Evaluation of the Effects of Corn Flour Consumption on Cardiometabolic Outcomes and the Gut Microbiota in Adults with High Cholesterol

by

Shannon L. Wilson

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Graduate Supervisory Committee:

Corrie Whisner, Chair
Dorothy Sears
Matthew Buman
Jared Dickinson
Qiyun Zhu

ARIZONA STATE UNIVERSITY

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ABSTRACT

High fiber diets have been associated with improved cardiometabolic health with specific efforts to lower circulating levels of low-density lipoprotein (LDL cholesterol). Whole grain and grain-based foods are major contributors of dietary fiber in the American diet, of which wheat has been extensively studied. Corn, however, has not been well studied for its cholesterol-lowering properties. Further, the mechanisms by which grains improve cardiometabolic health require further exploration with regard to the human microbiome. The objective of this single-blind randomized controlled, crossover trial was to assess the impact of three different corn flours (whole grain, refined, and bran-enhanced refined flour mixture) on serum LDL cholesterol and the gut microbiota diversity and composition. Twenty-three participants were recruited, between the ages of 18-70 with hypercholesterolemia (Male = 10, Female = 13, LDL >120 mg/dL) who were not taking any cholesterol-lowering medications. Participants consumed each flour mixture for 4 weeks prepared as muffins and pita breads. At the beginning and end of each 4-week period serum for cholesterol assessment, anthropometrics, and stool samples were obtained. Serum cholesterol was assessed using a clinical analyzer. Stool samples were processed, and microbial DNA extracted and sequenced based on the 16S rRNA gene. A generalized linear model demonstrated a significant treatment effect (p=0.016) on LDL cholesterol and explained a majority of the variance (R-squared= 0.89). Post hoc tests revealed bran-enhanced refined flour had a significant effect on cholesterol in comparison to whole grain flour (p=0.001). No statistically significant differences were observed for gut microbial community composition (Jaccard and weighted Unifrac) after corn consumption. However, relative abundance analysis (LEfSE) identified *Mycobacterium celatum* (p=0.048 FDR=0.975) as a potential marker of post-corn consumption with this microbe being differentially less abundant following bran-enhanced flour treatment. These data suggest that corn flour consumption may be beneficial for individuals with hypercholesterolemia but the role of gut microbiota in this relationship requires further exploration, especially given the small sample size. Further research and analysis of a fully powered cohort is needed to more accurately describe the associations and potential mechanisms of corn-derived dietary fiber on circulating LDL cholesterol and the gut microbiota.

DEDICATION

This dissertation is dedicated to all of the family, friends, colleagues, mentors, and peers who have encouraged and helped support me throughout every step of my academic journey.

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CHAPTER 1

INTRODUCTION

Background to the Study

Approximately one out of every three adults in the United States (US) will develop cardiovascular disease (CVD) during their lifetime.[1] Since 1900, CVD has remained the highest cause of mortality in the US,[1] the only exception being in 1918 due to the Spanish Flu Pandemic. Despite great improvements to both diagnosis and treatment, CVD still accounts for approximately 1 out of every 2.7 (~37.5%) deaths.[1] This averages to a staggering death rate that translates to one death each minute in America.[1] Even once discovered and treated, CVD has a large reoccurrence rate of nearly 50% within one year of initial myocardial infarction (MI) despite revascularization. [2,3] This reoccurrence rate increases to a staggering 75% after 3 years post initial MI.[2,4] This high reoccurrence rate combined with high prevalence places a high economic burden on the country. The annual cost of CVD alone reached approximately $351.2 billion from 2014 to 2015,[5] while reoccurrence alone made up 17% of all medical expenses in the US, and nearly 30% of Medicare expenditure.[6]

Through the use of large-scale epidemiological studies, such as the Framingham Heart Study,[7] the development of CVD has been linked to several key modifiable and non-modifiable risk factors dictated by either genetics or behavior. Some risk factors, including age, sex, and family history, are non-modifiable due to the cardioprotective effects of estrogen in females[8] as well as the genetic components of CVD, which are still not well understood. Modifiable risk factors, such as smoking status, diet, sedentary behavior, hypertension, dyslipidemia, and insulin resistance can be attributed to behavioral aspects of health.[9] Due to the behavioral nature of these factors, the clustering of modifiable risk factors are common. An analysis of the National Health and Nutritional Examination Study (NHANES) demonstrates that the majority of subjects surveyed had either one or two CVD risk factors (32.8 and 27.8% of subjects, respectively).[10] Each additional risk factor adds a steep increase in relative risk (2.2 to 3.1 with an additional risk factor).[10] Cardiometabolic risk factors do not just predict the risk of developing CVD, but are also associated with a 3-fold increased risk of developing type II diabetes mellitus (T2D).[11] These

factors also contribute to adverse effects on the patient's quality of life.[12,13] While CVD mortality is decreasing due to advances in the identification and treatment of CVD, the growing prevalence of multiple risk factors have allowed CVD to remain the highest cause of mortality.[14]

The majority of research has focused on modifying these risk factors through the use of medication, diet, and exercise. Pharmaceuticals can assist in the management of some factors (e.g. insulin resistance, hypertension, and dyslipidemia), but due to the strong behavioral link in most factors, behavior change has been demonstrated to be a powerful modifier as well.[15] The Activity, Diet, and Blood Pressure Trial (ADAPT) out of Western Australia enrolled over 200 participants and randomized them to either a usual care group, or a 4-month program incorporating healthy diet (low sodium, high in fruits and vegetables and fish) and physical activity. Researchers found that post-intervention, improvements in dietary behaviors, weight, and waist circumference were maintained after a year of follow-up.[15] The Trials of Hypertension Prevention, Phase II (TOPH II) found that their subjects' weight loss was regained after 3 years, while blood pressure remained lower in comparison to the usual care group.[16]

Strong epidemiological and clinical evidence links the consumption of whole grains with a reduced risk of CVD and attenuation of subsequent risk factors.[17,18] Although the exact protective mechanism is not known, it is believed to be linked to the bioactive compounds (e.g., nutrients and phytochemicals) provided by whole grains as well as their positive effects on the gut microbiota. Whole grains are a dietary source rich in fiber, resistant starch, and oligosaccharides; all forms of fermentable carbohydrates.[19] These indigestible carbohydrates reach the large intestine and are fermented by the microbiota to short-chain fatty acids, which thereby increases the abundance of beneficial microbial species.

Short-chain fatty acids (SCFAs), such as acetate, butyrate, and propionate are essential to human health. Essential roles of SCFA include gut barrier function, glucose homeostasis, immunomodulation, and it serves as a metabolic substrate for essential processes.[20-22] Emerging research demonstrates the numerous potential direct and indirect roles the production of SCFAs may have in modulating metabolic health and CVD risk factors. These include blood pressure regulation, lipid homeostasis, and the production of trimethylamine N-oxide (TMAO), a strong

microbiome-mediated risk factor for CVD.[23-25] Whole grains provide the substrates for SCFA production and appear to be a key mediator of the beneficial effects elicited by the gut microbiome. While associations are strong, more randomized controlled trials in human subjects are needed to determine the effects of both intact and isolated fiber in whole grains on cardiometabolic health via microbial mechanisms.

Statement of the Problem and Hypotheses:

In this study, we aim to investigate the effect of varying levels of dietary fiber from a commonly utilized whole-grain (corn) on cardiometabolic health and changes within the gut microbiome in adults with elevated LDL-cholesterol. Flours evaluated include: 1) whole grain corn flour, 2) excellent fiber mixture (corn bran derived from whole cornmeal mixed with refined corn flour), and 3) refined corn flour. Specifically, we sought to address the following aims:

Aim 1: To evaluate the cardiometabolic effects of the three corn flours (primary outcome LDL-cholesterol) after 4 weeks of consumption in adult males and females with elevated cholesterol utilizing a crossover design.

Ha1: We hypothesized that the whole grain and the excellent fiber mixture would lower LDL-cholesterol in adults with already elevated cholesterol in comparison to that of the refined flour mixture.

Aim 2: To evaluate changes to the gut microbiome with the consumption of 48 g/day of each of the three corn flours for four weeks utilizing a crossover design in men and women with elevated LDL cholesterol.

Ha2: We hypothesized that whole grain and the excellent fiber mixture would positively modulate the gut microbiota (i.e., increase alpha and beta diversity as well as differential abundance) in comparison to that of the refined flour mixture.

Significance of the study:

While it is well known that dietary fiber is beneficial for human health, the complexity of fiber and its effect on health are not well understood. Adequate fiber intake is defined as 14 g per 1000 kcals consumed, an amount that is strongly supported with CVD prevention.[26] However, it is estimated that Americans consume far less than the current recommendation, only averaging one

serving (16g of a whole grain ingredient) or less per day.[27,28] Cereal fibers (fiber derived from grains) have been shown to be the most effective at reducing CVD risk.[29] Whilst reducing the risk of chronic disease, dietary fibers contain prebiotics, defined as “non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health”.[30] These cereal fibers and prebiotics are fermented in the colon, creating an energy source for certain beneficial bacteria such as *Bifidobacterium* and *Lactobacilli,* both of which have been shown to increase in number with fiber consumption.[26] Whole grain corn is one of several carbohydrate sources that have been identified to have a prebiotic effect in humans.[26] The proposed research aims to add to the growing body of literature on the effects of fiber on both the gut microbiome and cardiometabolic outcomes such as cholesterol levels. While there is an established association between dietary fiber and positive effects on the gut microbiome and cardiometabolic factors, more research needs to be conducted to explore this potential therapeutic mechanism.

CHAPTER 2

REVIEW OF LITERATURE

Cardiovascular disease (CVD) is defined as a group of disorders that affect the heart and blood vessels within the body. These disorders include hypertension (HTN), atherosclerosis, coronary artery disease (CAD), cerebrovascular disease (stroke), peripheral vascular disease (PAD), congestive heart failure (CHF), as well as cardiomyopathies.[31] CVD is the number one cause of death globally.[31] An estimated 17.3 million people died of CVD-related causes in 2008, accounting for nearly 30% of all deaths globally.[31] Of that 17.3 million, approximately 7.3 million were linked to coronary artery disease alone, while 6.2 million were due to stroke.[31] While CVD mortality has been declining over the last 50 years,[32] it has remained one of the most costly and prevalent diseases in the United States for the last century.[5,33] Research suggests that the identification and reduction of risk factors has led to a nearly 40-60% decrease in CVD-related deaths.[34] While CVD mortality has been falling over the last 35-40 years, prevalence in Western countries is still increasing.[32] Despite the increases in scientific knowledge, prevention, and medical and pharmaceutical interventions, CVD still has a large reoccurrence rate of 50% within one year, and 75% within 3 years.[2,3] This not only places a large burden on the United States health care system, but CVD reoccurrence alone accounts for approximately 17% of all health care, and 30% of Medicare expenses.[35] Early identification and attenuation of risk factors are essential in the prevention of CVD development. Optimal management usually requires the assessment and treatment of several risk factors established from evidence-based guidelines.[14]

Large-scale epidemiological studies, such as the Framingham Heart Study have heavily contributed to our working knowledge of the disease, identifying factors relating to the development of CVD.[36] These factors have a strong correlation to CVD and are known as cardiovascular risk factors. These main risk factors include family history of CVD, sex, age, smoking status, hyperlipidemia, hypertension, physical inactivity, obesity, and diabetes mellitus.[37] Research has demonstrated that >70% of cardiovascular events and 90% of new Type II diabetes (T2D) cases are strongly attributed to these factors.[38,39] While these risk factors are not directly causal for CVD, research has demonstrated their influence on other pathophysiological,

metabolic and endocrine factors that play a larger causal role. These factors are mostly derived from either a genetic or behavioral component.

Table 1. *Cardiovascular risk factors and their primary mechanisms for atherosclerosis. Modified from Scott, 2004.*[40]

Risk Factors		Primary Pathogenetic Process
Not modifiable		
	Age	
	Ethnicity	
	Male gender	
	Genetics	
Modifiable by lifestyle change		
	Western Diet	High blood cholesterol, oxidative stress
	Type II diabetes	Insulin resistance
	Smoking	Oxidative stress
	Lack of Exercise	Poor perfusion, adverse lipid profile
Modifiable by drugs		
	Dyslipidemia	Oxidative stress
	Hypertension	Oxidative stress, enhanced vasoconstriction

Pathophysiology of the disease

Atherosclerosis, the major cause of cardiovascular disease, is defined as the buildup of lipids, cells, and connective tissue matrix on the walls of the arteries in the form of plaque. These plaques develop slowly over many years, narrowing, disrupting, and eventually blocking blood flow through the artery. While originally considered a cholesterol storage disease due to its close

relationship with low-density lipoprotein (LDL) cholesterol, atherosclerosis is now defined as an inflammatory disorder.[41]

Atherosclerosis begins to develop when circulating LDL cholesterol and other nutrients are transported into the vascular intima via caveolae in the endothelium. The LDL is then retained in the intimal layer by interaction with matrix prostaglandins.[42] LDL is then oxidized by reactive oxygen species (ROS) and subsequently engorged by macrophages, leading to a state of chronic inflammation.[40] Cytokines and chemokines promote adhesion molecule expression and attract more inflammatory and immune cells such as monocytes, leukocytes, and mast cells. Smooth muscle cells are then recruited and proliferate in the intima, secreting collagen via growth factors, forming the fatty-fibrous plaque seen in atherosclerosis.

Originally thought to grow inwards, research has demonstrated that these stenotic plaques grow outward rather than inward.[43,44] This results in stenosis (or the narrowing of the arteries) becoming the “tip of the iceberg” of atherosclerosis. The atherosclerotic lesions are already diffuse and widespread by the time stenoses are produced.[45] Thus, numerous primary and secondary prevention trials have shown that aggressive management of modifiable risk factors via drug therapy or lifestyle modification significantly reduce cardiovascular events, death, and the need for revascularization.[41] These studies and prevention methods will be discussed in this review.

Genetic Components of CVD

Genetics and genetic variants have been found to play a pivotal role in the development of CVD. Lloyd-Jones and colleagues demonstrated a 3-fold increase in risk for CVD in the children of parents who suffered a heart attack at a young age.[46] Historically, most genetics-based research in this area has investigated single gene, family-based, Mendelian inheritance. This has led to further understanding of rare cardiovascular diseases such as Marfan’s disease, long-QT syndrome, and several forms of congenital heart disease,[47] all of which can likely be attributed to a single causal gene. However, more common forms of CVD (such as coronary artery disease) as well as CVD risk factors have demonstrated a much more complex level of inheritance, suggesting complex interactions between multiple genes and/or mutations. With

recent advances in technology, researchers now have the ability to sequence and examine the entire human genome as well as identify single nucleotide polymorphisms (SNPs).

Researchers are just beginning to investigate and understand the genetic architecture of more common forms of CVD as well as underlying SNPs using techniques such as genome-wide association. Based on this approach, it is now agreed upon that individual single-gene mutations play a very small role in the development of CVD, but the summation of multiple SNPs add to the total genetic risk. This summation of SNPs are not fully causal in the development of CVD, but rather play a moderate role, leading researchers to dub CVD a multifactorial disorder.[48] More common forms of CVD (namely CAD) are strongly influenced by environmental, internal, and behavioral factors. To properly characterize a clinical phenotype, these other factors have to be documented, closely monitored, and considered.[49] Because of this, genomics may be best used to predict disease, assist in the understanding of the mechanisms of CVD, as well as identify therapeutic targets rather than looking for a causal role.[49]

Behavioral Components of CVD

Unlike genetic factors, behavioral components of cardiovascular disease are modifiable. Largely modifiable through non-pharmaceutical means, behavioral components include physical activity, diet, alcohol use, smoking habits, and nicotine use. Like genetic components, these behavioral factors are not directly causal of CVD, but influence other pathophysiological and metabolic constituents that have a greater causal link.

Smoking

Smoking is one of the major causes of avoidable death worldwide and is linked to nearly a third of all CVD deaths.[50,51] Cigarette smokers have been found to carry twice the risk of all-cause mortality in comparison to western non-smokers[52]; smoking is responsible for approximately 140,000 premature deaths from CVD annually [53]. In the United States alone, it is estimated that smoking accounts for 33% of CVD-related deaths and 20% of deaths from ischemic heart disease.[54] Research has demonstrated a large dose-response relationship between smoking and CVD morbidity and mortality.[52,55] This large effect on CVD morbidity and mortality likely is due to the strong connection between smoking and several known and novel

CVD risk factors, such as insulin resistance, hyperlipidemia, and endothelial dysfunction.[55,56] Being one of the most impactful preventative factors of CVD, the risks of morbidity and mortality are compounded with the accumulation of other cardiac risk factors, most of which have a synergistic effect with smoking.[57] While smoking is a behavioral factor of CVD, it has a great effect on pathophysiological, endocrine, and other metabolic aspects of the disease.

Sedentary Behavior and Physical Inactivity

Sedentary behavior has been considered an important risk factor for cardiovascular disease and other cardiometabolic outcomes such as abnormal glucose and lipid metabolism, type II diabetes, and all-cause mortality independent of moderate-to-vigorous physical activity.[58-60] The Sedentary Behavior Research Network defines sedentary behavior as any waking behavior utilizing $\leq$1.5 metabolic equivalents (METs) in a sitting, reclined or lying position.[61] This is distinctly different from physical inactivity. Physical inactivity has been used to describe people who have not met the American College of Sports Medicine (ACSM) and the World Health Organization (WHO) recommendations of at least 150 minutes of moderate-intensity physical activity, or 75 minutes of vigorous-intensity physical activity per week.[61-65] The WHO, ACSM, and the Centers for Disease Control (CDC) define moderate-intensity physical activity as any activity with a MET value of 3.0-5.9, while vigorous-intensity physical activity requires activity above 6.0 METs.[63,66] While these may not seem like two distinct concepts, they are each thought to play a unique role in the health and cardiovascular risk of an individual. For example, an individual may wake up, go for a 30 min run in their neighborhood, then shower, drive to work, sit at a desk, drive home, and then watch TV for the remainder of the night. This individual would not be classified as physically inactive, although they still engage in large amounts of sedentary behavior throughout the day.

The independent effects of physical inactivity alone have been documented. Most observational studies have demonstrated an association between the development of CVD and physical inactivity.[67] Research has estimated that physical inactivity is likely responsible for 6% of all CVD, 7% of type 2 diabetes, 10% of breast cancer, as well as 10% of colon cancers globally.[68] In the United States, it is estimated that both CVD deaths and all-cause mortality are advanced

by 4 and 2.4 years, respectively, due to physical inactivity alone.[69] While strongly associated with an increased risk of mortality, physical inactivity and sedentary behavior are closely associated with metabolic disturbances such as impaired glucose metabolism as well as vascular consequences including endothelial-dependent vasodilation and arterial stiffness[70,71], both of which dramatically increase the risk of CVD. A longitudinal study following children and adolescents in Finland for 31 years demonstrated the effects of chronic physical inactivity on glucose metabolism throughout life.[72] Subjects who were physically active had nearly half the relative risk of impaired glucose metabolism when compared to that of physically inactive individuals (relative risk 0.47).[72]

Vascular complications are one of the most robust predictors of CVD known to researchers. The role of both endothelial dysfunction and arterial stiffness in the pathogenesis of CVD will be addressed in a subsequent section of this review. However, bedrest studies such as that published by Boyle et al. have demonstrated significant reductions in endothelial dysfunction with just an acute exposure to an inactive lifestyle.[73] Boyle and colleagues took highly active participants (those who achieved >10,000 steps per day) and decreased activity level to <5000 steps per day for a 5-day period. This acute exposure to an inactive lifestyle took flow-mediated dilation (FMD) from an average baseline value of 4.7 $\pm$ 0.98% to 1.72 $\pm$0.68% ($p<0.05$) while also increasing $CD31^{+}/CD42b^{-}$, markers of endothelial cell apoptosis.[73]

The 2018 Physical Activity Guidelines Advisory Committee published a report examining 9 meta-analyses, including 20 original research articles examining the relationship between sedentary behavior and all-cause mortality.[74] The committee was able to show a strong relationship between sedentary behavior, cardiovascular disease, and all-cause mortality.[74] While there is more and more research being done demonstrating the negative effects of sedentary behavior on health and its strong link to cardiovascular disease, the majority of individuals still spend more than half of their waking hours engaged in sedentary behavior.[75,76] Analysis by Koster and colleagues of the National Health and Nutritional Examination Survey (NHANES) showed that even after adjustment for moderate to vigorous exercise, subjects in the highest quartile of sedentary time were found to have a 3.3 times increased risk for all-cause

mortality in comparison to those in the lowest quartile.[59] This demonstrates a need for not only increasing physical activity in subjects, but also a need to reduce sedentary time in subjects.[77-80]

Diet

Diet is another important behavior to examine in patients who are at risk of cardiovascular diseases. The reduction of excess calories and improving dietary composition has been demonstrated to assist in the primary and secondary prevention of cardiovascular events.[81] For CVD in particular, the typical dietary modifications seen in the literature as are follows: reduced intake of dietary fat, increased intake of dietary fiber, increased intake of fruit and vegetables, and reduced intake of dietary sodium.[82] The connection between these dietary behaviors, obesity and CVD is highly debated in some circles, with the majority of the literature demonstrating a paradoxical effect.[83] While several structured diets have been developed and considered "heart healthy" (e.g. Dietary Approaches to Stop Hypertension diet, the American Heart Association diet, and the Mediterranean diet), specific nutrients have been the focus of the majority of research.

The human body utilizes specific nutrients and food groups for a reason. Cholesterol, for example, is needed to build cell walls (lipid bilayer) and serves as a building block for hormones and neurotransmitters, necessary to human health. However, like anything, too much of something does not necessarily have a positive impact. As previously discussed, circulating cholesterol can become trapped within the intimal layer of the vessel walls and become oxidized.[84] Macrophages attend to the site, in an attempt to eliminate excess cholesterol, engulfing it and subsequently converting to foam cells. These foam cells then form the basis of the atherosclerotic plaques. While CVD is not directly caused by an excess or deficiency of certain nutrients, they play a highly important yet complex role and interact with both genetic and behavioral factors.

Fatty Acids

Throughout the mid 20th century, it was believed (mainly due to the work of Ansel Keys) that dietary cholesterol and total fat consumption were the main determinants of serum

cholesterol, and thusly the cause of CVD.[85] Later research has demonstrated that serum cholesterol is determined more by genetics,[86] the gut microbiome,[87] and the type of fat consumed. More specifically, trans fat and saturated fat molecules containing 12-16 carbons,[85,88,89] though this is still debated in the field.[90]

Keys and colleagues were the first to demonstrate the existence of a strong relationship between diet and CVD during their seven-country study.[91] They observed a lower incidence and death rate associated with cardiovascular disease in cohorts that consumed olive oil as their main source of dietary fat, demonstrating an inverse association between monounsaturated fat consumption (MUFA) and cardiovascular disease.[84] Randomized controlled trials have demonstrated a decline in CVD incidence with the partial replacement of saturated fat (SFAs) with polyunsaturated fatty acids (PUFA).[84] However, the strength of the effect of MUFAs are still debated. Nettleton et al. provided evidence demonstrating that the replacement of SFAs with PUFAs is associated with a reduction in CVD risk and mortality.[92] They also demonstrated that replacement of SFA with either PUFA or MUFA reduced lipid markers,[92] while MUFA alone was associated with decreases in Hemoglobin A1c (HbA1c).[93] Unsaturated fat consumption has been demonstrated to decrease cardiovascular risk, but has a greater effect when combined with other nutrients such as fiber and polyphenols.[84]

Fiber and Whole Grains

Fiber is defined as a food of plant origin that is resistant to digestion by human digestive enzymes.[84] Fiber can be separated into two groups, soluble and insoluble fiber. Most soluble fiber can be readily fermented by the gut microbiota while most insoluble fiber types remain more metabolically inert, but rather play a role in modulating the composition of the gut microbiota. Several insoluble fibers are fermentable such as resistant starches found in corn, oats, green bananas and other sources. Up until the 1990's, all fiber was considered inert while transiting through the bowel until several studies were able to demonstrate a relationship between fiber intake and decreasing cholesterol. Recent metanalyses have demonstrated a dose-dependent, negative association with CVD and CVD-associated mortality.[94] An increase of 10 g/day of fiber decreased cardiovascular mortality by approximately 10%.[84] Fiber has also been proposed to

reduce blood pressure. While it does not have a direct effect on blood pressure, fiber does have an unclear mechanistic effect likely relating to decreased cholesterol levels or consequential metabolites and their effect on endothelial vasodilation.[95] Current research has demonstrated a strong association between fiber consumption and a decrease in CVD risk, however the mechanisms for how this occurs are still unclear.

Health guidelines have changed to promote the use of fermentable fiber as it has a considerable effect on the gut microbiome and metabolic health as a whole. Three main health benefits from fiber have been identified: promoting weight loss, improvement of glycemic control, and improvement of LDL cholesterol.[96] Fiber plays an essential role in human health. While unable to be digested by the upper gut, fiber is fermented and metabolized by the gut microbiome, producing short-chain fatty acids (SCFAs). The main SCFAs produced by the human microbiome are acetate, propionate, and butyrate which serve as an energy substrate and signaling metabolite.[97] SCFAs have been linked to a reduction in inflammation,[98] improvements in skeletal muscle[99] and overall insulin sensitivity,[100] as well as maintenance of the gut barrier and immune function.[101]

Whole grains, more specifically cereal grains, have been shown to be the most effective dietary source for reducing the risk of CVD.[102] This additional benefit to the fiber contained in whole grains likely comes from the accompanying vitamins, minerals, and polyphenols. A meta-analysis performed on 15 prospective cohort studies found that just 10g of whole grain fiber per day decreased the relative risk of CVD mortality to 0.91 after adjustments for age, BMI, smoking, alcohol consumption, and physical activity.[94] This association has been demonstrated thoroughly in the literature, yet very little is understood about how whole grains and dietary fiber mechanistically protect against cardiovascular disease.

Metabolic, Endocrine, and Vascular Components of Cardiovascular Disease

While the genetic and behavioral factors previously discussed play a large role in the development of CVD, these components play a role in the metabolic, endocrine, and vascular components of cardiovascular disease as well. Identified as cardiovascular risk factors from

large-scale epidemiological studies, factors such as insulin resistance, hypertension and vascular function, as well as dyslipidemia need to be reviewed as well.

Insulin resistance

Insulin resistance is seen as a molecular and genetic abnormality involving a disrupted response in both insulin signaling and glucose transport which result in elevated cardiovascular risk.[103] Insulin plays the role of both a hormone and a metabolic constituent; controlling blood glucose levels within the blood stream (along with glucagon) as well as assists in the regulation of the metabolism of macronutrients in the body. While insulin resistance is the leading cause of type II diabetes, it also has been demonstrated to be linked to both hypertriglyceridemia as well as CVD.[103] Several large prospective studies have demonstrated this strong association of insulin resistance as a strong predictor of CVD.[104-108] While some research argues that it may be an independent risk factor,[108] others argue that insulin resistance may cause a cascade of physiological mechanisms triggering the appearance of subsequent CVD risk factors such as dyslipidemia, hypertension, and endothelial dysfunction.

Insulin resistance has been shown to cause an increase in the hydrolysis of triglycerides, releasing more fatty acids into circulation.[103] This increased hydrolysis puts an increased pressure on adipose tissue to store these triglycerides. When the adipose cells can no longer meet the demand and uptake is decreased, the burden is then placed on hepatic tissue. The excess fatty acids are carried back and forth from the fat cells to the hepatic tissue until they can be up taken or metabolized, after which the cycle begins again.

Insulin resistance is not only related to dyslipidemia, but also to other CVD risk factors such as hypertension. While it is not as commonly linked to insulin resistance as dyslipidemia and CVD risk alone, research has shown an association between hypertension and insulin resistance independent of weight or body mass index (BMI).[103] This association is not nearly as strong as that of dyslipidemia; it is approximated that only about 50% of hypertensive individuals are insulin-resistant.[103] Insulin given intravenously has been shown to cause vasodilation in normal subjects, while this effect is blunted in obese, insulin-resistant, and diabetic subjects.[109] Untreated

hypertensive patient have been found to have higher postprandial insulin levels when compared to normotensive subjects, regardless of body mass.[110,111]

It has been suggested that this blunting occurs due to the failure of insulin to stimulate the secretion of nitric oxide (NO). Increasing evidence has begun to demonstrate a direct role of insulin resistance with atherogenesis. Many early prospective cohort studies showed that hyperinsulinemia was most often linked with CVD. More recently, the Insulin Resistance Atherosclerosis Study (IRAS) tested 1625 subjects over multiple sites. Insulin sensitivity was compared to intimal-medial thickness (IMT) of the carotid artery,[112] demonstrating a direct role of insulin resistance to atherosclerosis even after adjustments for subsequent risk factors. This may indicate a direct effect of insulin not only on the endothelium but the vascular smooth muscle as well.

Hypertension

Hypertension has been identified as one of the strongest risk factors for CVD.[113] Being one of the most prevalent risk factors worldwide (approximately 30-45% of the general population in Europe[114]), this prevalence increases steeply with age. In 2017, the American Heart Association and American College of Cardiology adapted the hypertension guidelines, calling for a much more aggressive treatment of hypertension in the United States.[115] The National Center for Health Statistics reported that an estimated 29% of adults in the United States were considered hypertensive in 2015-2016.[116] With stage 1 hypertension now being defined as systolic blood pressure of 130-139 mmHg or a diastolic blood pressure of 80-89 mmHg, the prevalence of hypertension in the United States has likely increased dramatically. These new guidelines call for earlier pharmaceutical treatments through the use of beta-blockers, diuretics, and angiotensin receptor blockers/antagonists (ACEs/ARBs). It also calls for lifestyle intervention (e.g. low-sodium diet and physical activity). Research has demonstrated that a 1 mmHg decrease in blood pressure lowers the long-term risk of myocardial infarction by 2-3%.[41] While prevalent, hypertension is a largely modifiable risk factor with proper treatment and patient compliance.

Dyslipidemia and the Atherogenic Effects of Cholesterol

Secondarily to hypertension, hyperlipidemia is considered one of the most common chronic conditions treated in the United States.[117] While this can be genetic, it is more commonly an acquired condition in western society.[118] Due to their main role in the atherogenic process, circulating LDL (LDL-c) remains the primary target in lipid-lowering therapy and treatment of cardiovascular risk [119]. Clinical trials have demonstrated that a 25% decrease in LDL cholesterol lowers risk of CVD mortality by 30-40%.[120] The role of LDL cholesterol in the atherogenic process has been discussed previously in this review, but several other studies not discussed have demonstrated that LDL is the most atherogenic lipoprotein.[121] This is why statin therapy is so heavily studied as well as prescribed to patients. The Cholesterol Treatment Trialists' (CTT) Collaboration provided data on 90,000 individuals in 14 separate randomized control statin trials. These studies showed that statin therapy significantly reduces the 5-year incidence of major CVD events and the need for coronary revascularization by ~20% per mmol/L reduction of LDL cholesterol.[122] The CTT collaboration also demonstrated in a later meta-analysis that further reductions of LDL cholesterol (defined as 0.51 mmol/L at 1 year compared to standard care) led to a further 15% reduction in CVD events.[123] Statin therapy and control of dyslipidemia (with LDL as a primary target) has been demonstrated to be a powerful mediator of CVD risk. Along with LDL, other targets have been identified such as high-density lipoprotein (HDL).

HDL, largely deemed the "beneficial cholesterol" has been demonstrated to have anti-atherogenic properties such as reverse cholesterol transport (RCT). This ability allows HDL to transport cholesterol back from the peripheral tissue to the liver, where it can be excreted via the biliary system. RCT also provides a protective effect, that is not quite defined yet by the research, yet some studies have suggested that HDL may improve endothelial function by increasing the production of nitric oxide as well as promoting endothelial integrity.[124-126] HDL however may play a role in the atherogenic process as it serves as a transport vehicle for several proteins due to its extremely heterogeneous structure. Both acute and chronic inflammation may cause several structural alterations (mainly to Apolipoprotein A1), which cause HDL to become pro-atherogenic, impairing HDL's ability to perform RCT.[124,127] Murine models with a predisposition for early

atherosclerosis exhibited pro-inflammatory HDL particles while those resistant to atherosclerosis acted as an anti-atherogenic compound.[128,129] This has also been demonstrated in human studies comparing individuals with known CVD to healthy controls.[130] With these beneficial effects of HDL, their association with CVD has been extensively studied.

Large epidemiological studies have demonstrated that low plasma concentrations of HDL are independently correlated with the incidence of CVD.[131,132] This remains true even in patients treated with statin therapy.[133] Subsequent risk models have shown that a 1 mg/dl increase in HDL was associated with a 2-3% lower risk of cardiovascular events.[131,132] While lowering LDL cholesterol is standardized as the primary lipid goal in clinical care, HDL is the secondary.[134,135] Both LDL and HDL cholesterol play a large role in the development and prevention of CVD which is why it is essential to provide both pharmacologic and lifestyle interventions that adjust these values accordingly.

The Gut Microbiome and Microbiota

The gut microbiome is a broad term used in reference to the trillions of microbes (bacteria, fungi, archaea, protozoa, and viruses) that live symbiotically within the human gastrointestinal tract.[136] Commonly misused in reference to the organisms that inhabit the gastrointestinal tract themselves (correctly termed, microbiota), the microbiome refers to the genomes of these microbes. These microorganisms interact with both each other and the host,[137-139] providing traits that humans have not developed on their own.[140] Shaped by both genetics and environmental factors, behavioral practices have been demonstrated to manipulate and modulate gut microbial composition as well as its functionality [141,142]. Commonly referred to as a "forgotten organ",[143] the gut microbiota plays a major role in health and disease. Involved in metabolism (e.g. metabolism and fermentation of undigestible carbohydrates) and immune function, the gut microbiota have become of large interest in the health field.

Characterization of the "healthy" microbiome in humans has been of large interest to researchers. Large-scale studies such as the Human Microbiome Project[144,145] and MetaHIT[146] have demonstrated large variability in the composition of the microbial communities of healthy humans. Research has demonstrated that monozygotic twins share less than 50% of their

species-level bacterial taxa.[145] Despite the large variability between healthy subjects, research has uncovered a shared core structure, beginning to shape the picture of a healthy human microbiome.[15,147]

The human gut microbiota is dominated by five bacterial phyla (*Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Verrucommicrobia*) and one Archaea (*Euyarchaeota*). More than 90% of species in the human gut belong to Firmicutes or Bacteroidetes phyla, hence why the balance between these phyla is of large interest. When holding diet, disease, and environment constant, time-series data demonstrate that the microbiome in humans is relatively stable.[148-151] However, animal and human studies have demonstrated a rapid shift in the microbiota within 24 hours utilizing dietary interventions.[152,153]

Largely influencing metabolism, the gut microbiota also produces metabolites that play a role in disease and health. Unable to be digested by the host alone, the gut microbiota is largely responsible for the fermentation and metabolism of fiber and resistant starches, producing essential SCFAs such as butyrate, acetate, and propionate.[21] The amount of SCFAs produced have been shown to affect the pathogenesis of a variety of diseases ranging from allergies to cancers.[21] The production of SCFAs varies greatly in humans and is dependent on such factors as diet, microbial composition, site of fermentation (proximal vs distal colon), as well as host genotype.[154] Butyrate is commonly utilized as an energy source for colonocytes (providing approximately 60-70% of their energy requirements)[155] while propionate and acetate are typically shuttled to the liver through the hepatic portal vein, where they are either stored or metabolized by the liver.[156]

Other than SCFAs, the microbes that inhabit the human digestive tract produce other metabolites that have a large effect on systemic health. The gut microbiota also produces lipopolysaccharides (LPS) which may cause inflammation in peripheral tissues.[157] Plasma levels of LPS have been found to be higher in those with type II diabetes[158] and confirmed to play a role in insulin resistance in a mouse feeding study.[159] TMAO, produced from dietary trimethylamines in the gut,[160-162] has been demonstrated to be strongly associated with CVD risk, independent of CVD risk factors.[160,161,163] A crossover trial of 52 adults demonstrated that a low carbohydrate,

high resistant starch diet significantly increased plasma TMAO and LPS levels, demonstrating that both diet and the gut microbiota have a large role in the production of these metabolites.[164]

Diets effect on the gut microbiome

The above benefits have led to a strong interest in the gut microbiota. In order to test the effects of the gut microbiota on the human body, multiple intervention methods have been developed, including dietary interventions. Long-term dietary habits have been demonstrated to have a major effect on the gut microbiota, but acute changes have been seen in the gut microbiota within just 24 hours.[142] Researchers have also demonstrated a reversal back to baseline within 48 hours of cessation of the dietary intervention.[142] Over the years, different macronutrients have been shown to elicit different responses in the gut microbiota.

Protein has a strong effect on the gut microbiota as demonstrated in multiple studies since 1977.[165] Early culture studies demonstrated lower abundance of *Bifidobacterium adolescentis* while increased abundance of *Bacteroides* and *Clostridia* were found in subjects consuming a high beef diet in comparison to a vegetarian diet.[165] With advances in measurement techniques, later research has confirmed that heavily consumed animal protein (including whey, eggs, meats, and cheeses) has been demonstrated to increase the risk of Irritable Bowel Disorder (IBD) and TMAO production.[166,167] Vegetarian sources of protein (such as pea protein) have been demonstrated to increase SCFA production when compared to animal protein consumption in Italian subject who consumed a protein rich diet.[167] While important to note that diets typically high in animal protein are also high in saturated fat, it is important to note that protein itself has a high impact on the gut microbiota as a whole.

Fats have been linked to CVD through the increased amounts of LDL and total cholesterol found in the bloodstream.[168,169] However, consumption of healthy fats, such as unsaturated fats have been demonstrated to decrease the risk of chronic disease as discussed previously.[84] Several more recent studies have shown that high-fat diets may increase the abundance of total microbes as well as *Bacteroides* specifically.[152,170-172] Research performed by Fava and colleagues demonstrated that a low-fat diet led to reductions in fasting glucose and total cholesterol as well as an increase in the abundance of *Bifidobacterium.*[172] High-saturated fat

diet increased *Faecalibacterium prausnitzzi*, specifically while a diet high in monounsaturated fat did not demonstrate any change in microbial abundance or LDL and total cholesterol.[172] While both fat and protein exhibit an effect on the gut microbiota, carbohydrates are most commonly studied macronutrient and its effect on the gut microbiota.

Carbohydrates exist in two generalized forms: digestible and undigestible carbohydrates. These so-called "digestible carbohydrates" are generalized to contain starch and sugars that can be enzymatically degraded. This generalization typically includes simple sugars such as glucose, fructose, lactose, and sucrose. A study performed by Eid and colleagues fed dates (a source of high levels of fructose, glucose and sucrose)[173] to human subjects demonstrated an increase in abundance of *Bifidobacteria* and reducing the abundance of *Bacteriodes.*[174] Lactose supplementation, however, has been demonstrated to increase fecal concentrations of SCFAs as well as decreasing abundance of *Clostridia* species, a species notably linked to Irritable Bowel Disorder (IBD).[175,176]

Undigestible carbohydrates on the other hand, are generalized to include fiber and resistant starches. These more complex carbohydrates are relatively resistant to enzymatic digestion in the intestinal tract, but rather are fermented by the microbial population, creating a food and energy source for the microbiota.[177] Some of these fibers also have a prebiotic effect in that they benefit host health and selectively stimulate the activity and growth of certain beneficial microorganisms.[178] Common prebiotic substances include inulins, whole grain wheat and barley, raw oats, and undigestible oligosaccharides including fructans, polydextrose, fructooliogosaccharides, galactooliogosaccharides, xylooligosaccharides, and arabinooligosaccharides.[179] Diets low in these substances have been shown to reduce microbial abundance while increased intake of these carbohydrates increase microbial richness in human subjects.[180,181] The majority of the existing literature suggests that these types of undigestible carbohydrates most consistently increase bifidobacterial and lactic acid bacteria (e.g. lactobacilli).[180,182-186] However, corn and its effects as a whole grain and prebiotic have not been extensively studied in the literature.

The Microbiomes impact on cholesterol

Early studies conducted with germ-free mice studies have demonstrated that the gut microbiota plays a large role in lipid modulation.[187] Fu et al. provided recent insights into the effect of the microbiota has blood lipid levels in humans.[87] This group was able to demonstrate several interesting components associating lipid metabolism with the gut microbiota. Firstly, they demonstrated an inverse correlation between microbial richness and diversity with subject Body Mass Index (BMI) as well as triglyceride (TG) levels ($p=3.8x10^{-4}$ and $p=1.37x10^{-4}$, respectively). Microbial richness and diversity were found to be positively associated with HDL ($p=8.3x10^{-4}$) while no significant correlations with LDL or Total cholesterol were shown. This suggest that the gut microbiota may play a specific role in different lipoproteins. Secondly, the authors demonstrated several bacterial associations that were predominantly linked to lipid as opposed to being shared with BMI and obesity. For example, the *Clostridiaceae/Lachnospiracease* family was specifically associated with LDL cholesterol ($p=9.1x10^{-5}$) while not detected to be linked with BMI or other lipids. The most novel finding of the study was the strong association of the *Christensenellaceae* family and *Tenericutes* phyla with lower levels of triglycerides ($p=2.1x10^{-5}$ and $p=2.7x10^{-7}$, respectively) and high levels of HDL ($p=0.0047$ and $p=0.0006$, respectively). A further analysis in the paper demonstrated that gut microbial composition (including age, sex, and previous validated genetic risk factors) significantly increased the explained variance for each BMI, TG, and HDL by ~5%. While these findings are promising, further research needs to be done to understand the underlying mechanism(s) as well to determine if these association are causal.

While Fu and colleagues' findings are not causal, previous literature can assist in understanding what aspects of the gut microbiota may play a mechanistic role in lipid levels and metabolism. Certain bacteria in the bowel produces secondary bile acids from bile salts that are secreted into the intestines. When these secondary bile acids are absorbed back into the blood stream, they may play a modulatory role in both hepatic and systemic lipid metabolism through either nuclear or G protein-coupled receptors.[188-190] Several of the associated bacteria identified by Fu et al. have been known to be involved in bile acid production and metabolism, suggesting

this may be a factor in the association.[191] The production of trimethylamine N-oxide (TMAO) may also likely play a mechanistic role. TMAO has been increasingly linked to increases in CVD risk through its disruption of RCT, its effect on sterol and cholesterol metabolism, as well as its compromising role in the composition and quantity of bile acids.[160,161,163,192] While secondarily derived in the liver, the gut microbiota is responsible for the production of TMA (precursor of TMAO) via metabolism of choline and L-carnitine.[161,162]

While there is still a large gap in the knowledge, understanding of the broad impact the gut microbiota plays in human health is essential to the development of therapeutic targets and methods. While still in its infancy, the ability to manipulate the gut microbiota for the improvement of health and the prevention of disease is a promising area and requires further research.

CHAPTER 3

METHODS

Study Participants and Recruitment

This study targeted healthy male and female adults (n=45) between the age of 18-70 years who exhibit elevated circulating low-density lipoprotein (LDL-c) without the use of lipid-lowering medication (e.g., statin therapy). Participants were recruited through the use of flyers, word of mouth, and digital advertisement (e.g., social media, university e-newsletters) within Arizona State University Downtown and Tempe campuses as well as the Maricopa Integrated Health System (MIHS) clinics utilizing physician referral. Local libraries, community centers, clinics, and primary care physician offices were utilized for recruitment as well.

Potential participants were asked to complete an anonymous, online pre-screening survey administered using the Qualtrics online platform (Salt Lake City, Utah) to determine if they met initial enrollment criteria. Participants who qualified were asked to provide contact information for research staff to schedule an in-person laboratory screening. This laboratory screening was conducted to verify the required LDL-c $\geq$ 120 mg/dL. Participants with a LDL-c>190 mg/dL were permitted to participate upon written approval from their physician.

Participants were excluded from this study for the following reasons: a) weight fluctuation > 5 lbs within the past 3 months, b) following a restrictive diet (e.g., carbohydrate restriction, veganism), c) use of dietary supplements (e.g., antioxidants, fiber, botanicals), c) allergies to dairy, egg, wheat, corn, or gluten, e) use of antibiotics within the past 2-3 months, f) use of lipid-lowering medications, g) obtain $\geq$ 30 min/d of physical activity $\geq$ 5 days/week, h) history of thyroid disorders, diabetes, heart disease, cancer, hepatitis, inflammatory conditions, and/or gastrointestinal disorders that may alter gut metabolism and function. In addition, participants were excluded if pregnant or lactating.

Study Design

This study was a randomized, single-blinded, 3x3 crossover trial (Figure 1) that took place over the course of 16 weeks (for each participant). This design allows participants to serve as their own control- participating in three, randomly assigned active interventions. Each

intervention phase lasted 4 weeks, with a 2-week wash-out period. During each intervention, participants were asked to consume two, 24 g servings (48 g/day total) of either 1) whole-grain corn flour, 2) refined corn flour, or 3) excellent fiber mixture (bran-enriched refined corn flour) sourced from whole-grain cornmeal. Participants were also asked to maintain their normal diet, substituting corn-based snacks (in the form of muffins and/or pita bread) provided by research staff for similar foods consumed. All participants were randomized immediately following the collection of demographic information, survey questions, anthropometrics, biomarkers (i.e., glucose, total cholesterol, LDL and HDL cholesterol), as well as a baseline fecal sample.

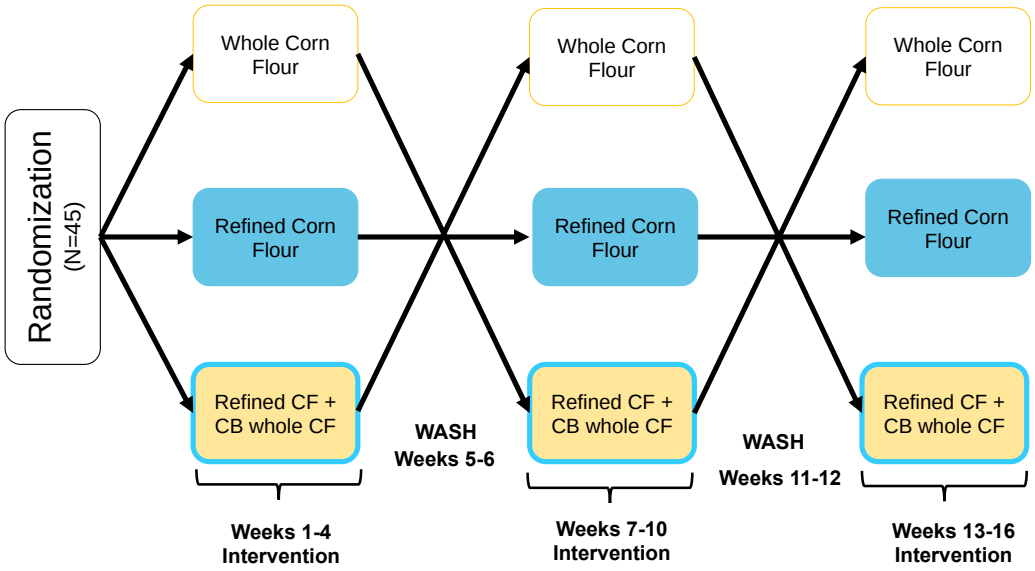


Figure 1. Schematic of study design

Study Protocol

All eligible and consented participants were subjected to all three intervention conditions in a randomized order. After completion of the laboratory screening, participants were fully consented, provided a stool sample collection kit (for baseline fecal sample prior to first laboratory visit) and scheduled for their first laboratory visit.

The first laboratory visit was composed of a fasting blood draw, baseline questionnaires, anthropometrics, and blood pressure measures. All participants presented to the laboratory in a

fasted state. A trained nurse/phlebotomist then drew approximately 7 ml of blood from the antecubital vein in the arm, which was subsequently examined for blood lipids via Randox Analyzer (Randox Laboratories Limited, Crumlin UK). A 3-day diet record (2 weekdays and one weekend day) was provided to the participant at this visit to record dietary habits prior to the intervention. Baseline questionnaires regarding gastrointestinal symptoms were provided to participants as well. Height and weight was measured via SECA scale and stadiometer (Chino, CA) and waist circumference was measured. Blood pressure was measured 3 times while the participant as seated in a quiet position. All values were recorded, and the participant was thanked for their time.

A least one day after the first laboratory visit, the participants were asked to return to lab in a fasted state for a confirmatory visit. The participant was asked to bring his/her stool sample to this visit if the sample had not been previously picked up by the research staff. This visit also contained a confirmatory blood draw (in which another biomarker panel was run to confirm elevated lipids) to assist in accounting for the day-to-day variability, which was averaged with measurements from the previous visit. A short physical activity questionnaire was completed as well. At the end of this visit, participants were provided corn-based foods prepared by the research staff. Participants were instructed to consume 2 servings (serving = one pita or one muffin) a day, at least 3 hours apart as well as fill out a weekly compliance calendar. Participants were advised to not alter their diet during the intervention periods. In order to adhere to habitual carbohydrate intake, we asked participants to substitute the corn-based products for other grain-rich products consumed in their typical diet.

Study food supplies were replenished on a weekly basis during each 4-week intervention phase via home delivery and/or participant pick-up. This was based on convenience for the participant. Each week, participants were asked to complete a weekly gastrointestinal symptom report and acceptability and satisfaction of test foods via an online survey (REDCap survey). If these had not been completed at the time of food delivery by study staff, a paper copy was provided for immediate completion before more study food was given. At the time of food drop-off, any uneaten food was returned along with a completed compliance calendar. At week 3 of

each intervention, a stool sample kit and 3-day food log were provided to the participant to collect a post-corn treatment sample towards the end of the 4th intervention week. The food logs were used to evaluate caloric, macro-and micro-nutrient consumption during the study intervention period (not presented in this dissertation).

After completion of the 4th week of the intervention, the participant was scheduled for another lab visit. As the previous lab visits entailed, this visit was comprised of a week four gastrointestinal symptom questionnaire as well as acceptability and satisfaction surveys. A blood draw (used to complete a blood lipid panel), anthropometrics, and blood pressure measurements were also completed. At least one day later, another laboratory visit was completed as a confirmatory visit. If not already picked up by research staff, the participant was asked to bring in stool samples at this time. Afterward, a blood draw, anthropometrics, and blood pressure were measured as a confirmation. After a 2-week washout period, the second and third intervention phases respectively follow the previous protocol outlined.

Food preparation

All food was prepared by research staff in the ABC1 metabolic kitchen under standardized food safety guidelines put forth by the Food Safety and Inspection Service division of the United States Department of Agriculture (USDA). The corn products produced for this study included both muffins and pitas. Both were formulated to contain 24g of carbohydrates (two daily servings provided a total of 48 g of carbohydrate) as well as the same amount of dietary fiber. The excellent fiber treatment (bran-enriched refined flour mixture) provided 12 g of fiber (6 g per serving) per day, allowing for the classification of an ‘excellent source of fiber’. Refined flour study foods provide <1 g of fiber per day, while whole grain corn flour products will provide 2 g of fiber per serving. Muffins were limited to 4 provided per week, due to the higher caloric intake (~300 kcals per muffin vs ~140 per pita).

Outcome measures

Biomarker panel

This biomarker panel was conducted using serum obtained from the blood draws conducted at each laboratory visit. All blood samples were centrifuged immediately post collection and stored at -80 degrees Celsius until analysis. Following the completion of the study, samples were run in batches, assessing LDL-c, HDL-c, triglycerides, total cholesterol, and glucose. Respective assays were run with proper reagents in accordance with manufacturer guidelines using the Randox RX Daytona+ clinical chemical analyzer (Randox Laboratories Ltd., Crumlin UK).

Microbiome Analyses.

DNA Extraction

DNeasy Powersoil Isolation Kits (Catalog No. 12888-100, QIAGEN, Germantown, MD, USA) were used to extract microbial DNA from fecal samples. These kits combine a series of salt and ethanol-based solutions as well as heating, cooling, filtering, and centrifugation methods to first decrease the amount of fecal matter in the sample then break the cell membranes of microbial cells to release the DNA. Once the DNA was isolated, it was tested using a QIAGEN spectrophotometer machine (Catalog No. 9002340, QIAGEN, Germantown, MD, USA) to test for the appropriate quality and concentration of the samples. A quality of at least 1.7 (ng/microL) and a concentration of roughly 10 (A260/A280) were considered adequate. Samples were tested for quality and concentration by putting 2 microliters of the DNA solution into a QIAxpert Slide-40 (Catalog No. 990700, QIAGEN, Germantown, MD, USA) and inserted into the QIAxpert spectrophotometer. If samples were of the appropriate quality and concentration, they were placed into the DNA box and stored at -80 degrees Celsius. If they were not, it was noted, and they were later reprocessed and extracted again.

DNA Sequencing

Samples were sequenced at The Biodesign Institute at Arizona State University Tempe Campus in the Genomics Core Lab. At the lab, sequences were quantified using Quant-iT PicoGreen/ assay (Catalog No. P7589, Invitrogen, Carlsbad, CA, USA). Sequencing methods

began with amplification through triplicate PCR in 96 well plates to distinguish the presence of archaea from the bacteria, and next-generation sequencing to identify bacterial species. This was done through amplification of the 16S rRNA gene sequence using primers for the conserved V4 region of the bacterial genome. The V4 region was identified through the use of the forward 515F primers and 806R reverse primers containing Illumina adaptor sequences.[193] Purification and quantification materials used for PCR in the Genomics Core Lab included QIAquick PCR Purification Kit (Catalog No. 28106, Qiagen, Germantown, MD, USA), and the KAPA Library Quantification Kit (Catalog No. KK4824, Kapa Biosystems, Wilmington, MA, USA). After PCR was completed, the Illumina MiSeq instrument, (Catalog No. SY-410-1003, Illumina, Inc., San Diego, CA) was used for sequencing. All protocols were completed in accordance with best practices established by the Human Microbiome Project guidelines.

Sequence Analysis

Quantitative Insights Into Microbial Ecology 2 (QIIME2) was the bioinformatics software in which statistics were performed on the sequences.[194] After sequences were demultiplexed, they were added into the QIIME2 pipeline where they were denoised by using the DADA2 command to account for inherent errors produced through sequencing. Samples were then rarified to determine a workable sequencing depth. Phylogeny and taxonomy were performed next. The FastTree command was used to analyze phylogeny of the sequences while a naive-Bayes classifier from the GreenGenes 13.8 database was used to assess taxonomy.[195,196] With sequences now categorized, diversity measures and statistics were performed to test the hypothesis.

Microbiome Analyst was also utilized to complete abundance analysis. Microbiome Analyst is an online platform and R shell capable of running complex abundance analyses, such as LEfSE.[197,198] While Microbiome Analyst is capable of running core metrics such as alpha and beta diversity measures. QIIME2 was chosen to be the platform of choice for our main analyses.

Statistical analysis

To estimate sample size, we utilized effect size estimates from previous literature[199], evaluating the effects of wheat fiber on cardiometabolic outcomes. This study suggests that we

will be able to detect a 7.8% (9.82 mg/dL) difference in LDL-c between treatments, assuming a 10% within-person standard deviation. In previous gut microbiome studies, we have been able to detect a significant treatment difference with 24-31 subjects. To address our specific aims and hypotheses, we calculated a sample size of approximately 37 subjects (power=0.9, significance level $p< 0.05$) will be needed. Accounting for a 20% dropout rate, we planned to recruit a total of 45 subjects for this study.

All participant characteristics (demographics, anthropometrics, etc.) are described through frequencies, means (SD) for normally distributed variables, and median (IQR) for non-normally distributed variables. Variables were tested for normality using the Shapiro-Wilk test and transformed as needed to assume normal distribution of model residuals in general linear models, with the exception of microbiota data. All data and statistical processing were performed using SPSS (Version 27, IBM Corp, Armonk, NY), Microbiome Analyst, and Quantitative Insights Into Microbial Ecology 2 (Version 2020.8, QIIME2; Flagstaff, AZ). Statistical significance was set a priori at $p<0.05$. Microbial models were adjusted for multiple comparisons using the Benjamini-Hochberg correction.

To address our specific aim of examining LDL cholesterol, we utilized a general linear model to test our hypothesis. This statistical model allowed for control of the sequence and phase of treatment as well as for examination of the relationship between cholesterol and other variables. Further, each participant served as their own statistical control by nesting sequence within-participant (participant(sequence)) to allow observations within subjects. Bodyweight was examined as a covariate, as we observed weight gain in several participants (<5% weight change), in an attempt to statistically control for these external factors. Final models included the following independent variables: participant, participant(sequence), sequence, period, time (pre and post measures), treatment as well as the interaction between treatment and time. Interactions only remained in the final model if they were statistically significant.

Alpha and beta diversity analysis

Several metrics of alpha diversity (Shannon diversity, Pielou's evenness, observed Taxa) were calculated in QIIME2 with the q2-diversity plugin. Differences between pre-and post-treatment diversity values were assessed for each response variable using pairwise-differences from the q2-longitudinal plugin[200] with corn treatment as a fixed effect. A separate Mann-Whitney U test was used to test alpha diversity measures between each treatment individually.

Changes in beta diversity between day pre-and post-treatment values, as measured by Bray–Curtis dissimilarity, Jaccard distances, and unweighted and weighted Unifrac, were tested using pairwise distances (Qiime2) with corn treatment as a fixed effect. Principal coordinates analysis (PCoA) was performed and visualized with Emperor plots.

Differential abundance analysis

To assess abundance difference between treatments, Linear discriminant analysis of Effect Size (LEfSE) was used. LEfSE is able to systematically identify species or taxa as a possible biomarker using class comparisons, effect size estimation and other tests of biological consistency. This method has been published in the literature and validated.[201]

We used Microbiome Analyst to perform our LEfSE analysis[197,198]. Features were filtered at a minimum count of 4 while 10% of species with the lowest variance across samples were removed. False discovery rate adjustments were made for multiple comparisons and set at 10%. Comparisons from differential abundance analyses were considered significant at $p \leq 0.05$ and $q \leq 0.1$.

CHAPTER 4

RESULTS

Participant Characteristics

A total of 23 participants were enrolled in the study (men=10, women= 13; age=35.74±15.44) that completed at least the initial baseline and one stool sample. Of that sample, 15 participants (men=7, women=8) completed all three interventions. Participants were mainly of Caucasian descent (65.2%) and overweight (mean BMI=29.95±5.17 kg/m^2). A further summation of participant characteristics is provided in **Table 2**.

Table 2.

Participant characteristics (N=23)

Variable		Mean±SD, count(%)
Age		35.74±15.44
Sex		
	Male	10 (43.4%)
	Female	13 (56.5%)
Race		
	Asian	3 (13%)
	Black or African American	2 (8.7%)
	Caucasian	15 (65.2%)
	Hispanic/Latino	4 (17.4%)
	More than one	1 (4.3%)
	Preferred not to state	2 (8.7%)
BMI (kg/m^2)		29.95±5.17
LDL		155.99±44.69
HDL		50.70±10.26
TG		108.75±13.84
TC		228.45±44.91

Note: all values are from the initial baseline visit

Cholesterol Analysis and Statistical Modeling

A generalized linear model was used to determine the strength of certain predictors on circulating cholesterol levels, specifically LDL cholesterol. Factors taken into account in each model included sequence (assigned order of treatments given), period (specific treatment phase one, two or three), treatment, and time (pre/post). A nested variable, participant(sequence), was also included to account for the crossover design. Treatment*time was an additional predictor examined in the model to test if cholesterol was affected by a combined effect of both treatment and time.

The LDL cholesterol model most notably had a significant treatment effect (p=0.016). While median LDL cholesterol for the excellent fiber mixture treatment was the lowest (136.91 mg/dL in comparison to whole and refined 145.21 and 154.47 mg/dL, respectively) mean LDL cholesterol was lowest in the whole grain flour treatment (143.46 mg/dL vs. excellent = 151.38 mg/dL and refined = 151.576 mg/dL). Bonferroni corrected post-hoc comparisons demonstrated that whole grain corn flour had a significantly greater effect on lowering LDL cholesterol when compared to both the excellent fiber flour (p=0.024) and refined flour (p=0.047). Overall, this model explained a majority of the variance (R squared= 0.894) in LDL cholesterol.

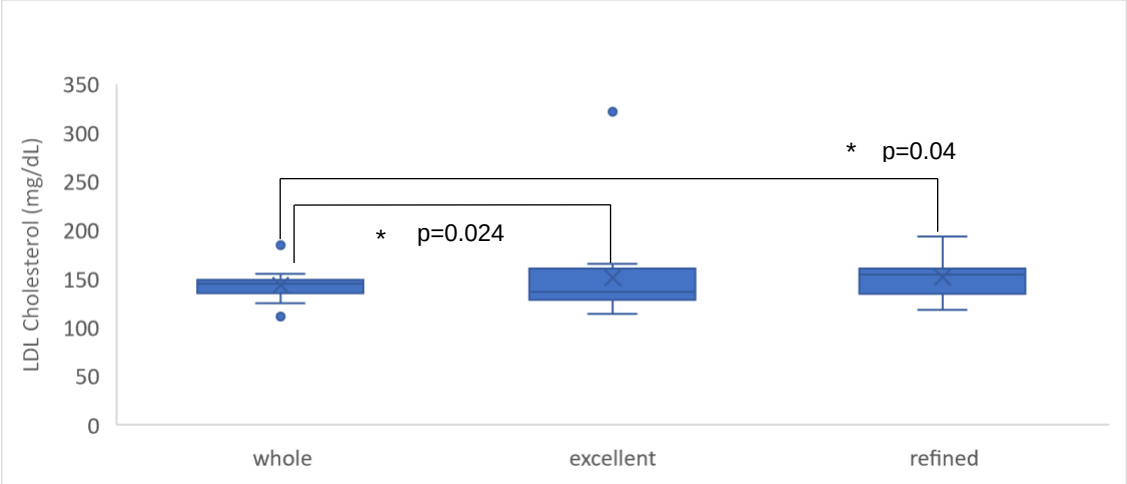
Table 3.
General Linear Model with LDL cholesterol

Source	Type III sum of Squares	Mean Square	P-value
Corrected model	88402.636	3400.101	**<0.001**
Intercept	1840861.78	1840861.78	**<0.001**
treatment*time	508.509	254.255	0.179
participant(sequence)	55985.121	3998.937	**<0.001**
Sequence	44263.704	8852.741	**<0.001**
period	606.088	303.044	0.13
Treatment	1269.421	634.711	**0.016**
time	98.18	98.18	0.412

R Squared= 0.894

Figure 2.

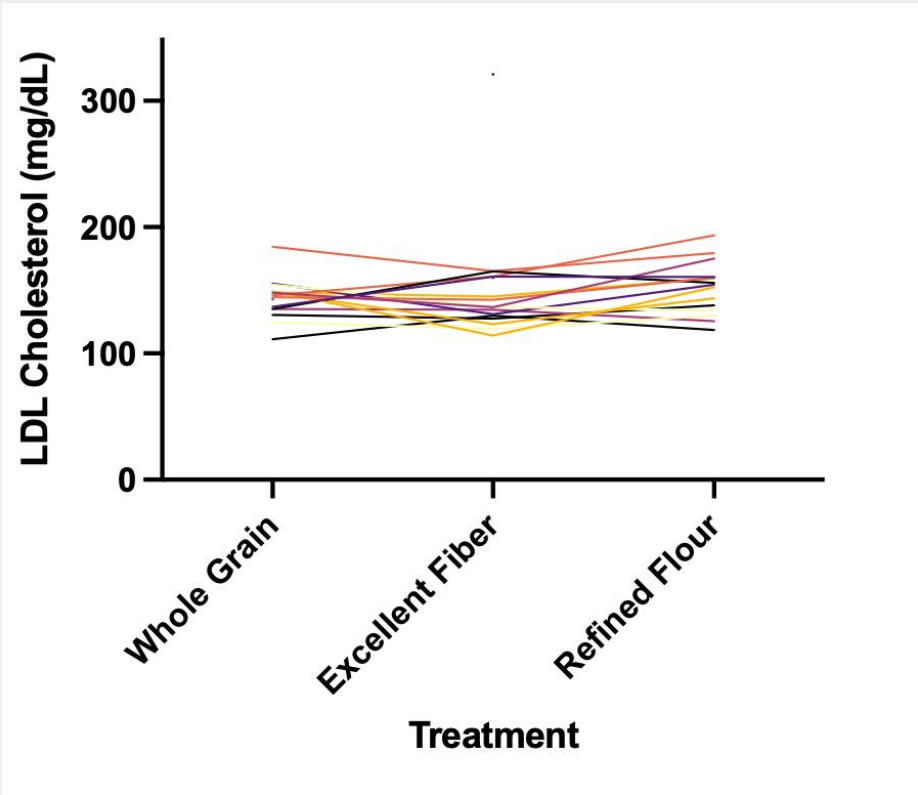
Box and Whisker plot demonstrating the effect of each treatment on LDL cholesterol levels.



* indicates statistical significance ($p<0.05$). Whole= whole grain corn flour (mean LDL cholesterol= 143.46 mg/dL, median LDL cholesterol= 145.21 mg/dL), Excellent= excellent fiber mixture of bran-enriched refined corn flour (mean LDL cholesterol= 151.38 mg/dL, median= 136.91 mg/dL), refined= refined corn flour (mean LDL cholesterol= 151.57 mg/dL, median= 154.47)

Figure 3.

Spaghetti plot of individual responses to flour treatments



One particular participant had naturally high, but still physiologically feasible serum LDL (333 mg/dL at baseline). This participant unfortunately only completed the excellent fiber mixture treatment before having to withdraw from the study for personal reasons. As the median for the excellent fiber mixture was lowest, it was thought that this participant's data may be biasing our results, despite improvements in LDL cholesterol. When this participant was removed from the data set, the overall model worsened (treatment= 0.017, R squared= 0.694) but still remained moderately strong. Bonferroni post-hoc comparisons revealed no significant differences between the whole grain flour treatment and the excellent fiber mixture (p=1.00) but a trend towards significance for differences between whole grain flour and refined (p=0.093).

While there was no treatment effect for HDL (p=0.423) or triglycerides (p=0.689), the HDL model explained a substantial portion of the variance (R squared= 0.892) while the Triglyceride model provided substantially less explanation (R squared= 0.665). Tables 4 and 5 summarize the results of these models.

Table 4.

General Linear Model with HDL cholesterol

Source	Type III Sum of Squares	Mean Square	P-Value
Corrected Model	7072.254	272.01	**<0.001**
Intercept	196753.972	196753.972	**<0.001**
treatment*time	4.4	2.2	0.829
participant(sequence)	4859.238	347.088	**<0.001**
sequence	1554.67	310.934	**<0.001**
period	12.708	6.354	0.584
treatment	20.453	10.226	0.423
time	35.326	35.326	0.087

R squared= 0.892

Table 5.

General Linear Model with Triglycerides

Source	Type III sum of Squares	Mean Square	P-value
Corrected model	165860.83	6378.109	**<0.001**
Intercept	963414.038	963414.038	**<0.001**
treatment*time	1357.12	678.56	0.555
participant(sequence)	125833.974	8988.141	**<0.001**
sequence	14535.443	2907.089	**0.036**
period	3989.328	1994.664	0.182
treatment	858.386	429.193	0.689
time	58.562	58.562	0.822

R Squared= 0.665

Total cholesterol had a near treatment effect, trending towards significance (p=0.052), while the model explained much of the variance (R squared=0.898). The model is summarized below in Table 6.

Table 6.

General Linear Model with Total Cholesterol

Source	Type III sum of Squares	mean Squares	P-value
Corrected Model	95372.279	3668.165	**<0.001**
Intercept	3986653.19	3986653.19	**<0.001**
treatment*time	309.836	154.918	0.357
participant(sequence)	59819.07	4272.791	**<0.001**
sequence	49274.035	9854.807	**<0.001**
period	1480.728	740.364	**0.009**
treatment	913.9	456.95	**0.052**
time	205.11	205.11	0.243

R squared= 0.898

Some fluctuations in bodyweight were observed during treatment periods for some participants (<5% of total body weight). In order to assess whether these changes influenced blood lipid concentrations body weight was added to all models as a covariate. The addition of body weight did not add additional explanation to the variance and findings for blood lipids were not changed (data not shown).

Mediation and Moderation Exploratory Analysis

As an exploratory measure, we also completed a mediation and moderation analysis to test the effect that the gut microbiota (Shannon Diversity and Pielou's Evenness) may have on the relationship between LDL cholesterol and the corn flour treatments. Unfortunately, no mediation or moderation effect was found and both models explained very little variance (R-squared= 0.1693 and 0.1791, respectively). Results of this analysis can be found in Appendix 1.

Gut Microbiota Analysis

Forward and reverse reads were trimmed for quality control, denoised, and filtered using the minimum sampling depth of 38,389 bases. Data cleaning was then confirmed using an alpha rarefaction curve. Alpha diversity analysis and pairwise testing of treatment effects (including baseline diversity) using Kruskal-Wallis tests revealed no significant differences (Pielou's Evenness: H=1.037, p=0.792; Faith's Phylogenetic Diversity: H=0.989, p=0.804, Observed

Operational Taxonomic Units: H=0.877, p=0.831; Shannon's Diversity Index: H=1.04, p=0.791). A summary of pairwise comparisons is provided below in Table 7.

Table 7.

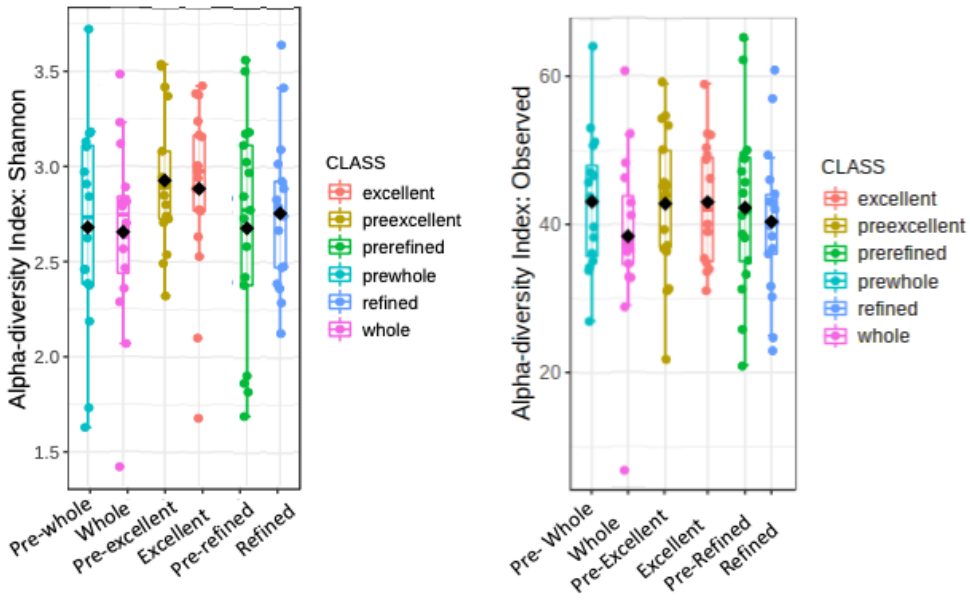
Summation of pairwise comparisons of Alpha Diversity measures

Measure			H	p-Value
Pilous Evenness				
	baseline			
		whole	0.135	0.713
		excellent	0.397	0.528
		refined	0.253	0.615
	whole			
		excellent	0.686	0.407
		refined	0.064	0.801
	excellent			
		refined	0.771	0.379
Faiths Phylogenetic Diversity				
	baseline			
		whole	0.182	0.67
		excellent	0.512	0.474
		refined	0.076	0.782
	whole			
		excellent	0.946	0.331
		refined	0.332	0.564
	excellent			
		refined	0.131	0.717
Observed Operational Taxonomical Units				
	baseline			
		whole	0.337	0.561
		excellent	0.231	0.63
		refined	0.013	0.909
	whole			
		excellent	1.169	0.279
		refined	0.021	0.885
	excellent			
		refined	0.343	0.558

Shannon's Diversity Index			
baseline			
	whole	0.296	0.586
	excellent	0.45	0.501
	refined	0.07	0.782
whole			
	excellent	0.747	0.387
	refined	0.05	0.829
excellent			
	refined	0.655	0.418

Figure 4.

Box and Whisker plots of Shannon Diversity and Observed OTUs



To examine differences in alpha diversity, post-treatment (baseline excluded) a Mann-Whitney U test was chosen. Again, no significant treatment effects were observed. Table 8 below summarizes the findings.

Table 8.

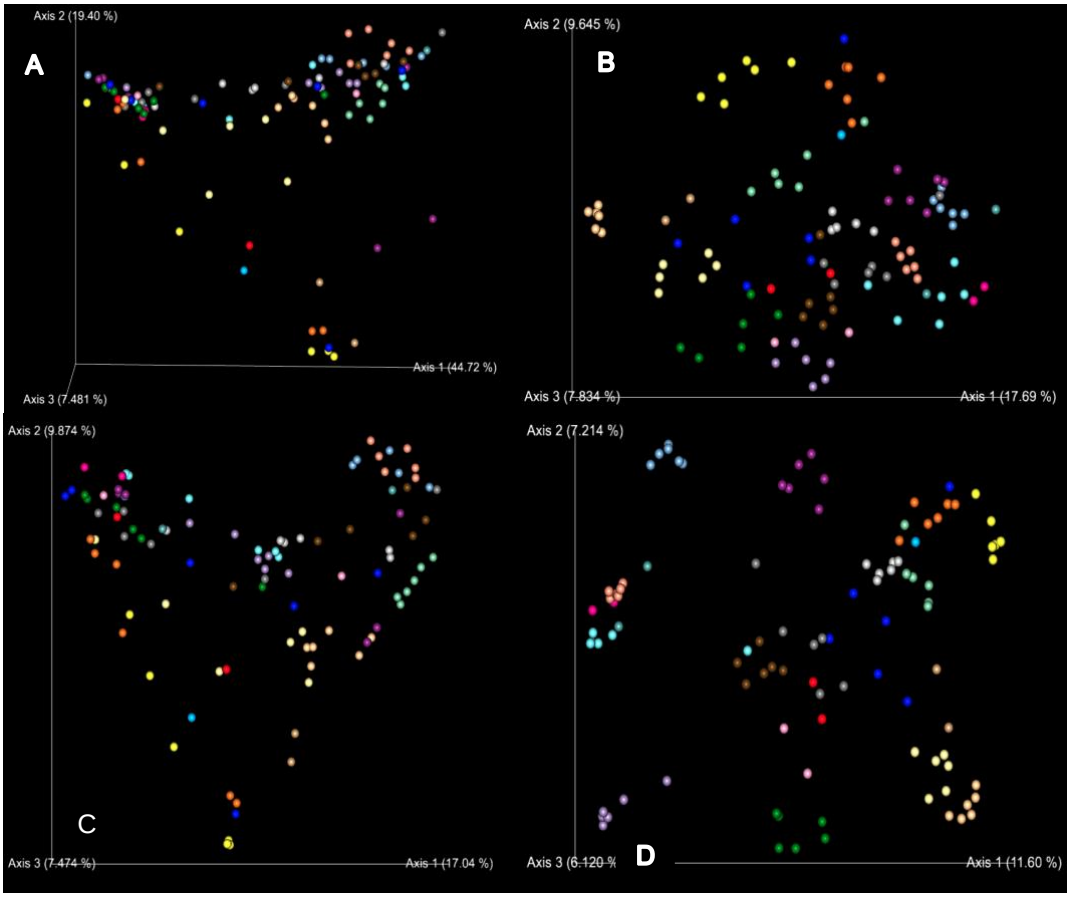
Summation of post-treatment pairwise analysis

		Mann-Whitney U	p-value
Whole v. Excellent			
	Pilous Evenness	113	0.423
	Faith's PD	109	0.345
	Observed OTU	106	0.292
	Shannon's Diversity	112	0.402
Whole v. Refined			
	Pilous Evenness	129	0.817
	Faith's PD	120	0.581
	Observed OTU	132	0.901
	Shannon's Diversity	130	0.845
Excellent v. Refined			
	Pilous Evenness	119	0.394
	Faith's PD	134	0.734
	Observed OTU	127.5	0.563
	Shannon's Diversity	121	0.433

Principal coordinate analyses of beta diversity demonstrated visual groupings by participant (Figure 4). PERMANOVA findings suggested no significant differences by treatment. A summation of pairwise treatment comparisons for Beta Diversity can be found in Table 8 in Appendix 1.

Figure 5.

PCoA plots of Beta Diversity Measures



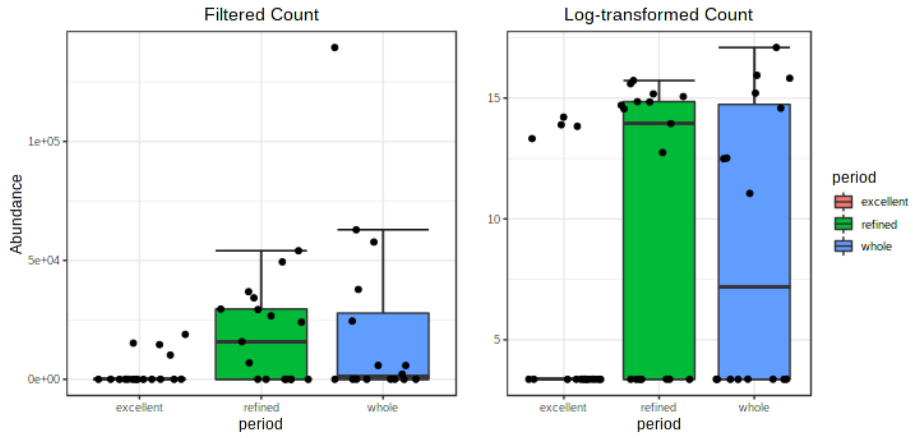
*Dots differentiated by participant. A.Weighted Unifrac, B. Unweighted Unifrac, C. Bray-Curtis, D. Jaccard

Abundance analysis

A test of Linear Discriminant Analysis Effect Size (LEfSE) was preformed to identify potential microbial biomarkers at the species level for each corn flour treatment. Only one species was found to be mildly significant (*Mycobacterium celatum* p=0.048), but after false discovery rate adjustment it was no longer significant (p=0.975).

Figure 6.

Abundance of Mycobacterium celatum compared between treatment groups



CHAPTER 5

DISCUSSION

High fiber diets have been associated with improvements in cardiovascular disease risk, specifically lowering serum LDL cholesterol. While wheat has been the focus of this research, other grains, such as corn have not been studied for their cholesterol-lowering properties. The current study implemented a 16-week, 3-period, single-blind, randomized crossover trial to test the cholesterol-lowering effects of three different corn flours: whole grain, refined, and bran-enhanced refined flour (herein referred to as excellent fiber flour mixture). As the mechanism by which corn improves serum cholesterol levels remains less clear when compared to other grains, the influence of corn consumption on the human microbiome is largely unexplored. Previous literature has shown that increased whole wheat consumption improves both diversity and abundance of microbial species.[202-204] This investigation aimed to explore whether a similar relationship could be observed for corn flour.

A Generalized Linear Model was used to test the impact of corn flour treatment on cholesterol. Treatment was found to have a significant effect on LDL cholesterol ($p=0.016$). Interestingly, whole grain corn fiber consumption resulted in a significantly greater decrease in LDL cholesterol when compared to the excellent fiber mixture and refined flour. Previous literature has established that 1 mmol/L decrease (38.7 mg/dL) of LDL cholesterol has a clinically significant effect, reducing relative risk for all cause mortality by 15.6%.[205] The median concentration of LDL cholesterol was lower following the excellent fiber flour mixture (136.91 mg/dL) when compared to both whole grain (145.21 mg/dL) and refined (154.468 mg/dL) flours, whereas mean LDL with the excellent fiber (151.38 mg/dL) was higher than whole grain (143.46 mg/dL). However, when we examine the median differences from pre to post treatment individiually, no treatment was able to establish a clinically meaningful decrease in LDL cholesterol (whole= -3.252 mg/dL, excellent= -3.53 mg/dL, refined= 8.353). While a 4 week interevention did not have a clinically meaningful impact on LDL cholesterol, the median differences for both the whole grain and excellent fiber flours demonstrate a small impact on LDL cholesterol, while median LDL increased for refined flour.

Differences in our results could be explained due to one participant who had incredibly high LDL cholesterol (333 mg/dL). This partcipant only completed the excellent fiber treatment before having to withdraw from the study for personal reasons. As the participant qualified as a statistical outlier (1.5xIQR), we removed them from the model and found the overall model worsened (R squared= 0.694, previous model R squared= 0.894). Regardless of outlier exclusion, the treatment effect still remained (p=0.017) but Bonferroni post-hoc comparisons revealed no significant differences between the whole grain flour treatment and the excellent fiber mixture (p=1.00). However, a trend towards significance was found for differences between the whole grain flour and refined (p=0.093) flours. As this person's cholesterol was high in comparison to the other participants, it was still biologically plausible. Of the other blood lipids measures, only total cholesterol trended towards a treatment effect (p=0.052) Overall, it seems that the three corn flours may be effectively altering blood lipids, but may not have a significant clinical effect on health within the dose or timeframe of our current intervention.

Prior literature has shown that whole grain consumption has the potential to lower LDL cholesterol [206,207] while cardiovascular benefits of bran consumption remain controversial. A review by James W. Anderson showed that cereal fiber intake (such as bran derived from wheat or corn) did not provide the same cardio-protective effects of whole grains, [206] which is in line with our current findings. Another, more recent, systematic review found that bran intake was associated with decreased CVD and all-cause mortality after controlling for lifestyle variables.[207] Due to the fact that we are underpowered and recruitment is ongoing, it is too early to rule out such an effect with corn bran, especially since median LDL happened to be lower for this group.

Further, research that specifically targeted bran saw significant decreases in total and LDL cholesterol, [208] which supported our original hypothesis. Gold and Davidson published a similar study to ours, feeding oat bran muffins, whole wheat muffins, or wheat/oat bran muffins (1:2:3 ratio of whole wheat flour, wheat bran, and oat bran, respectively) to healthy participants for 28 days. The total dietary fiber provided in the muffins was relatively similar over all three treatments (5.5-5.0 g), whereas our intervention foods varied (1.0 g – 6.0 g). Gold and Davidson saw a significant reduction in LDL (8.7%) and total cholesterol (5.3%) in participants who

consumed the oat bran muffins which contained the lowest dietary fiber (5.0 g per muffin) out of all the treatments.[209] On average, our participants had a 0.53% reduction in LDL cholesterol when consuming the bran-enhanced flour (6 g of fiber) while whole grain corn flour (2 g of fiber) averaged a 0.77% reduction in 4 weeks. While we did not see as potent of an effect in comparison to Gold and Davidson, this could be due to the fact that oat flour/bran has a superior effect in comparison to corn flours, or our population (those who have high-cholesterol and are unmedicated) are more resistant to dietary impact than healthy participants.

It has been well established that soluble fibers, such as psyllium, β-glucan, pectin, and guar gum lower both LDL cholesterol in humans. [210] However, substances such as rice bran (containing oil, negligible amounts of soluble fiber, and larger amounts of insoluble fiber) have also been shown to equally lower LDL cholesterol. [208] Further, research utilizing just the rice bran oil after removal of all fiber has demonstrated a greater effect at lowering serum LDL in humans when compared to defatted rice bran. [211] This is believed not to be due to the fatty acid profile of the rice bran oil, but of the other components such as phytochemicals and unsaponifiable (oily substances unable to form soaps) compounds. [211] This could suggest that phenols, phytochemicals, and unsaponifiable substances are more important than fiber content for lowering cholesterol.

While corn bran has the highest content of dietary fiber and phenolics in comparison to other cereal brans, [212] corn fiber oil (derived from corn bran) contains high levels of phytosterols,[213] which are known to interfere with the uptake of both digestive and biliary cholesterol in the intestinal tract, allowing for further excretion.[214] While our current findings demonstrate whole grains have a more potent impact on serum LDL cholesterol, our excellent fiber, bran-enriched flour mixture may still have the potential to demonstrate a meaningful impact on cholesterol upon completion of the study and recruitment.

Corn Flour Effects on Gut Microbiome Diversity and Abundance

Unfortunately, no statistical differences were observed for gut microbiome diversity after consuming the corn flour products. Alpha diversity measures (Pilou's Evenness, Observed OUT's, Shannon's Diversity, and Faith's Phylogenetic Diversity) resulted in no significant findings.

Beta diversity analysis also had no significant findings. PCoA plots of these metrics, however, established a visual clustering of participants. The lack of microbial diversity findings was unexpected as fiber from grains is known to induce alterations in the gut microbiome. One potential reason for lack of findings is due to the effects of fiber on gut microbiota being highly individualized.[215] Another potential reason for our lack of findings on the gut microbiome may be related to how the corn-based study foods were prepared, as grain processing has been shown to have a significant impact on microbial ability to ferment non-digestible carbohydrates [216]. Processing of grains begins with milling processes and extends to downstream product applications including extrusion and baking. Different applications alter the structural and chemical characteristics of the non-digestible carbohydrates, making them either more or less accessible for microbial fermentation. Smith et al. found that extrusion of wheat bran resulted in the highest levels of microbe-accessible carbohydrates, whereas baked sourdough bread resulted in the lowest accessibility [216]. Corn flours in the present study were baked to form palatable and visually appealing products but this may have diminished amount of available non-digestible carbohydrates, which could play a role in our non-significant findings.

In regard to abundance, only *Mycobacterium celatum* showed potential as a microbial biomarker following LEfSe analysis. While not significant after adjustments for multiple comparisons, visualizations demonstrated a large visual difference in abundance between the excellent fiber flour mixture and the other two flour treatments. The excellent fiber flour mixture appeared to result in far less abundant levels of *Mycobacerium celatum* in comparison to the other two treatments. As *Mycobacterium celatum* is associated with infections in immunocompromised individuals [217,218], the decreased abundance seen with the excellent fiber mixture could potentially be seen as a protective measure.[219] Despite this study not evaluating corn bran in concentrated amounts as was done in this study, a difference in methodology for assessing the gut microbiome could explain our lack of observed differences in bifidobacterial. Carvalho-Wells et al. used fluorescence in situ hybridisation with 16S rRNA oligonucleotide probes specific for Bifidobacterium spp. while we sequenced all microbes based on the 16S rRNA gene.

Research concerning arabinoxylan (a type of fiber derived from corn bran) has shown strong modulation microbiota composition.[220,221] Ngygen et al. showed that a 6-week high-dose arabinoxylan supplementation improves the composition and function of gut microbiota. Both community structure and composition was changed, showing two distinct temporal patterns.[220] While prior research on a corn bran derived fiber (arabinoxylan) has demonstrated a change in composition, this may be due to the fact that we used intact corn bran/fiber vs. concentrated arabinoxylan, are not as biologically available to be metabolized by the colon. This concentrated component of corn bran is not isolated as it is in these previous studies in our trial. We utilized intact corn fiber as we are trying to appeal to the FDA and USDA guidelines for nutrition labels and "heart healthy food guidelines". [222] This may be the difference between our results and the results of these previous literature as the fiber we utilized was not as microbial accessible as arabyinoxyin.

To date, this is the first study examining different corn flours/mixtures on both serum cholesterol and the microbiome. However, further parallels may be able to be drawn from previous literature concerning wheat. Similar to our study, Costabile and colleagues conducted a double-blind, randomized, crossover trial in 32 healthy individuals consuming whole grain wheat cereal and wheat bran-based cereal for 3 weeks. Fecal *bifidobacteria* and *lactobacill*i were found to be significantly higher during the whole grain wheat treatment and exerted a more pronounce pre-biotic effect in comparison to wheat bran[203] while our findings did not support that. Like us, Costabile et al. also saw no significant differences between treatments in SCFA production, glucose, insulin, or cholesterol values, though total cholesterol was reduced in those in the highest quartile with both treatments.[203] These non-significant differences shown by Costabile and colleagues demonstrate the possibility that whole grain cereal grains, such as corn, could have an equal or superior effect on both cholesterol and gut microbiota diversity and abundance in healthy populations. In the hypercholesteremic, unmedicated individuals used in this study, modulation of the gut microbiota from dietary supplemention may be more difficult. This has not been studied in previous literature and is an area that requires further investigation.

While whole grain corn flour showed the most potent effect for lowering LDL cholesterol, no significant changes were found in gut microbiota diversity or abundance after four weeks of corn flour consumption. The main limitation to this analysis is the small number of participants. After pausing due to COVID-19, the study is again enrolling and this analysis represents preliminary results from less than half of the expected number of participants. The final analysis will be done when the study is statistically powered, giving better insights into the differential impact of each of the corn flours. However, current insights could inform a larger, longitudinal trial in healthy populations, focusing on the differences between corn bran and whole grain corn flour.

Corn fiber has the potential to decrease both LDL and total cholesterol as well as positively modulate the gut microbiota. While our study showed limited results, we are continuing with ongoing recruitment. Once we achieve power, we predict we will be able to see a clearer picture. A strength of our current study is the crossover design: each participant serves as their own control. As we are currently underpowered, dropouts and those who have participated in only one treatment (N=8) may be biasing our data. This bias will be removed once we enroll enough participants. Weaknesses of this study include the fact that we did not reform metagenomic sequencing, but relied on 16S sequencing. Metagenomic may have given us a fuller, species level view of what was going on in the microbiota, as well as tell us more about function.

High fiber products that may not include a whole grain (i.e. bran-enriched products) may play a role in the reduction of cholesterol and positive modulation of the gut microbiota. Further research is required to determine the effectiveness of these interventions as well as the completion of this study with full power. If a non-pharmaceutical, nutritional intervention can be applied for less cost than statin medication, millions upon millions of dollars could be saved in the American medical system as well as economic effects of chronic disease. Further research needs to be completed in this area to demonstrate the potentially potent effect a non-pharmaceutical, nutritional intervention has on the United States in our current chronic disease crisis.

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