	-0.495	0.161	-3.085	0.005	-0.827	-0.164
f_Streptococcaceae	-4.283	1.852	-2.312	0.030	-8.106	-0.460

HFD, High Flavonoid Diet
LFD, Low Flavonoid Diet

PICRUSt metagenome function

PICRUSt is a program that infers the estimation of metabolic capabilities of the gut microbiome based on microbiome composition. The cutC gene group was not able to be analyzed during the PICRUSt analysis due to the lack of specific characterization of this gene cluster. Comparison of the PICRUSt Metagenome analysis between the HFD and LFD showed that there was significantly lower functional gene expression for Nucleotide Metabolism, Lipid Metabolism, Amino Acid Metabolism, Carbohydrate Metabolism, Butanoate Metabolism, and Type II Diabetes in the HFD as compared to the LFD (**TABLE 12**).

TABLE 12 Effect of high flavonoid diet on metagenome function

Functional Gene Group	**HFD Mean**	**LFD Mean**	**(HFD-LFD)**	**p**
Nucleotide Metabolism	14711±959	16144±967	-1433±1362	0.028
Lipid Metabolism	43614±2020	47914±2415	-4300±3148	0.006
Amino Acid Metabolism	63107±2570	69144±3485	-6037±4330	0.02
Carbohydrate Metabolism	66220±3366	70695±3573	-4475±4909	0.02
Butanoate Metabolism	185591±7847	199184±8323	-13593±11439	0.014
Type II Diabetes	15809±635	16819±643	-1010±904	0.015

Values are relative gene abundance.
Significance determined through Wilcoxon Signed-Rank Test.
Data are Mean ± SEM.
n=29
HFD; High Flavonoid Diet
LFD; Low Flavonoid Diet

DISCUSSION

Traditional CVD risk factors

This study showed that there were no significant differences in blood pressure, plasma lipids, or CRP between the HFD and LFD. Epidemiological studies have suggested that flavonoids may exert a protective effect against CVD, specifically cardiovascular events such as myocardial infarction, stroke, and death from cardiovascular causes [5-6]. The results contradict several studies that have sought to determine the effects of flavonoids on traditional CVD risk factors. Studies have shown that flavonoid rich diets and flavonoid diet interventions were effective in reducing systolic and diastolic blood pressure [7-9]. Similar beneficial results have also been demonstrated for plasma lipids and plasma CRP [10-17]. More recently, several studies have demonstrated that dietary flavonoids may not produce the beneficial effects on traditional CVD risk factors that earlier studies have posited as the reason for decreased cardiovascular events in epidemiological studies [18-20]. The results of this study reinforce the position that dietary flavonoids do not exert beneficial effects on traditional CVD risk factors and suggest that other mechanisms, such as affecting plasma TMAO concentrations may play a role in the reported beneficial effects of flavonoids on the incidence of CVD [21-24]. Since the production of TMAO is largely dependent on the activity of several phyla of bacteria, there may be a relationship between gut microbiota, plasma TMAO, and CVD [3, 21, 25-28, 34].

Microbiota analysis

The results of the microbiota analysis are consistent with the findings of other studies in regards to the effects of flavonoids on alterations to the gut microbiota [13, 30, 32-33]. Many studies have shown that flavonoids result in a reduction of members in the Firmicutes phylum and increases in members of the Bacteroidetes phylum. The results of this study reinforced the findings of previous studies due to the significant increases in Bacteroidetes and significant decreases in Firmicutes that were observed. It has also been reported that increases in Bacteroidetes and decreases in Firmicutes are associated with lower BMI and weight loss [40-41]. The effects of shifts in microbiota composition on weight change could not be determined from this study due to the fact that caloric intake was adjusted for participants in order to prevent weight changes. Studies have shown that flavonoid consumption can result in the increased abundance of members of the *Bifidobacterium* genus [13, 17, 32], however, the results from this study indicated that *Bifidobacterium* genus had a significant decrease in response to the dietary intervention.

The microbiota analysis demonstrated that there were significant decreases in the percentage abundance across two bacterial phyla which have been implicated in the production of TMAO (Actinobacteria and Firmicutes) [34]. These results are of particular interest due to the fact that these phyla have been shown to contain the Cut gene cluster, specifically CutC, which is essential for the initial cleavage of the C-N bond of choline, resulting in the formation of TMA and acetaldehyde [34]. The phylum Proteobacteria has also been implicated in the production of TMA from dietary choline, however there was not a significant difference observed for this phylum between the HFD and LFD [34].

The class Gammaproteobacteria has been shown to contain the CutC gene cluster and has been implicated in the production of TMAO [34]. There was an increase observed in the percentage abundance of Gammaproteobacteria in the HFD vs. LFD of 0.103±0.182%, however it did not reach significance ($p=0.08$). The class Deltaproteobacteria has also been shown to have the ability to produce TMA from choline, however, there were no significant changes in the percent abundance for this class between the HFD and LFD. The phylum Bacteroidetes showed significant increases in response to the dietary interventions. This is of potential significance due to the fact that the Bacteroidetes phylum has not been shown to contain genes necessary for the metabolism of choline and subsequent formation of TMA [34].

The significant increase observed in the percent abundance of c_Chloroplast between the HFD and LFD is likely the result of an increase in the consumption of high flavonoid containing plant foods.

The results from alpha diversity measures showed that there were no significant differences between the HFD and LFD for chao1 or PD_whole_tree estimates. These results suggest that there was no significant difference in total species richness (chao1) between the HFD and LFD. The results from the beta diversity analysis showed that there were no significant shifts in the principal coordinates (PC1; PC3) for the unweighted Unifrac distances, however there was a significant shift in the principal coordinate PC2 for the unweighted Unifrac distance. These results indicate there was a change in the appearance/disappearance of a common cluster of OTUs across the study participants as a result of the HFD. The results of the weighted unifrac distance showed that there were no

significant shifts in the principal coordinates (PC2; PC3), however there was a significant shift in PC1. Weighted unifrac distance takes into account the relative abundance of different taxa within the gut microbiome. These results indicate there was a change in the relative abundance of a cluster of OTUs across the study participants as a result of the HFD. The results of the beta diversity analysis demonstrated there was a common shift in a cluster of bacteria across study participants, specifically the cluster of bacteria that were represented by the principal coordinate PC2 for unweighted unifrac distance and the principal coordinate PC1 for the weighted unifrac distance.

TMAO analysis

The results of the TMAO analysis demonstrated that there were no significant differences in the plasma TMAO concentrations of participants between the HFD and LFD. A post-hoc power analysis determined that the minimum detectable difference in plasma TMAO at 80% power was 0.394 µM. This contrasts with our observed difference of 0.590 µM. Following linear regression model selection, it was determined that the model of best fit for the prediction of plasma TMAO concentration from percent abundance of gut microbiota was TMAO = 2.566 + 2.524 * f_Christensenellaceae + 0.248 * g_Pseudobutyrivibrio + 3.237 * c_Deltaproteobacteria. This model was selected based upon various 'Goodness of Fit' criteria such as adjusted R^2 (0.377), Akaike's Information Criterion AIC (99.174), and Schwarz Bayesian Criterion SBC (107.416). Based upon the selected model, for each percentage increase in the relative abundance of the family Christensenellaceae, there was a corresponding increase of 2.524 µM TMAO.

For the class Deltaproteobacteria, there was a corresponding increase of 3.237 µM TMAO. For the genus *Pseudobutyrivibrio*, there was a corresponding increase of 0.248 µM. These results in the correlation between microbiota population and TMA production are consistent with other research due to the fact that the family Chirstensenellaceae and genus *Pseudobutyrivibrio* are members of the class Clostridia which has been implicated in the production of TMA from choline [34]. The class Deltaproteobacteria has also been implicated in the production of TMA from choline due to the fact that members of this class were among the first to be identified as containing the CutC gene that has been demonstrated as being necessary for the cleavage of the C-N bond of choline which results in the formation of TMA [34]. The three variables selected by linear regression modeling as the most significant predictors for plasma TMAO concentration (Christensenellaceae, Pseudobutyrivibrio, and Deltaproteobacteria) showed no significant differences in percent abundance between the HFD and LFD. This fact may provide an explanation for the HFD having no effect on plasma TMAO concentrations. Based upon the prediction equation, it appears that the class Deltaproteobacteria is the strongest predictor for plasma TMAO concentrations, however, this class of bacteria does not seem to be influenced by the habitual consumption of a HFD. The adjusted R^2 of the model (0.377) suggests that the three predictor variables explain 37.7% of the variation in plasma TMAO concentrations which indicates that there may be other factors outside of the gut microbiota that significantly contribute to the circulating TMAO concentrations in individuals.

A second model showed that changes in plasma TMAO were significantly correlated with changes in c_Bacilli, c_Erysipelotrichi, and f_Streptococcaceae. The equation for the model of best fit was TMAO = 0.897+5.049*c_Bacilli 0.700*c_Erysipelotrichi -4.86*f_Streptococcaceae. The class Bacilli has previously been shown to have the ability to produce TMA from choline [34]. This model was selected based upon 'Goodness of Fit' measures such as adjusted R^2 (0.295), AIC (50.556), and SBC (57.392). These results indicate that changes in c_Bacilli were significantly correlated with increased plasma TMAO concentrations while changes in f_Streptococcaceae were significantly correlated with decreases in plasma TMAO concentrations. The adjusted R^2 indicates that this model explains 29.5% of the variance observed in changes in plasma TMAO. This could possibly be due to the fact that the gut microbiota is theorized to only play a role in the cleavage of the C-N bond of choline to form TMA [34]. In order to form TMAO, TMA must cross the intestinal border and travel to the liver where it is oxidized to TMAO by FMO3 and later contributes to the development of atherosclerosis [3, 21, 25-28, 34]. This process leaves many areas that may contribute to the concentrations of TMAO beyond the influence of the gut microbiota composition of individuals. Potential contributing factors might include the efficiency of transportation of TMA across the intestinal border as well as the activity of FMO3. Potential areas of future research could include the determination of other factors that influence the fate of TMA after it is produced by the gut microbiota from dietary choline. Also, a more in-depth classification of the CutC gene could enable a better picture of the TMA producing capabilities of different taxonomies of bacteria, as well as

the overall shift in TMA producing capabilities of the gut microbiota as a whole on a person to person basis. These areas of research would enable researchers to better understand the link between plasma TMAO, gut microbiota and cardiovascular disease.

PICRUSt analysis

The CutC homolog, which has been implicated as a necessary component for the microbial dependent conversion of choline to TMA, was not able to be characterized by the metagenome analysis conducted with the PICRUSt program. There were significant decreases in the expression of butanoate metabolism with the HFD as compared to the LFD. The reduction in gene abundances in the butanoate pathway are consistent with findings from the Flavonoid Study which showed a reduction in fecal butyric acid and total short chain fatty acids. The Clostridia class has previously been shown to contain bacteria which are able to ferment fiber to butyrate [40]. A human study also determined there are reductions in butyrate production following the addition of apple proanthocyanins [39]. These previous findings are consistent with results from the microbiota analysis which showed a significant decrease in the abundance of the class Clostridia between the HFD and LFD.

CONCLUSION

The results of this study indicate that a HFD can promote changes in the gut microbiota composition in human subjects. Specifically, a HFD promotes increases in the percent abundance of p_Actinobacteria, p_Bacteroidetes, and p_Cyanobacteria while promoting decreases in the percent abundance of p_Firmicutes. There were no significant effects observed for the phyla p_Proteobacteria, p_Tenericutes, and p_Verrucomicrobia. The results also indicated that a HFD did not promote beneficial changes in traditional risk factors for CVD such as systolic and diastolic blood pressure, lipid profile, and CRP. Also, there were no significant effects observed for changes in plasma TMAO concentrations between the HFD and LFD. Based on linear regression modeling, plasma TMAO was most highly correlated with percent abundance of c_Deltaproteobactera. Changes in plasma TMAO concentrations were most highly correlated with changes in c_Bacilli.

In conclusion, the results of this study supported the hypothesis that a HFD will promote changes in the gut microbiota of study participants. However, the results of this study did not support the hypothesis that a HFD will result in decreased plasma TMAO concentrations in study participants. It appears that the beneficial effects of a HFD, in regards to CVD risk, are not the result of promoting beneficial effects on traditional risk factors. Finally, it appears that a HFD does not significantly affect the plasma TMAO concentrations in study participants.

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