# Prebiotic supplementation with inulin and exercise influence gut microbiome composition and metabolic health

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## ABSTRACT

Development of type 2 diabetes (T2D) is preceded by prediabetes, which is a metabolically "atypical" state associated with chronic low-grade inflammation, overweight and obesity, lack of exercise, and detrimental changes to the gut microbiome. Dietary intake and exercise are modifiable lifestyle factors for reducing T2D risk; however, several questions remain unanswered related to the efficacy and role of prebiotics and exercise, and their respective influences on gut microbiome composition, intestinal permeability, insulin sensitivity and metabolic flexibility. Sedentary to recreationally active overweight and obese adults 40-75 years old at-risk for T2D were recruited (n=22) and randomized to either supplementation with inulin, a prebiotic dietary fiber, (10g/d) or maltodextrin while consuming a controlled diet for six weeks. At baseline and week 6, participants completed a stool collection, a 4-sugar probe test, an intravenous glucose tolerance test (IVGTT), and high-fat meal challenge with skeletal muscle biopsies to evaluate changes in the gut microbiome composition, intestinal permeability, insulin sensitivity and metabolic flexibility, respectively. There were no baseline group differences (all $p>0.05$). Following the intervention, *Bifidobacteria* operational taxonomic units increased in the intervention group ([placebo: $\Delta$ 9.5 ± 27.2 vs inulin: 96.3 ± 35.5][$p=0.03$]). There were no other group differences over time in any other outcome variables with the exception of changes in metabolic flexibility. Secondarily, a systematic review of literature was conducted to determine the influence of exercise engagement on gut microbiome composition. Overall, exercise interventions

appeared to diversify taxa within the Firmicutes phylum, and specifically in several taxa associated with butyrate production and gut barrier function. Due to unclear risk of bias in all studies and low quality of evidence, additional research is needed using well-designed trials. In summary, the respective influences of prebiotics and exercise on human gut microbiome composition and their subsequent effects on metabolic function and disease risk are not well understood.

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## GENERAL AUDIENCE ABSTRACT

Type 2 diabetes (T2D) is common in the United States. Prediabetes occurs before T2D, and goes frequently undiagnosed, yet lifestyle changes (e.g. dietary changes and exercise engagement) may prevent or delay the development of T2D. Gut bacteria is a newer area of research that may have an important role in disease prevention. Several dietary supplements, such as pre- and probiotics, and their influence on gut bacteria have been studied, but the effectiveness of the prebiotic inulin for delaying or preventing T2D is unknown. Additionally the effects of exercise on gut bacteria and its role for T2D prevention is still not well understood. To address these questions, sedentary to recreationally active overweight and obese adults 40-75 years old at increased risk for T2D were recruited (n=22) and randomly assigned to either supplementation with inulin (10g/d) or maltodextrin and all consumed a six week standardized diet. At baseline and week 6, all participants completed a stool collection, a 4-sugar probe test, a high-fat challenge (HFC), and intravenous glucose tolerance test (IVGTT), and changes were evaluated in the gut microbiome, intestinal permeability, and indicators of metabolism, respectively. At week 6 *Bifidobacteria*, which is associated with improved gut health, increased in the inulin group ([placebo: $\Delta$ 9.5 ± 27.2 vs inulin: 96.3 ± 35.5][p=0.03]). There were no other differences over time for any other measurements with the exception muscle metabolism meal response. A systematic review of currently available research was also conducted to determine the influence of exercise engagement on gut

microbiome composition. Overall, exercise engagement appeared to increase bacteria that is associated with better gut health. These findings are preliminary, and most evidence is from animal studies. Therefore, more research is needed to confirm these changes in humans. In summary, the roles of prebiotics and exercise on gut bacteria and human health are not well understood.

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## ATTRIBUTIONS

Several co-authors aided in the experimental design, collection of data, and preparation of manuscripts for chapters two, three, and four. A brief description of their contributions are outlined below.

**Chapter 2. The effect of prebiotic supplementation with inulin on cardiometabolic health: Rationale, design, and methods of a controlled feeding efficacy trial in adults at risk of type 2 diabetes.**

Brenda Davy, Ph.D,, RDN, Matthew Hulver, Ph.D., Andrew Neilson, Ph.D., Monica Ponder, Ph.D., were all co-investigators on the grant that funded this project, and each contributed to their expertise to the study design and conceptual framework of this study. Kevin Davy, Ph.D. was the Principle Investigator on the grant for this investigation and contributed his expertise and all testing visits occurred in the lab he funds. Tanya Halliday, Ph.D., RDN developed the controlled diet for this project. All authors contributed to manuscript preparation.

**Chapter 3. The effect of prebiotic supplementation with inulin on metabolic health in adults at elevated risk of type 2 diabetes: a pilot randomized controlled trial.**

Brenda Davy, Ph.D., RDN, Matthew Hulver, Ph.D., Andrew Neilson, Ph.D., Monica Ponder, Ph.D., and Kevin Davy, Ph.D. each contributed their expertise, materials and laboratory personnel to assist in processing samples collected during this study. Brenda Davy, Ph.D., RDN, Matthew Hulver, Ph.D., Andrew Neilson, Ph.D., and Kevin Davy, Ph.D. were involved throughout the recruitment and data collection phases of this study, and all authors will continue to assist in the preparation of this manuscript.

**Chapter 4. Does exercise alter gut microbial composition? A systematic review.**

Brenda Davy, Ph.D., RDN chaired the examination that resulted in the development and preparation of this manuscript. Matthew Hulver, Ph.D., Andrew Neilson, Ph.D. and Brian Bennett, Ph.D. provided valuable feedback and contributed to preparation of the manuscript. Kevin Davy, Ph.D., served as senior author on this manuscript.

## CHAPTER 1: Introduction

An estimated 7.2 million U.S. adults, or 23.8%, are living with undiagnosed type 2 diabetes (T2D).[1] Currently, 30.2 million people in the U.S., or 9.4%, are living with T2D.[1] In 2015, T2D remained the 7th leading cause of death in the U.S. and is one of the most frequently contributing factors to mortality. In 2015, an estimated 193,000 individuals ≤ 20 years of age had a diagnosis of diabetes (including type 1 diabetes mellitus). The 2015 data represented an alarming increase when compared to the 2012 data, where an estimated 23,200 individuals ≤ 20 years of age had a diagnosis diabetes in 2012.[2] U.S. adults ≥ 65 years of age had the greatest frequency with 25.2% predicted to have T2D.[3] Not only are the prevalence and incidence of T2D increasing, but T2D is associated with costly complications or co-morbid behaviors such as: poor dietary quality, lack of exercise, hypertension (HTN), dyslipidemia, renal disease, and vascular damage resulting in increased cardiovascular disease (CVD) and/or amputation.[1] Therefore, the prevention and treatment of T2D represents a significant public health concern.

Prediabetes is a condition characterized by impaired fasting glucose (IFG), impaired glucose tolerance (IGT), elevated hemoglobin A1c (HbA1c), or a combination of these factors (see Table 1).[1,4,5] In 2015, approximately 84.1 million U.S. adults, or 33.9%, 20 years of age and older had prediabetes, and only 11.6% reported that they received a diagnosis by a health care provider.[1] Thus, prediabetes is often undiagnosed yet treatable with lifestyle therapy interventions.[1,6,7] Approximately 70% of individuals living with prediabetes will develop T2D.[8] The scope as well as the preventability of this health problem demonstrates the need for a translational understanding of T2D development and effective lifestyle therapies to reduce risk.

***Lifestyle and diabetes risk***

T2D is a chronic disease with non-modifiable and modifiable risk factors.[9] Non-modifiable risk factors include age, sex, race, and family history.[10] Modifiable risk factors include smoking status, overweight and obesity, consumption of a western diet (e.g. high-fat and/or high-sugar diets),[11] and lack of exercise.[10,12] Modifiable risk factors are treatable with lifestyle therapies such as weight loss, and improved dietary intake and exercise engagement. Dietary intake and exercise have a direct impact on body weight, and in combination may work to reduce a person's weight and overall risk for T2D development.[13] It is widely accepted that exercise, such as aerobic training and resistance training, improves insulin sensitivity.[14] However, many questions surrounding the ways that changes in dietary composition and exercise engagement alter diabetes risk remain.

***Diet, exercise and diabetes risk***

The Diabetes Prevention Program (DPP) was a landmark multicenter trial in the United States that aimed to determine the role of weight loss through dietary modification and initiation of physical activity compared to metformin (a commonly prescribed drug for diabetes management).[12] Results from the DPP indicated that adults with prediabetes could slow or prevent the onset of T2D through weight loss, consumption of a low-fat diet, and engagement in regular physical activity.[12] The results of this trial were influential in guiding current recommendations and guidelines for prevention of T2D. Currently, dietary recommendations for T2D prevention include consumption of whole grains, lean sources of meat and dairy, fruits and vegetables, small servings of seeds and nuts, and a decrease in overall caloric intake.[15-17]

Many physiological changes, mediated in part by diet, may contribute to the pathogenesis of prediabetes and T2D.[7,12,18] For example, human and animal models have shown links between

consumption of a westernized diet and alterations in bacterial colonization in the gut (i.e. changes in the microbiome).[19-22] Diet-induced changes to the microbiome have been linked to intestinal permeability and metabolic endotoxemia.[19,20,23,24] These physiological changes contribute to chronic low-grade inflammation and may increase risk for development of T2D.[25-28]

Lack of exercise also uniquely contributes to the pathogenesis of prediabetes and T2D in several ways. Firstly, exercise prevents age-related loss in muscle mass (e.g. sarcopenia). Sarcopenia is accelerated in sedentary adults, and the rate of loss is greater particularly after the age of 60. Sarcopenia can become a barrier for exercise engagement due to associated loss of functional ability, decrements in muscular strength, decreased quality of life, increased risk for cardiometabolic disease, and increased prevalence of morbidity.[29,30] Secondly, exercise stimulates GLUT4 translocation and expression in humans, which promotes overall glucose homeostasis and insulin sensitivity.[31-33] Lastly, engagement in exercise may mitigate increased abdominal adiposity that is associated with aging and decreased insulin sensitivity.[34,35] Although research investigating mechanisms by which diet and exercise mitigate disease risk is rapidly growing, many factors are still not well understood. Supporting theories and potential mechanisms are expanded upon below to further describe the novel roles of the microbiome, intestinal permeability, metabolic endotoxemia, and insulin resistance in the pathogenesis of T2D.

***The gut microbiome***

The gut microbiome is an all-encompassing term that refers to colonization of bacteria, fungi, archaea, protozoa, and viruses within the gastrointestinal (GI) tract.[36,37] The GI tract is estimated to comprise approximately 100 trillion microorganisms and 1000 species of yeast,

bacteria, and parasites[36,38,39] and contains more genes than the entire human genome by at least 100-fold.[39] The microbiome facilitates fermentation and extraction of micro- and macro- and phytonutrients that may otherwise not be bioavailable.[40-42] Fecal studies on the human microbiome have determined that, in general, the bacterial fecal composition of healthy adults consist of the five following phyla: Firmicutes, Bacterioidetes, Actinobacteria, Proteobacteria, and Verrucomicrobia;[43] however, Firmicutes, Bacterioidetes, and Actinobacteria are the most predominant and well-studied phyla.[44]

*Bifidobacterium* is a species of Actinobacteria.[43,45] *Bifidobacteria* are frequently implicated as essential bacteria for normal metabolic functioning in the GI tract due to their capability to maintain or improve gut barrier function.[45-47] Specifically, they are gram-positive rod-shaped bacteria that are capable of being isolated singly, in chains, or clusters, and are generally branched (i.e. polymorphic).[45,48] While *Bifidobacteria* are both versatile and essential in preventing dysbiosis (e.g. disruption of gut symbiosis) in the gut microbiome, the ways that these bacteria interact with (or counteract the effects of) other phyla in the presences of obesity are not well understood. For example, some evidence indicates that Firmicutes are increased in the presence of obesity, may consequently suppress Bacteroidetes and *Bifidobacteria*, and in rodents resulted in an enhanced capability to harvest energy from the diet during diet-induced obesity.[49-51]

The relationship between Firmicutes and *Bacteroidetes*, and how their respective proportions influence gut health has still not been fully elucidated. Previously, both low and high ratios of Bacteroidetes:Firmicutes were associated with both health and chronic disease.[49,50,52] For example, many bacterial taxa within Firmicutes (e.g. Lactobacillus, Faecalibacterium, and *Roseburia spp*.) facilitate short chain fatty acid (SCFA) production of butyrate.[53,54] Whereas

propionate, another SCFA thought to reduced GI inflammation and promote gut-barrier function, production appears to be largely facilitated by bacterial taxa within Bacteroidetes (e.g. Bacteroides and Prevotella species) and Verrucomicrobia (e.g. *Akkermansia mucinphila*).[55]

It has been postulated that changes in the microbiome (such those described above) may be responsible for alterations in metabolism and glucose sensitivity due to diet's ability to influence bacterial profile, colonization, and potentially weight status.[44,56,57] Additionally, a shotgun sequencing protocol (e.g. a sequencing protocol to analyze long strands of DNA) was developed in a metagenome-wide analysis study and has been subsequently utilized among other investigations to validate microbial markers for the quantification and classification of gut dysbiosis and its association with glucose control.[58-61] Therefore, GI dysbiosis can occur from lifestyle factors, such as dietary intake and lack of exercise, and can lead to greater disease risk such as metabolic syndrome and insulin resistance.[62,63] These factors directly influence intestinal permeability (IP), and consequently contribute to metabolic endotoxemia and insulin resistance.

Given the rapid evolution of gut microbiome research, sequencing techniques warrant discussion.[64] The two most heavily relied upon bacterial sequencing technique to date are 16s ribosomal ribonucleic acid (rRNA) sequencing and metagenomics shotgun sequencing of deoxyribonucleic acid (DNA).[65] Specifically, 16s rRNA is a DNA bacterial coding gene for rRNA which allows for bacterial differentiation.[66,67] Shotgun sequencing allows for simultaneous analysis of all genes present in an organism, and therefore differs from 16s rRNA sequencing which specifically targets the variable region of DNA between each organism.[64]

Sequencing of 16s rRNA will be used in this project, and will be the subsequent focus of the next two paragraphs. Following the development and refinement of 16s rRNA techniques, high throughput sequencing evolved. An example of high throughput sequencing is barcoded

pyrosequencing, which allows for examination of individual phylogenetic trees and allows for evaluation of gut microbial community structure.[68] To use barcoding techniques, polymerase chain reaction (PCR) primers must be used.[68] Prior to 16s sequencing each PCR primer is given a unique identifier, or tag, and then sample DNA can be amplified. Through DNA amplification, the bacterial gene of 16s rRNA for each sample can be identified for community sequencing in the gut bacteria (e.g. how many bacteria from a given phyla or species are present, where are they located, etc.).[69] During sequencing each bacterial strain will be given an operational taxonomic unit (OTU). These OTUs can then be analyzed through a bioinformatics platform, such as QIIME (Illumina, Inc), which will then assign each OTU to a bacterial phyla (or class, order, family, etc.) and can provide visual representations of bacterial diversity.[69]

***Intestinal permeability and metabolic endotoxemia***

Intestinal permeability (IP) may be defined as impaired epithelial barrier function along the upper and/or lower portion of the GI tract. Epithelial barrier function is maintained by the microvilli (i.e. enterocyte brush border membrane). The microvilli serve as a transport regulator allowing certain cells to flow freely through the brush border membrane and have secretory and absorptive functions, making them integral to digestion.[70] IP occurs when the intracellular junctions of the epithelium become separated, which allow endotoxins (e.g. bacterial lipopolysaccharides) to pass from the intestine to the bloodstream.[70] When this occurs, the transport regulatory function of the microvilli loses its "selectivity" and induces metabolic endotoxemia.

Specifically, when proportions of gram positive and gram negative bacteria shift (e.g. suppression of *Actinobacteria* and *Bacterioidetes* and an overgrowth of *Firmicutes*), the bioavailability of lipopolysaccharide [LPS (i.e. endotoxin)] shifts also such that as gram-negative

bacteria increases, bioavailability of LPS in the intestinal lumen also increases.[71,72] Increases in LPS are associated with increases in inflammation via activation of toll-like receptor-4 (TLR4).[72,73] Additionally, correlative data suggests that increases in LPS and LPS binding protein increased inflammatory responses associated with obesity,[19,20,71,74] metabolic syndrome (MetS),[74] prediabetes, and T2D.[71,74,75] As gram negative bacteria increase, LPS availability increases leading to increased IP;[76] consequently, metabolic endotoxemia occurs and can result in impaired skeletal muscle insulin sensitivity and varying degrees of metabolic inflexibility.[73,77-79] Furthermore, metabolic endotoxemia activates TLR4 and consequently promotes insulin resistance via metabolic inflexibility (see ***Metabolic Flexibility*** below).[73,78,79] Therefore, IP resulting in metabolic endotoxemia may be one intertwined mechanism by which insulin resistance, and potentially T2D, occur.

***Metabolic flexibility***

Metabolic flexibility may be defined as a tissue's ability to modify its oxidative response to fuel availability.[79] In humans, metabolic flexibility is characterized as the body's ability to cycle between fat and carbohydrate utilization for energy, and to store glucose in muscle and fat tissue. Therefore, metabolic *inflexibility* can be broadly defined as a blunted or impaired response of the body to utilize or store consumed nutrients (e.g. glucose and lipids) efficiently.[78] Metabolic inflexibility is frequently seen in conjunction with insulin resistance and T2D.[78,79]

Some of the underlying pathways and mechanisms have been identified and are explained briefly herein. In insulin sensitive humans, insulin secretion from pancreatic β-cells signals GLUT4 (a glucose transporter protein).[80] GLUT4 facilitates the storage of glucose in myocytes and adipocytes as either glycogen or triglycerides, respectively.[80,81] Increased accumulation of stored triglycerides in skeletal muscle is a characteristic of obesity and contributes to skeletal

muscle inflexibility.[82,83] In insulin resistant individuals, cell-signaling function between the pancreatic β-cells and GLUT4 is impaired and which suppresses glucose uptake and reduces glycogenesis.[79-81,84,85,86] Therefore, metabolic inflexibility can be further, and more specifically, characterized by the impaired mitochondrial capacity for cellular respiration, impaired glucose uptake, and intramyocellular lipid accumulation.[79-81,84] Additionally, metabolic endotoxemia has been associated with increases in inflammation and decreases in insulin sensitivity;[19,21,87,88] In the presence of metabolic endotoxemia toll-like receptor 4 is activated which increases reliance on glucose as a primary fuel substrate while suppressing fatty acid oxidation in skeletal muscle[77] which further contributes to metabolic inflexibility, via initiation of a localized inflammatory process in the muscle.[89]

Previous research has attempted to understand the relationship between skeletal muscle, metabolic flexibility, and diet patterns such as the consumption of a high-fat diet.[78,79] Consumption of a low-fat diet is associated with beneficial changes in insulin sensitivity and T2D risk reduction.[12] Conversely, a high-fat diet reduces capacity for fat oxidation in insulin-resistant individuals when compared to their insulin-sensitive counterparts and caused detrimental alterations cardiometabolic health including reduced insulin sensitivity. [78,90,91] The available evidence in rodents[92] and in humans[93] has determined that acute,[21,94] short-term,[94] and chronic[92] consumption of a high-fat diet (percentages ranging from 50-63% total fat) has a direct impact on endotoxin concentration,[21,92-94] particularly when diets are high in saturated fat.[95-97] Available evidence supports that dietary quality, not just overall energy intake, is important in the etiology and development or prevention of metabolic diseases such as T2D. In sum, dietary intake can impact peripheral insulin sensitivity, and therefore whole body insulin sensitivity and overall T2D risk.

***Prebiotics and their potential health benefits***

Prebiotics are generically categorized as a type of non-digestible polysaccharide. There is no universally agreed upon nomenclature or definition for discernment between inulin and its other prebiotic counterparts.[98-100] For the purposes of this study, prebiotics will be defined as:

*"a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confers benefits upon host well-being and health."*[99]

Appetite regulation and satiety to promote weight management are frequently implicated as a potential benefits of supplementation with inulin or inulin type fructans (e.g. short-chain fructooligosaccharides, oligofructose, or inulin); however, positive findings for appetite regulation and satiety have not been consistently reported and study populations have varied from infants to obese adults.[98,101-103] While some studies reported no significant differences in satiety and hunger,[104-106] others reported decreased hunger and increased satiety,[107] increased satiety but no change in energy intake,[108] or no changes in energy intake and satiety.[103] Additionally, many studies on supplementation with inulin and inulin-type fructans have reported mixed findings regarding weight. While some found no significant changes in intervention groups when compared to their control group counterparts,[109-112] others found significant reductions in the intervention groups;[113,114] however, it is important to note that changes in weight were not primary outcomes in these studies nor were weight stability parameters specified. Emerging research has begun to explore the role of supplementation with inulin on risk reduction for chronic diseases such as cardiovascular disease,[115,116] prediabetes,[117,118] and T2D.[119-123]

Prebiotics can also enhance *Bifidobacteria* along the GI tract by promoting and inhibiting

growth of specific bacteria.[46,124-127] Not only do changes in the microbiome, IP, and metabolic flexibility contribute to risk for T2D development via facilitation of chronic low-grade inflammation, but many aspects of dietary modification and supplementation also have yet to be investigated. Diet is frequently identified as a modifiable lifestyle factor for prevention of T2D;[12,16-18] however, the efficacy of prebiotic consumption on modifying risk for T2D are not well understood. Prebiotics are not yet widely accepted or utilized in clinical practice, and dose-response effects are also unknown.

***Inulin supplementation on diabetes risk and T2D***

To date, only one study done more than 20 years ago attempted to quantify average U.S. consumption on inulin,[128] and only a handful of studies have researched the effect of prebiotic consumption in adults with prediabetes.[117,118,129] Two trials with published findings have tested the effect of inulin supplementation (as opposed to oligofructose-enriched inulin) in adults with prediabetes.[117,118] Both studies were conducted by Guess et al. and were published in 2015 and 2016. In their 2015 study, the effect of inulin supplementation (10g, 3x/d for 18 weeks) was evaluated on ectopic fat and weight management in adults (≥ 18 years old) with clinically diagnosed prediabetes. Inulin supplementation was effective for weight reduction (-1.8 ± 0.4 kg from weeks 9-18), weight management, and decreased body fat (-3.7 ± 0.6%); however, no significant changes were seen in FBG after the first 9 weeks of supplementation.[117] In 2016, Guess et al. studied the effect of 6-weeks of supplementation with inulin (10g, 3x/d) or cellulose in adults (≥ 18 years old) with impaired fasting glucose (IFG) or impaired glucose tolerance (IGT). In the IFG phenotype, significant improvements were detected for insulin sensitivity and increased early insulin secretion; however, no significant changes were observed for adults with IGT.[118] Side effects, which included gas and bloating, and participant withdrawal attributed to

inulin side effects were reported in these investigations.[117,118] Collectively, these results that suggests that dietary changes alone, specifically supplementation with inulin, may not be sufficient for the delay or prevention of T2D in adults with an IGT phenotype, and the dose utilized to elicit changes may not be feasible for some individuals. Further research should examine the mechanistic novelties associated with this phenotype.

Several studies have evaluated the efficacy of supplementation with inulin on adults with T2D; however, outcome measures are not consistent across studies. Outcome measures reported include fasting blood glucose, HbA1c, lipid profile, antioxidant capacity, and blood pressure.[119-123,130] These various studies have consistently determined that inulin supplementation is effective in decreasing FBG, [119-123,130] and HbA1c,[119-123,130] improving lipid profile,[119-121] increased antioxidant capacity,[119,123] decreasing systolic and diastolic blood pressures,[120,130] and improving anthropometric measures (e.g. body mass index [BMI]).[119-123] All trials that have evaluated the effect of inulin on adults with T2D used a 10g dosage for inulin; however, not all studies used inulin as the sole prebiotic source. Sources of inulin included oligofructose-enriched inulin derived from chicory root [120-123,130] and inulin from companies who reported proprietary ingredients.[117,118]

Exercise and PA are additional strategies frequently recommended for weight management and prevention of T2D.[131] Both exercise and PA may facilitate this by reshaping the gut microbiome.[132] However, the mechanisms by which exercise and PA shape the gut microbiome are preliminary and not well understood. To date most studies are qualitative in nature, have been conducted in animal models, and have only characterized the changes within the gut microbiome associated with exercise engagement and PA accumulation. While these

studies do provide insight, as many observable shifts were in SCFA producing bacteria, the current evidence has not been systematically summarized and evaluated.

The objectives for this dissertation are two-fold. Firstly, this research seeks to test the efficacy of a prebiotic in overweight and obese adults at elevated risk for T2D as a preventive strategy. The specific aims and methods will be detailed in Chapter 2, and results and interpretations will be discussed in Chapter 3. Secondly, this dissertation seeks to systematically review and synthesize current evidence surrounding how exercise and PA influence the gut microbiome composition. Chapter 4 will summarize the current evidence and remaining questions on the relationship between these behaviors and gut microbiome composition. Chapter 5 will discuss the cumulative implications and future research directions.

**CHAPTER 2: The effect of prebiotic supplementation with inulin on cardiometabolic health: Rationale, design, and methods of a controlled feeding efficacy trial in adults at risk of type 2 diabetes.**

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**Abstract**

Prediabetes is associated with low-grade chronic inflammation that increases the risk for developing type 2 diabetes (T2D) and cardiovascular disease (CVD). An elevated lipopolysaccharide concentration, associated with dysbiosis of the intestinal microbiota, has been implicated in the development of both T2D and CVD. Selective modulation of the intestinal microbiota with prebiotics reduces intestinal permeability and endotoxin concentrations, inflammation, and metabolic dysfunction in rodents. The primary aim of this trial is to determine the influence of prebiotic supplementation with inulin on insulin sensitivity and skeletal muscle metabolic flexibility in adults at risk for T2D. We hypothesize that prebiotic supplementation with inulin will improve insulin sensitivity and skeletal muscle metabolic flexibility. We will randomize 48 adults (40-75) with prediabetes or a score $\geq 5$ on the American Diabetes Association (ADA) risk screener to 6 weeks of prebiotic supplementation with inulin (10g/day) or placebo. Subjects will be provided with all food for the duration of the study, to avoid potential confounding through differences in dietary intake between individuals. Intestinal permeability, serum endotoxin concentrations, insulin sensitivity, skeletal muscle metabolic flexibility, endothelial function, arterial stiffness, and fecal bacterial composition will be measured at baseline and following treatment. The identification of prebiotic supplementation with inulin as an efficacious strategy for reducing cardio-metabolic risk in individuals at risk of T2D could impact clinical practice by informing dietary recommendations and increasing acceptance of prebiotic by the scientific and medical community.

## 1. Introduction

In 2012, approximately 86 million U.S. adults aged 20 years and older had prediabetes.[133] Low-grade chronic inflammation plays an integral role in the pathogenesis of atherosclerosis,[134-136] and may increase risk for development of type 2 diabetes (T2D)[25-28] and cardiovascular disease (CVD)-related events.[25,136] Results from numerous studies in animal models [19,23,51,92,137] and humans [50,61,113,138,139] have implicated the intestinal microbiota (i.e., bacteria residing in the gastrointestinal tract) in the pathophysiology of obesity and T2D.

Studies in both human and rodent models suggest that the consumption of a high fat/high sugar, westernized diet may lead to changes in the composition/activity of the gut microbiota (e.g., depletion of *Bifidobacteria*), increase intestinal permeability to luminal antigens, and cause metabolic endotoxemia, i.e., elevated endotoxin concentrations.[19,21,23,140] In turn, metabolic endotoxemia is associated with the development of a low-grade chronic inflammatory state, obesity and insulin resistance in rodents.[19,23] In humans, endotoxin concentrations are higher in prediabetes and T2D compared with normoglycemic individuals. [141,142] In addition, elevated endotoxin concentrations are associated with an increased risk of incident T2D.[143] Importantly, Frisard et al. [77] demonstrated that skeletal muscle, the primary site of insulin stimulated glucose disposal, is a target for circulating endotoxin. Furthermore, low dose endotoxin concentrations, consistent with metabolic endotoxemia, activate skeletal muscle TLR4 resulting in a state of metabolic inflexibility consistent with that observed in insulin resistance and T2D.[78 79]

Several lines of evidence implicate the gut microbiota in mediating CVD risk.[144] First, serum endotoxin increases progressively with the number of metabolic syndrome components [143] as with the 10-yr. CVD.[145] Second, endothelial dysfunction, an early step in atherogenesis, occurs following endotoxin exposure in humans.[146-148] In addition, elevated serum endotoxin is

associated with arterial stiffness.[149] Finally, chronic elevations in endotoxin have been associated with carotid intima media thickening and incident CVD.[150,151] These findings suggest that gut dysbiosis and the subsequent increase in circulating endotoxins may lead to adverse changes in vascular function and structure, and elevated CVD risk.

Consumption of non-digestible polysaccharides (i.e. prebiotics) is an effective way to improve overall gut health.[125,126] Prebiotics are "selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring health benefit(s) upon host health."[100] Commonly used prebiotics include inulin-type fructans, fructo-oligosaccharides, xylo-oligosaccharides and galacto-oligosaccharides.[152] In rodents, the proliferation of a targeted bacterial species (i.e. *Bifiodobacterium* spp. and *Lactobacillus* spp.) contributes to host cardio-metabolic health by reducing intestinal permeability, endotoxin concentration, and pro-inflammatory cytokines.[152-155] In humans, the prebiotic inulin appears to be particularly efficacious in increasing the abundance of Gram-positive *Bifidobacteria,* while decreasing the proportions of Gram negative bacteria (see reviews [46,127]). However, the potential benefits of prebiotic supplementation on cardiometabolic dysfunction in humans are unclear.

The identification of inulin supplementation as a simple and efficacious strategy for reducing cardio-metabolic risk in individuals at risk for T2D could impact clinical practice by informing dietary recommendations and increasing acceptance of prebiotics by the scientific and medical community. In turn, our findings could lead to enhanced adoption and maintenance of inulin supplementation as a cardio-metabolic risk-reducing behavior in individuals at risk for T2D.

## 2. Aims and Hypotheses

The general aim of this clinical efficacy trial is to determine the effect of prebiotic supplementation with inulin on cardio-metabolic function in those at risk for T2D. For reference, a conceptual framework figure has been provided (Fig. 1)[156] along with the specific aims and hypotheses:

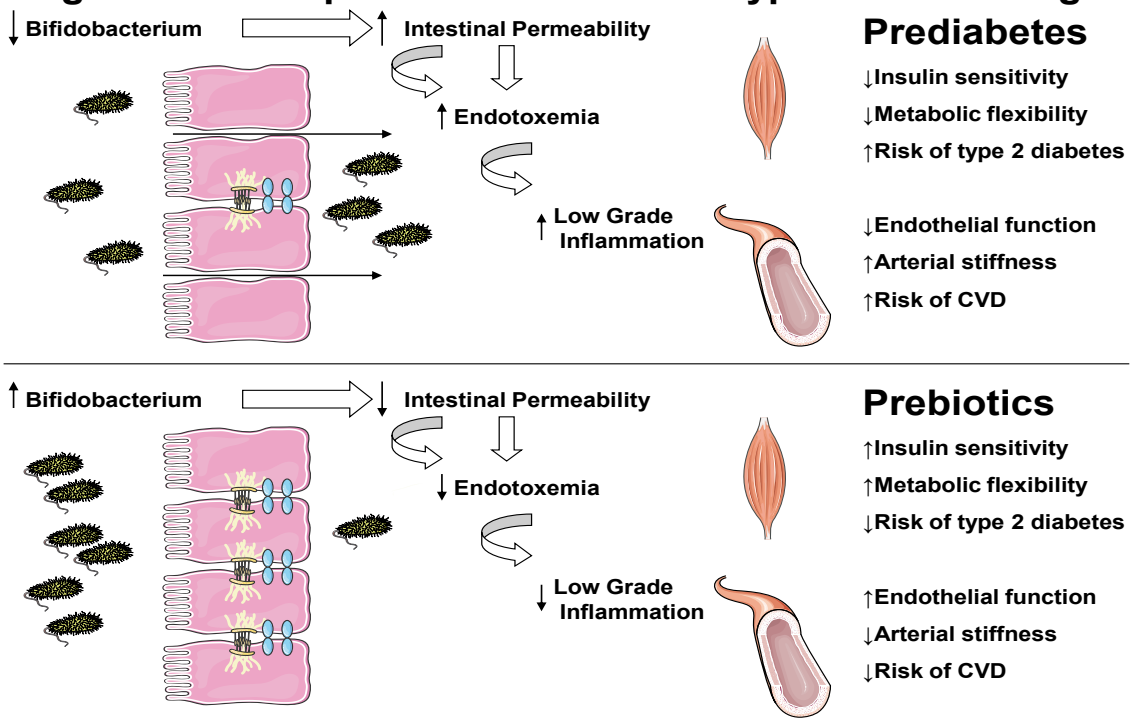
Aim 1: To determine whether prebiotic supplementation with inulin improves insulin sensitivity and skeletal muscle metabolic flexibility in individuals at elevated risk of developing T2D. We hypothesize that prebiotic supplementation with inulin will improve insulin sensitivity and skeletal muscle metabolic flexibility in these individuals.

Aim 2: To determine whether prebiotic supplementation with inulin will reduce arterial stiffness and improve endothelial function in individuals at elevated risk of developing T2D. We hypothesize that prebiotic supplementation with inulin will reduce arterial stiffness and improve endothelial function in these individuals.

Aim 3: To determine the relationship between changes in the gut microbiota (i.e., the abundance of important groups of bacteria such as *Bifidobacteria*), intestinal permeability, and endotoxin concentration with prebiotic supplementation. We hypothesize that the magnitude of change in Gram-positive gut microbiota, intestinal permeability, and endotoxin concentrations with prebiotic supplementation will be correlated with the magnitude of change observed in the aforementioned metabolic and cardiovascular variables.

The primary outcome is change in insulin sensitivity following treatment. Secondary outcomes include skeletal muscle metabolic flexibility, arterial stiffness, and endothelial function.

**Figure 1. Conceptual Framework for Hypothesis Testing**




## 3. Study design

### *3.1. Overview*

The Virginia Tech Institutional Review Board has approved the study protocol. The nature, purpose, risks and benefits of the study will be explained to each potential participant before obtaining written and verbal informed consent. We will include 48 adults with prediabetes or at increased risk for T2D, as determined by the American Diabetes Association (ADA) Diabetes Risk Screener.[9] Eligibility and exclusion criteria are presented in Table 1. Participants will be randomized to 6 weeks of prebiotic supplementation (inulin) or a placebo (maltodextrin), and will be provided with all of their food and beverages for the 6-week intervention period to avoid the potential confounding effects of differences in dietary intake on the gut microbiome. Measurements of outcome variables will be performed at baseline and following 6 weeks of treatment (Fig. 2).

A double-blind, placebo-controlled, parallel group design will be utilized for the present study. All participants who successfully complete the screening process will be enrolled in the study. Following baseline measurements, individuals will be randomized to one of two groups: supplementation with 10g/d of inulin, or with 10g/d of maltodextrin (placebo) for 6 weeks. Product details are provided in the following section. The supplements will be mixed into water and provided with the supervised breakfast meal consumed in the laboratory dining facility. An inulin dose of 10g/d was selected as the prebiotic type and dose because it is well-tolerated[157] and increases fecal *Bifidobacteria* within as few as 2 weeks.[46,127] Importantly, *Bifidobacteria* are the most common prebiotic target [46,127] and have been shown to reduce endotoxin concentrations and improve gut barrier function.[20,47,158,159] The 6-week feeding period duration was selected to allow adequate time for changes in the gut microbiota to exert its hypothesized effects and to improve the feasibility of diet adherence while considering the participant burden associated with a controlled feeding study. Randomization will be stratified by gender under the supervision of an individual not involved in the collection or analysis of the study data. Separate randomization schemes will be developed for males and females to ensure equal numbers within each gender strata. Individuals performing outcome measurements will be appropriately blinded.

*3.2 Product standardization and palatability testing*

Participants will be supplemented with 10g/d of either inulin (Frutafit® IQ, Sensus American, Inc, Lawrenceville, NJ; 2 kcal/g) or placebo (maltodextrin; 4 kcal/g) for 6 weeks. All participants will begin taking inulin or the placebo at a 5g loading dose the first 7 days prior to taking to full 10 g amount. Frutafit® IQ is composed of 100% inulin from chicory root. The nutrient composition of the supplements will be accounted for in the standardized diets (S*ection 3.3*) by using an average of 30 kcal and 10g carbohydrate per day. By comparison, usual intake

of inulin in the US population is 2.6g/d, primarily from wheat products and onions.[128] Fruitafit IQ was obtained from a single lot for the entire study along with a certificate of analysis documenting the composition and purity of the product (Appendix C).

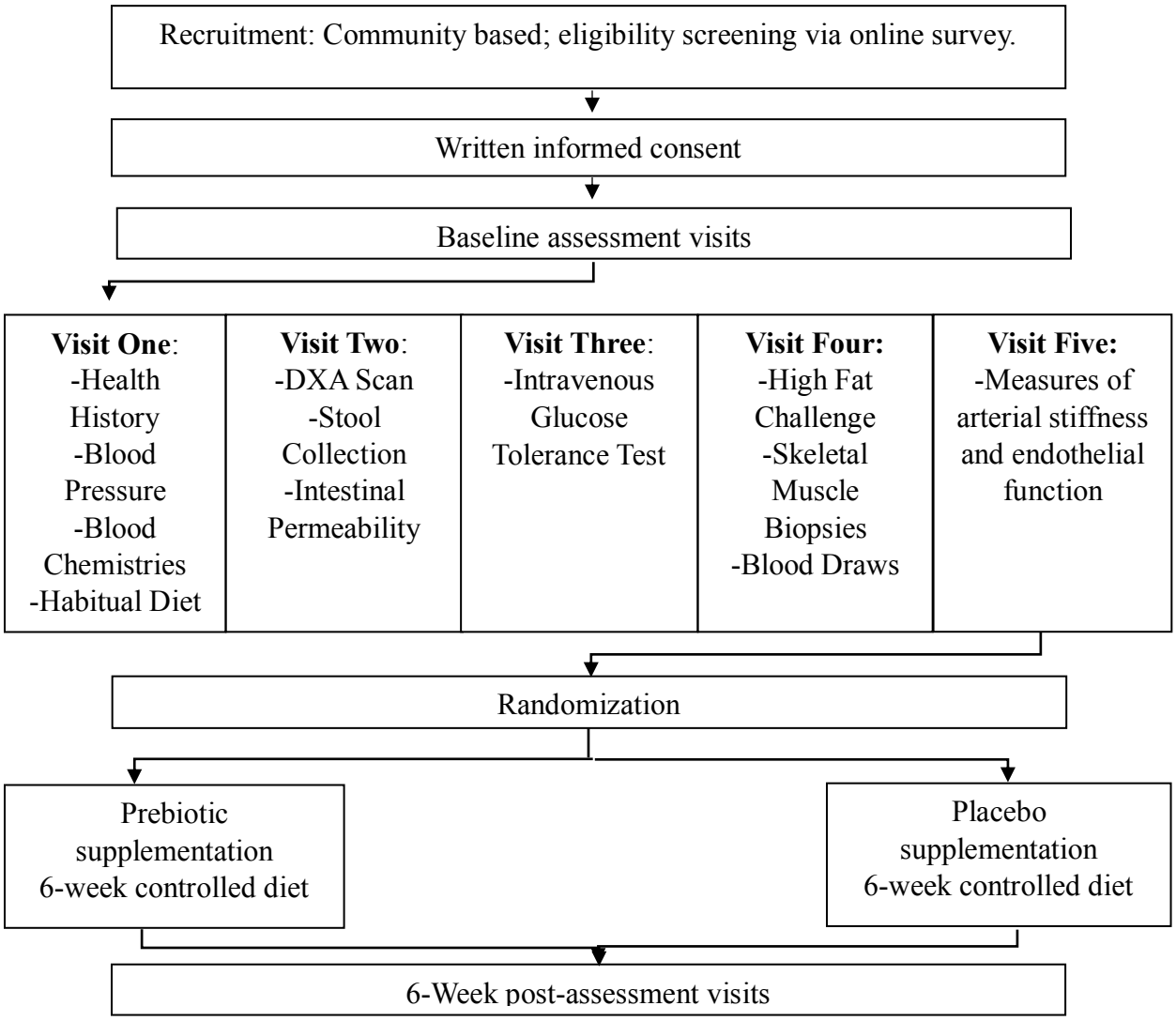



**Figure 2.** Study Design Schematic

Prior to the onset of the trial, the palatability and solubility of both supplements (10g dose) in water and in orange juice (one cup) were assessed by the research staff to determine the optimal supplement delivery mode. When mixed into either water or orange juice, both

supplements were tasteless and acceptable, and both dissolved well in either beverage. Due to the calorie-controlled nature of the study diets, water was selected as the beverage for supplement delivery.

*3.3 Diet design and standardization*

All participants will be fed a standardized diet (55% carbohydrate, 30% fat [8% saturated fat], 15% protein) isocaloric to their individual energy requirements for 6 weeks to avoid the potential confounding effect of individual differences in dietary intake on the gut microbiota. The standardized diets will be prepared in the Metabolic Kitchen and Dining Laboratory at Virginia Tech, by ServSafe®-certified research assistants, and consist of a 7-day cycle of menus with 3 meals and a snack. Menus will be developed for each day for each of the following 5 cal levels: 1500 kcal, 2000 kcal, 2500 kcal, 3000 kcal, 3500 kcal. An example of a one-day menu for the 2000 kcal level with nutrient information is provided in Table 2. Probiotic foods (e.g., yogurt) will be excluded from the diet. Daily soluble fiber intake will be maintained at $\leq$2g/1000 kcal and total dietary fiber will be maintained at or below the US daily average intake of 8g/1000 kcal.[160-163] Sodium intake will be maintained at less than 3000mg/d, except for the 3500 kcal level, which will be under 3500mg/d.[164,165]

The process used for menu development was as follows. First, a list of readily available food items (i.e., from local grocery stores, commercial food suppliers) was selected for inclusion in the controlled diets by a research dietitian. The label information for each food item selected was then matched to a comparable item in the nutritional analysis software's database (Nutrition Data Systems for Research; NDS-R, Nutrition Coordinating Center, University of Minnesota), that provided detailed nutrient composition information on each food item used in the controlled diet. The research dietitian then developed 7 days of menus to meet the daily calorie and nutrient

targets for each of the 5 kcal levels. Individual macronutrients were considered acceptable if they were ±5g of the daily targeted amount, with the exception of soluble fiber, that was ±1g of the daily targeted amount. The menus were then reviewed by a second research dietitian to verify that the daily energy and nutrient target levels were achieved, and that the proposed food portions were reasonable. After verification that daily energy and nutrient target levels were achieved, daily food preparation forms were developed for each day of the controlled diet that provided gram amounts for each food item and preparation instructions for the metabolic kitchen research assistants.

**Table 2.** Sample 2000 kcal menu

| **Food Item (Approximate Volumetric Quantity)** | **Gravimetric Quantity (g)** | **Energy (kcal)** | **Protein (g)** | **Carbohydrate (g)** | **Fat (g)** | **Saturated Fat (g)** | **Fiber (g)** | **Sodium (mg)** |
|---|---|---|---|---|---|---|---|---|
| **BREAKFAST [Bagel with Cream Cheese, and Apple Juice]** | | | | | | | | |
| Plain, White Bagel (1 bagel) | 81 | 207.7 | 8.1 | 40.9 | 1.3 | 0.3 | 1.8 | 418.8 |
| Strawberry Cream Cheese (1.8 Tbs) | 28 | 78.8 | 0.9 | 4.4 | 6.4 | 4.0 | 0.04 | 97.4 |
| 100% Apple Juice, from Concentrate (1 cup) | 240 | 137.1 | 0.2 | 33.4 | 0.3 | 0.1 | 0.2 | 10 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **LUNCH [Sandwich with Cookies, and Lemonade]** | | | | | | | | |
| White Bread (2 slices) | 86 | 231.4 | 6.6 | 43.6 | 3.4 | 0 | 2 | 586 |
| Low Sodium Roast Beef, deli slices (4 slices) | 100 | 189.4 | 36.1 | 0 | 5 | 1.7 | 0 | 44.6 |
| Low Sodium Swiss Cheese, deli slices (1 slice) | 28 | 106.2 | 7.5 | 1.5 | 7.8 | 5.0 | 0 | 4 |
| Mayonnaise (2 Tbs) | 25 | 182.2 | 0.2 | 0.8 | 19.8 | 3.0 | 0 | 142.3 |
| Vanilla Wafer Cookies, Mini (19 cookies) | 33 | 149 | 1.2 | 20.3 | 7.0 | 2.0 | 0.6 | 82 |
| Lemonade (2 cups) | 480 | 213 | 0.4 | 52.4 | 0.2 | 0 | 0 | 20 |
| **DINNER [Pasta with Alfredo Sauce]** | | | | | | | | |
| Penne Pasta, cooked in unsalted water (1 cup) | 139 | 215.7 | 8.1 | 42.9 | 1.3 | 0.2 | 2.5 | 1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Alfredo Sauce (0.75 cup) | 61 | 94.5 | 3.9 | 5.1 | 6.5 | 4.0 | 0.8 | 282 |
| **SNACK** | | | | | | | | |
| Graham Crackers, Honey-Flavored (2 crackers) | 30 | 129 | 2.3 | 23.2 | 3.0 | 0.5 | 1.2 | 183.8 |
| **SUPPLEMENT** | | | | | | | | |
| Inulin/Placebo | 10 | 30 | 0 | 10 | 0 | 0 | 0 | 0 |
| **TOTAL** | **1341** | **1944** | **75.5** | **278.5** | **62.0** | **20.8** | **9.1** | **1872** |
| **CONTROLLED DIET TARGETS (% total energy)** | - | **2000** | **75 (15%)** | **275 (55%)** | **67 (30%)** | **18 (8%)** | **16 (<8g/ 1000 kcal)** | **<3,000** |

Energy requirements for each participant will be determined using estimated resting energy expenditure based upon age, weight, height, and sex multiplied by an activity factor based on self-reported physical activity levels.[166] Participants will consume breakfast in our dining facility and be given their daily dose of inulin or placebo (mixed into water) at this supervised meal for each day of the controlled diet. The remainder of their meals for the day will be taken with them in a large portable cooler bag. The menu and instructions (i.e., for food preparation; to consume all foods provided, etc.) for the day will be included. Any uneaten items

will be returned to the metabolic kitchen the following day, weighed by the research assistants, and recorded on the food preparation sheet for that participant. Participants will be blinded from their weight and weighed at each visit during the controlled feeding period, and any trend of >1.0 kg weight loss or gain over a 3-day period will be countered by the addition or subtraction of 250 kcal food modules (e.g., 45g low-sodium saltines, 20g Swiss cheese) with the same macronutrient composition as the overall diet. Participants will be permitted to consume no more than three 6 fl oz. caffeinated beverages daily.[167,168] Caffeinated beverages allowed during the 6-week controlled feeding period will consist of unsweetened tea and coffee, which will be brewed in the metabolic kitchen. At each visit, participants will be asked to report if any non-study food or beverage were consumed since the preceding laboratory visit. Participants were instructed not to consume food and beverages (excluding water) outside the study diet. Participants who repeatedly (>3d/week) fail to consume 100% of the prescribed diet will be excluded.

*3.4. Participant recruitment, and screening*

Recruitment will take place over a 2-year period. Direct mailers, advertisements in local newspapers, campus email listservs, and posted flyers will be utilized as recruitment methods. Individuals who contact the research coordinator will be emailed a link to an online screening survey to determine if they meet basic eligibility criteria, (age, body mass index and medical/supplement use). Those who meet these basic eligibility criteria will be sent the informed consent form that provides details about the study requirements and an initial in-person visit will be scheduled. During this visit verbal and written consent, and subsequently, a detailed health history will be obtained from each eligible volunteer. If a participant meets all eligibility criteria (Table 1), subsequent baseline testing visits will be scheduled (Fig. 2).

*3.5. Procedures*

Participants will be instructed to arrive for laboratory testing between 7:00 am and 10:00 am after a 12h overnight fast (including abstinence from caffeine containing foods/beverages) and having performed no vigorous physical activity for the previous 48h. In addition, participants will report being free of acute illness/infection for the prior 2 weeks and a supplementary infection and inflammation questionnaire will be answered. Participants will undergo 5 laboratory visits at baseline and again following the 6-week intervention (Fig. 2).

*3.5.1. Body mass and composition*

Participants will be weighed on a digital scale accurate to ± 0.1 kg, and height will be determined using a scale-mounted stadiometer (Scale-Tronix Inc.; White Planes, New York). Body composition will be assessed using dual energy X-ray absorptiometry (Prodigy Advance, GE Healthcare) by a limited licensed radiologic technician as required by the Virginia Department of Health.

*3.5.2. Plasma lipids and lipoproteins concentrations*

Plasma lipid and lipoprotein concentrations (i.e., total cholesterol, high- and low-density lipoprotein cholesterol, and triglycerides) will be measured in a Clinical Laboratory Improvement Amendments-certified laboratory (Solstas Lab Partners) (Table 1).

*3.5.3. Resting blood pressure*

Mercury sphygmomanometry will be used to measure blood pressure (BP) according to American Heart Association guidelines.[169] Participants will be instructed to remain seated and resting for 10 min prior to the first BP measurement with a minimum of 1 min between each BP measurement. Participants will be instructed to keep both feet on the floor without crossing legs, and the right arm will be supported at heart level. Blood pressure will be measured twice on

participants who display a normal or pre-hypertensive value on the first measurement. The average of two additional measurements will be used for participants with an initial blood pressure in the hypertensive range.

*3.5.4. Habitual physical activity and dietary intake*

Physical activity levels will be assessed during screening via the Godin Leisure Time Questionnaire to insure participants are sedentary to minimally active.[170] Upon study enrollment, habitual physical activity level will be assessed at baseline and follow-up using the ActiGraph GT3x accelerometer (ActiGraph LLC, Pensacola, Florida, USA). The ActiGraph GT3x is a triaxial accelerometer designed to measure physical activity for extended periods of time and will be utilized in this study to monitor activity over four consecutive days (3 weekdays and 1 weekend day) to capture 60% of the workweek and 50% of the weekend. The ActiGraph will be initialized to record continuously at 15-second epochs (i.e. time intervals) and will be analyzed using the Freedson cut-point equations.[171] In addition to wearing the accelerometer, participants will be sent home with a form and instructions to note the wear time and non-wear time and any pertinent notes regarding why the accelerometer was removed (i.e. sleeping, showering, etc.). All non-wear times will be excluded from the analyses conducted in the accompanying manufacturer software. Participants will wear the accelerometer for 4 consecutive days on the right hip at each assessment point to ensure compliance with our instructions to maintain baseline levels of habitual physical activity.[172] Assessment will occur concurrently with habitual dietary intake assessment.

Baseline dietary intake, including total energy and macronutrient intake, will be assessed using detailed 4-day food intake records. Participants will be instructed on procedures for measuring and recording food intake for 4 consecutive days. Participants will be provided with

detailed recording forms, and a booklet of 2-dimensional food models to assist with accurate portion size determination. Returned records will be reviewed with the participant by research staff to ensure clarity and completeness of the food intake record. Records will be analyzed by trained research assistants using the NDS-R software (v. 2014) to determine participant's habitual dietary energy and macronutrient intake. Participants with who are taking prebiotic supplementation will be asked to discontinue for a minimum of 3-months prior to participating in the study.

**Table 1.** Prebiotic supplementation and cardiometabolic health: participant eligibility criteria

Inclusion Criteria:

- **Population: Men and women 40-75 years of age**
- **Sedentary to Recreationally Active (i.e., self-reported physical activity < 150 minutes/week)**
- **BMI: 25-40 kg/m$^2$**
- **Prediabetic or at increased risk: ADA Screener (score ≥ 5), HbA1c (5.7-6.4 mg/dl), FBG (100-125 mg/dl) or OGTT (140-200 mg/dl)**
- **Doctor's Note for patients with CHD**
- **Blood pressure: ≤ 160/100 mm/Hg**
- **Total Cholesterol and Triglycerides: ≤ 300 mg/dl and ≤ 450 mg/dl**

Exclusion Criteria:

- **Current smokers**
- **Diabetic or use of diabetes medications (i.e., metformin or insulin)**
- **Health history of: respiratory disease, unstable CHD (i.e., chest pain or heart failure), inflammatory bowel disease, cancer, neurological or hematological disorders, or substance abuse**
- **Antibiotic use in the past 3 months**
- **Current enrollment in other research studies**
- **Currently pregnant or intent to become pregnant**
- **Currently taking prescribed NSAIDs, antioxidants, or fiber supplements**
- **Recent surgery**
- **Food allergies, intolerances, and/or religious, cultural, or other dietary restrictions**

Note: BMI=body mass index; ADA=American Diabetes Association; FBG=fasting blood glucose; OGTT=oral glucose tolerance test; CHD=coronary heart disease;
NSAID= non-steroidal anti-inflammatory drugs

*3.5.5. Intestinal permeability*

Intestinal permeability will be assessed using the four-sugar probe procedure.[173-175] Following an overnight fast, participants will ingest 40 g sucrose (Spectrum Chemical MFG. Corp., Gardena CA and New Brunswick, NJ), 5 g lactulose (Qualitest Pharmaceuticals, Huntsville, AL), 1 g mannitol (Spectrum), and 1g sucralose (Spectrum) in 500 ml water.[173-175] Participants will be instructed to consume an additional 500 ml of water within a 2-h timeframe following consumption of the sugar probe. In addition, participants will be provided a standardized sucrose-free meal to consume within the first 5 hours of the urine collection period. All of the food consumed that day will be free of artificial sweeteners. Urine will be collected from 0 to 5 and 6-24h in containers with 5 ml 10% thymol (VWR, Radnor, PA) to inhibit bacterial growth. Total urine volume will be recorded, and aliquots frozen at -80ºC for later analysis. Urinary sugars will be measured on a Waters Acquity UPLC-TQD.[176] Permeability will be defined as % urinary excretion and excretion ratios for sugars from hours 0-5 (upper GI) and ours 6-24 (lower GI).[173-176] Fig. 3 shows LC-MS chromatogram of an internal standard and each of the 4 sugars recovered from urine of 1 of the Co-I's (AN) 5h after ingestion.[156]

*3.5.6. Plasma endotoxin and inflammatory cytokine concentrations*

Plasma endotoxin concentration will be determined using the PyroGeneTM Recombinant Factor C Endotoxin Detection Assay (Lonza International). Pro-inflammatory cytokines (TNFα, IL-6, and MCP-1) in plasma will be measured by ELISA (American Diagnostica Inc., New York, NY).

*3.5.7. Glucose tolerance and insulin sensitivity*

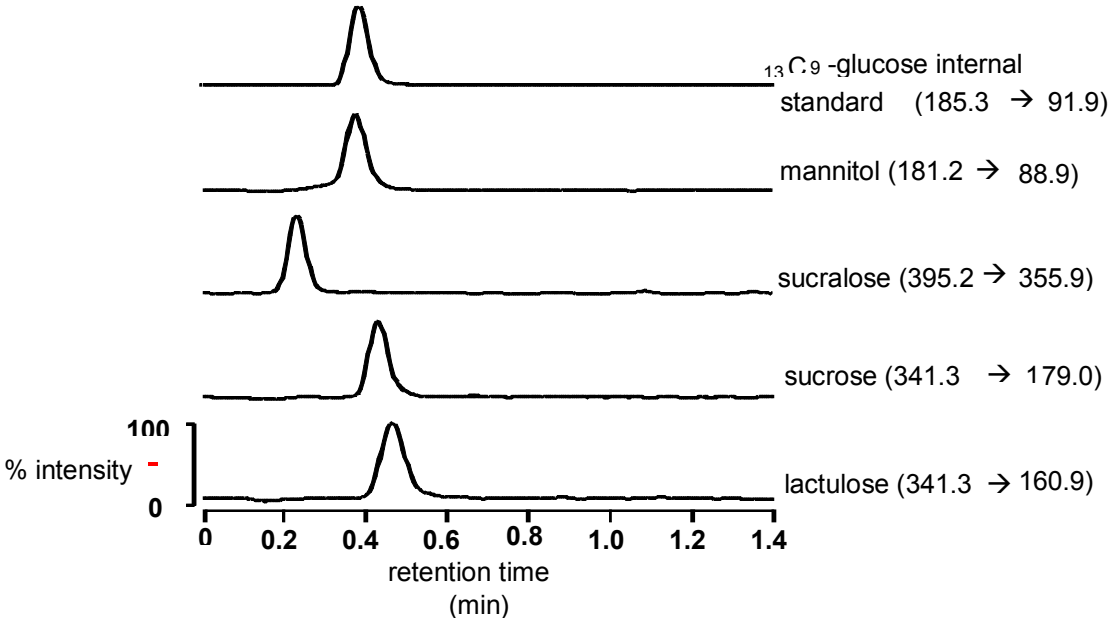
Oral glucose tolerance tests (OGTT) will be used to determine eligibility during baseline screening. Following baseline blood sampling, participants will consume a single 10 fluid ounce

beverage containing 75g glucose (Sun-Dex, Fisherbrand, Fisher Scientific, Hanover Park, IL). A second blood sample will be obtained 120 min following consumption of the beverage. Thresholds for normal, prediabetic and diabetic at the 2h time points are: <140 mg/dl, 140-200 mg/dl, and >200 mg/dl, respectively.[177]

Whole body insulin sensitivity will be estimated using Bergman's minimal model (MINMOD Millenium) during a modified frequently-sampled Intravenous Glucose Tolerance Test (IVGTT).[178] Intravenous catheters will be inserted in an antecubital vein of each arm for blood collection or glucose and insulin injection. Two baseline plasma blood

**Figure 3. Gut Permeability UPLC- MS Assay**

(subject 1, 0-5 h urine sample)




samples (*(t)* = -10 and -1 min) will be drawn. Glucose (0.3 g/kg; 50% solution) will be injected at time 0 and insulin (0.025 U/kg) will be injected at *(t)* = 20 min. Additional blood samples (3 ml) will collected at *(t)* = 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 22, 23, 24, 25, 27, 30, 40, 50, 60, 70,

80, 90, 100, 120, 150, and 180 min during the 3-h protocol. Plasma glucose concentration (mg/dl) will be analyzed immediately using a YSI Glucose Analyzer (Yellow Springs, OH). Insulin (μU/ml) will be determined later via an ELISA (ALPCO Diagnostics, Salem, NH). Samples will analyzed in duplicate.

*3.5.8. Skeletal muscle biopsies, substrate metabolism, and metabolic flexibility*

Participants will arrive for testing after a 12-h overnight fast. Muscle biopsies in the vastus lateralis will be obtained using a modified Bergstrom needle technique. Following local anesthesia (2% lidocaine), a 1.0 cm incision will be made through the skin and fascia. Biopsy tissue obtained will be obtained using a multi-pass approach. The incision will be closed with a steri-strip.

A catheter will be inserted in an antecubital vein for a baseline blood sample following the first biopsy. Participants will then be provided with a high-fat test meal (Breakfast-style sausage biscuits; 850 kcal; 63% of energy from fat; 21% saturated fat), which will be consumed within a 10-min period. The test meal will be followed by hourly blood sampling for 4 h, in addition to the initial baseline blood sample, and a second muscle biopsy will occur at the 4-h mark. After the second biopsy, participants will be given a snack and standardized discharge instructions.

Fatty acid, glucose, and pyruvate oxidation will be assessed in skeletal muscle homogenates, prepared from ~100 mg of biopsied tissue, using [1-$^{14}$C]-palmitic acid, [U-$^{14}$C]-glucose, and [1-$^{14}$C]-pyruvate, respectively, as previously described. [77,179,180] Briefly, complete and incomplete fatty acid oxidation will be assessed by measuring $^{14}C$-$CO_2$ and acid soluble metabolites, respectively. Glucose and pyruvate oxidation will be assessed by measuring $^{14}$C-CO2 production. Metabolic flexibility will be assessed by measuring $^{14}$C-CO2 production from

the oxidation of [1-$^{14}$C]-pyruvate with/without the presence of non-labeled palmitic acid. Reductions in pyruvate oxidation in the presence of palmitic acid will be examined to assess metabolic flexibility.

*3.5.9. Cardiovascular outcomes*

Flow mediated dilation (FMD) of the brachial artery will be assessed using duplex ultrasonography (HP Sonos 7500) with a high resolution linear array transducer according to published guidelines [181] and recent recommendations.[182] Reactive hyperemia will be produced by inflation of a pediatric BP cuff around the forearm for 5 min. Off line analysis of baseline and post-reactive hyperemic diameters and velocities will be performed using edge detection software (Vascular Analysis Tools, Medical Imaging Applications, Inc). Endothelium independent vasodilation (EID) will be assessed by measuring brachial arterial dilation for 10 min following administration of 0.4 mg of sublingual nitroglycerine. Both FMD and EID will be expressed as mm and % change from baseline diameter.

Carotid-femoral (C-F) pulse wave velocity (PWV), our primary measure of arterial (aortic) stiffness, will be obtained by serially measuring carotid and femoral artery waveforms using a high fidelity, non-invasive applanation tonometer (NIHem, Cardiovascular Engineering, Inc.) as previously described.[183,184] Briefly, subjects will be studied in the supine position after ~10 min of rest. A semi-automated computed controlled device will be used to auscultate brachial arterial pressure between 3 and 5 times at 2-min intervals, in order to obtain BP stability (± 5 mmHg difference for both systolic and diastolic blood pressure). Next, a high fidelity finger probe tonometer will be used to obtain carotid, brachial, radial, and, femoral artery waveforms over 10-20 cardiac cycles. Arterial waveforms will then be saved to a computer device for later analysis. Body surface measurements will be made from the suprasternal notch (SSN) to the

carotid, brachial, and radial recording sited using a Gulik tape measure and to the femoral recording site using a large caliper.

Tonomtery waveforms will be signal-averaged and the electrocardiogram R wave will serve as a fiducial point.[184] BP values will be read by an experienced reviewer and the average of the systolic and diastolic BP will be used to calibrate the peak and trough of the signal averaged brachial waveform. Brachial diastolic and mean arterial pressures will then be used to calibrate the other arterial waveforms. C-F PWV will be calculated by dividing the travel distance (SSN to carotid recording site – SSN to the femoral recording site) by the travel time obtained from the foot to foot of the signal-averaged carotid and femoral pulse waves.

β-Stiffness index, a relatively BP independent index of carotid artery stiffness, will be measured using an ultrasound unit (Sonos 7500, Phillips Medical Systems) equipped with a high-resolution linear array transducer (3-11 MHz) and applanation tonometry (NIHem, Cardiovascular Engineering, Inc.) as previously described.[185] Briefly, after resting in the supine position for ~20 min, longitudinal B mode images of the cephalic portion of left common carotid artery, 1-2 cm proximal to the carotid bulb, will be obtained over 15 cardiac cycles by placing the transducer at a 90° angle over the artery. When clear visibility of the near and fall walls are obtained, the images will be stored on an optical disk for offline quantification. The maximal and minimal carotid artery diameters of 3 consecutive cardiac cycles will be acquired with commercially available software (Vascular Research Tools 5, Medical Imaging Applications, LLC). Carotid artery BP will be acquired from applanation tonometry of the carotid artery and brachial artery auscultation as described above. β-Stiffness index will be calculated as: $\beta = \ln\left(\frac{P1}{P0}\right)/((\frac{D1}{D0}/\ D0)$, where P1 represents carotid artery systolic pressure, P0 represents carotid artery diastolic pressure, D1 represents the maximal diameter recorded during

systole, and D0 represents the minimal diameter recorded during diastole.

*3.5.10. Assessment of gut microbiota*

Stool samples will be collected daily for 3 days at baseline and the final 3 days of supplementation during the intervention. Participants will be provided with sterile plastic containers intended for stool sampling (Omnigene gut for microbiome, Owatonna, ON, Canada). Samples will be kept in a portable refrigerated cooler, and delivered to the Human Integrative Physiology Laboratory within 24 h of collection. The sample will be immediately frozen at -80°C until final processing and analysis. Total bacterial DNA will be extracted from fecal samples using QIAamp DNA Stool Mini kit (QIAGEN California). Total fecal bacterial copies will be assessed using real-time quantitative polymerase chain reaction (qPCR) of the housekeeping gene rpoD and aliquots from the 3 composite days mixed equally. Abundance of select bacteria (*Bifidobacterium, Lactobacillus*), previously shown to be modulated by inulin will also be quantified by qPCR with previously published primers. Fecal bacterial community composition will be assessed using Tag-Encoded Pyrosequencing. The V4 region of the bacterial 16S rRNA gene will be amplified from fecal microbial DNA using barcoded PCR primers.[186] Additionally a specific barcoded primer for *Bifidobacterium* will be used to amplify this group of bacteria, which has been shown to be under-represented by standard primer sets.[187] The amplicons from each reaction will be mixed in equal amounts based on concentration and will be subjected to sequencing using the Illumina MiSeq platform. Baseline fecal bacterial compositions will be compared to fecal samples obtained after 6 weeks supplementation with inulin and a detailed characterization of the gut microbiota performed via bioinformatics pipelines including MG-RAST.[188]

*3.5.11. Side effect monitoring*

Participants will be asked to report any side effects they experience during the intervention period. Gastrointestinal side effects related to the diet and/or treatment will be recorded using a standardized questionnaire, and addressed by study personnel. The side effect questionnaire will rate gas/bloating, nausea, flatulence, cramping, diarrhea, constipation, and GI rumbling from none (rating of 0) to severe (rating of 3).[157]

**4. Data Analysis**

*4.1. Power Analysis*

Sample size/power calculations were based on the number of participants needed to detect statistically ($P<0.05$) and physiologically/clinically significant differences in the magnitude of change in insulin sensitivity and aortic PWV with prebiotic supplementation compared with placebo. With 2 groups, 2 repeated measures, and $\alpha=0.05$ we will have greater than 80% power to detect significant group by time interactions for an effect size as small as 0.50 with minimum sample of n=20 participants per group. Our conservative estimate is for 20% attrition and hence we plan for n=24 per group or a total n=48 participants. Using our prior published and unpublished data, we estimated an effect size (prebiotic-placebo/S.D.) of 0.61 for insulin sensitivity (+20±32%) and 0.54 for aortic PWV (-20±37% difference in the reduction). As such, we will have greater than 90% power to detect significant group by time interactions. If dropouts exceed the 20% level, we will recruit additional participants to achieve our desired sample size.

*4.2. Statistical analysis*

We will conduct descriptive univariate analyses on all study variables. Data will be examined for the presence of outliers, violations of normality and missing data. Major violations

of normality will be corrected with an appropriate transformation procedure. In case of an outlier, rather than transform the data, the outlier will be "Winsorized," that is, replaced by the most extreme value in the tail of the distribution.

To test our hypothesis that prebiotic supplementation will improve insulin sensitivity (primary outcome) and skeletal muscle metabolic flexibility in prediabetic individuals, we will use a multiple-sample repeated measure analysis of variance with between-subject factors approach. This is a common design in randomized controlled trials, where subjects are randomized to different treatment and control groups and followed across time. Because the data are repeated, we will treat the multiple observations as nested within individuals. This will allow us to make a direct comparison between the time points while accounting for the correlation in the data to make the correct inference regarding group differences. For our analysis, the group by time interaction will be of primary interest. A compound symmetry error structure will be chosen for this model. We will use an identical approach for testing our hypothesis associated with specific aim 2. Our study is not designed nor is it powered to detect significant differences in intervention efficacy across the stratification variables (i.e., age, gender, etc.). However, these subsamples will be compared on the main dependent variables at baseline. If the groups are found to be different from each other then they will be entered in the model as a covariate.

Aim 3 is exploratory in nature so we will begin with correlational analyses. We will use analysis of covariance (ANCOVA) with insulin sensitivity (or arterial stiffness) as the dependent variable, the treatment as the independent variable, and endotoxin concentration, serving as the covariate. If changes in endotoxin concentration with inulin supplementation are: 1) directly correlated with changes in insulin sensitivity among the individual subjects, and 2) the treatment is no longer significant in the ANCOVA, this will be interpreted as support for the concept that

changes in insulin sensitivity (or arterial stiffness) with inulin supplementation are mediated, at least in part, by reductions in endotoxin concentration. We will use the same approach using percent abundance of bacterial taxonomic groups and intestinal permeability as covariates. Similarly, we will explore whether total fecal bacteria or changes in abundance of other bacterial members are significant covariates. We will also explore this using other approaches, such as mediation analysis or structural equation modeling.

*4.3. Missing data*

We will use an intention-to-treat analysis as our primary analytic approach. We will examine the missing data patterns and utilize maximum likelihood algorithms with the mixed linear model ANOVAs to longitudinally compare our outcomes across the three groups. Maximum likelihood algorithm estimations use all available data to construct weighted averages across the different patterns of missing data to provide valid point estimates and confidence intervals for population parameters.[189] As a secondary analysis, we will conduct a completers-only analysis and restrict the analysis to only those individuals who complete the interventions. In our experience, the two approaches yield similar results. However, if the results differ we will interpret the findings based on the intention-to-treat analysis, but report both so that the readers can reach their own conclusion.

*4.4. Data management and quality control*

The Principal Investigator (PI) will ultimately be responsible for the quality of the data. The project coordinator will be responsible for handling all data, entering data on the study computer, performing data editing, and maintaining a secure filing system for the study data forms that will serve as an ultimate backup (and the source for data random re-entry). Before a form is entered, the data entry staff and PI will inspect the form for completeness and legibility.

Each form will be logged into a microcomputer-based system for tracking, validation of assignment, and checked against duplication of visits or forms. This will identify problems in subject records and enable clarification. The data will then be entered into a data entry system that will be constructed to check (1) the validity of the subject identification, (2) each field entered for allowable response (range checks), and (3) validity of examination dates. Data will be duplicate keyed, verified for accuracy, and accumulated and managed using MS Access. Once all data are received, entered, and completeness verified, analysis will proceed with a listing of all data for each subject, summary statistics for all variables at each measurement period, a listing of subjects who are in noncompliance with the study protocol, and statistical analysis.

The PI will ultimately be responsible for quality control of study procedures and measurements. He will supervise performance of all of the various study protocols, questionnaires, forms, and measurements. An operations manual will be developed and the procedures strictly followed. Training sessions will cover all study procedures, including recruitment, informed consent, measurements, and specimen handling. Adherence to the procedures in the operations manual will be assured by periodic assessment and retraining.

**5. Discussion**

There is currently little information regarding if or how prebiotics improve cardio-metabolic function in humans, particularly in prediabetic individuals who are at high risk for developing T2D and experiencing cardiovascular events. Although the concept that dysbiosis of the gut microbiota leads to metabolic endotoxemia and increased risk of cardio-metabolic disease is not novel, very little information is available in humans. The significance of this trial includes providing proof of concept efficacy of prebiotic supplementation with inulin on cardio-metabolic

dysfunction and assessing its relation with changes in gut bacterial communities, intestinal permeability and metabolic endotoxemia in individuals at increased risk for T2D. Our findings could lead to identification of inulin supplementation as a simple and efficacious adjunctive therapy for reducing cardio-metabolic risk in prediabetes, which could change clinical practice by informing dietary recommendations and increasing acceptance of prebiotics by the scientific and medical community.

There are several innovative aspects of this clinical trial. First, we are testing hypotheses involving a novel concept for which there are little data in humans. Second, we have linked our ideas to the important physiological problem of metabolic endotoxemia that has been implicated in T2D and CVD. Third, we are focusing on individuals at high risk for T2D, including those with prediabetes, who are a growing segment of the population at high risk for adverse CVD-related events that may precede T2D onset. Fourth, hundreds of prebiotic products are available, yet little is known about their cardio-metabolic health benefits. Finally, we may identify a simple and efficacious adjunctive lifestyle approach to reduce T2D and CVD risk. Importantly, if our hypotheses are supported, our findings could have a significant impact on clinical practice and public health.

*5.1.Potential challenges and limitations*

A primary challenge of our study will be participant recruitment, enrollment, and retention. Recruitment will be ongoing and challenges will be addressed and managed via weekly research team meetings. Participant enrollment and retention will be managed by study personnel and the project coordinator. All participants will visit with study personnel a minimum of 3 times/week and any concerns or issues will be immediately addressed by the project coordinator.

Strict adherence to the controlled diet may also pose a challenge to enrollment and retention. To overcome barriers related to the controlled diet, daily menus are designed to reflect the "average U.S. diet." Therefore, the foods consumed during this study will be similar in composition and volume to the participant's habitual intake. All participants will be given menus to review prior to initiating the controlled diet, in order to familiarize them with what they will be expected to consume. Any questions or concerns related to the diet will be addressed at that time by the project coordinator. All participants will be informed of the expectations of the controlled diet component prior during the consenting process. For example, all food must be consumed each day and difficulty consuming any of the food must be reported to study personnel. Strategies such as re-portioning the pre-designated amounts may be utilized. Participants who cannot or will not comply with the controlled diet will be excluded from participating.

Blinding subjects to inulin may be difficult if there are gastrointestinal side effects. However, the nature of the outcomes (biochemical and physiological) and the utilization of a controlled feeding paradigm will minimize any potential for this as a limitation. It is possible that any observed improvement in certain outcomes (e.g. blood lipids) could be attributed to the low saturated fat content of the diet provided. However, we are employing a randomized controlled trial design, and all of the participants will be receiving the same diet. Thus, the impact of the treatment on our outcomes should be preserved.

This investigation should provide vital preliminary data for a larger, more comprehensive trial that would serve to establish the efficacy on the effects of inulin supplementation on cardio-metabolic function in prediabetic individuals. One of the hallmarks of science is replication of

study findings. As such, it will be important to replicate the findings of the proposed small clinical trial that may have limited generalizability to a broader context.

## 6. Conclusions

To date, the potential benefits of prebiotic supplementation on cardio-metabolic dysfunction in humans has received little attention. This trial will address this important research gap, by exploring the role of the prebiotic inulin in modifying cardio-metabolic risk among adults at increased risk for T2D. The results of this trial could impact clinical treatment approaches, and contribute to the evidence base for developing dietary guidelines that address the amount and types of dietary fiber to consume to maximize health benefits.

## Chapter 3. The effect of prebiotic supplementation with inulin on metabolic health in adults at elevated risk for type 2 diabetes: a pilot randomized controlled trial.


### Abstract

Prediabetes affects nearly 84.1 million adults and approximately 58.8 million of these individuals will progress to develop type 2 diabetes (T2D). Supplementation with prebiotics is a potentially efficacious strategy to reduce inflammation and promote insulin sensitivity by modulation of the gut microbiome. This investigation sought to determine whether the *Bifidogenic* effect of supplementation with inulin could improve intestinal barrier function and thereby improve whole body insulin sensitivity and skeletal muscle metabolic flexibility in adults at-risk for T2D. Twenty-two sedentary/recreationally active, overweight/obese (BMI: 31.3 ± 2.9 $kg/m^2$) adults (age: 54.4 ± 8.3 years) were randomized to six weeks of prebiotic supplementation with inulin (10g/day) or placebo. Diet was controlled and assessments of gut microbiome composition, intestinal permeability, whole body insulin sensitivity, and skeletal muscle metabolic flexibility were completed at baseline and week 6. There were no baseline group differences (all $p>0.05$). Following the intervention *Bifidobacteria* operational taxonomic units (OTUs) increased in the intervention group [(placebo: Δ 9.5 ± 27.2 vs. inulin: Δ 96.3 ± 35.5) ($p=0.03$)]. There were no other group differences over time in any other outcome variable with the exception of changes in metabolic flexibility. Our preliminary findings suggest that inulin supplementation increases *Bifidobacteria* in the gut microbiome but does not improve metabolic function in adults at elevated risk of T2D. Future investigations will need to assess the benefits of inulin to mitigate chronic disease risk in other populations.

## Introduction

The prevalence of type 2 diabetes (T2D) among U.S. adults is estimated to be 9.4%, and T2D is currently the 7th leading cause of deaths in the U.S.[1] Prediabetes is a condition defined by impaired glucose tolerance (IGT), impaired fasting glucose (IFG), or elevated hemoglobin A1c (HbA1c).[1,4,5] In 2015 prevalence of prediabetes rose to 33.9%, with only one-third (or 11. 6%) of individuals reporting a formal diagnosis by their primary care provider.[1] An estimated 70% of adults with prediabetes will develop T2D, making prediabetes a preventable yet frequently undiagnosed metabolic condition.[1,6-8] The rising prevalence of prediabetes and T2D demonstrates a national health problem that warrants research focused upon T2D prevention strategies.[1,6,7]

Lifestyle modification strategies for prevention of T2D include weight loss of 5-10% total body weight and 30 minutes of moderate physical activity (PA) at least 5 days/week.[12] However, specific dietary modifications and their subsequent influence on T2D risk are not well understood. Human and animal investigations suggest that consumption of a western diet, characterized by a high intake of dietary fat and sugar, may negatively impact gut microbiome composition by reducing taxa associated with gut health.[19,21,23,135] Some evidence suggests that prebiotic consumption may influence gut microbiome composition and function by selectively targeting bacteria beneficial for gut health and barrier function.[46,121] One way that prebiotics may improve gut health is by promoting increases in several bacterial taxa, such as *Bifidobacteria*. The potential functional outcome of these shifts in gut microbiome composition have been associated with improved gut-barrier function, reduced endotoxin concentration, and lower levels of pro-inflammatory cytokines.[46,118-121] Additionally, the *Bifidogenic* effect of inulin

has been well established in a wide span of age groups, ranging from infants to elderly adults.[46,190]

Supplementation with inulin, a prebiotic dietary fiber, may be a simple and effective strategy to decrease risk for T2D. However, there is limited evidence evaluating the mechanisms by which prebiotic supplementation with inulin may alter glucose metabolism and diminish T2D risk.[117,118] Therefore, the objective of this pilot randomized controlled trial (RCT) was to validate conceptual framework previously described.[156] We hypothesized that inulin supplementation would be associated with improved insulin sensitivity and skeletal muscle metabolic flexibility.[156] Secondarily, we hypothesized that inulin supplementation would be associated with increases in *Bifidobacteria* and reduced intestinal permeability (e.g. improved intestinal barrier function).[156]

**METHODS**

*Participants*

Eligible individuals were between 40-75 years, with a body mass index (BMI) between 25-39.9 kg/m$^2$ and sedentary to recreationally active.[170] Body weight must have been stable for at least six-months prior to study enrollment. Additionally, individuals had to meet 1 or more of the following criteria established for elevated T2D risk: American Diabetes Association (ADA) risk screener score ≥ 5; hemoglobin A1c (HbA1c) between 5.7-6.4 mg/dl; fasting blood glucose (FBG) between 100-125 mg/dl; or 2-hr oral glucose tolerance (OGT) value between 140-200 mg/dl.[4,9]

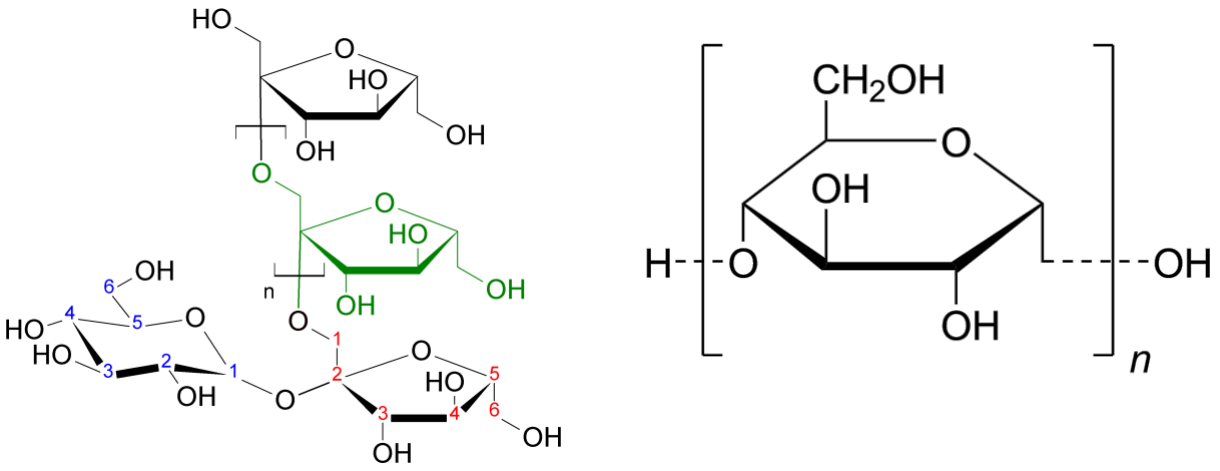
Exclusion criteria included a total cholesterol value ≥ 300 mg/dl, triglycerides ≥ 450 mg/dl, and a blood pressure ≥ 160/100 mmHg. Individuals who were currently enrolled in other research studies, currently smoked, had a history of substance abuse, a diagnosis of T2D or were

prescribed anti-diabetic medications or non-steroidal anti-inflammatories, were currently taking antioxidant or fiber supplements, or had food allergies and/or dietary restrictions were excluded. Additional exclusions included a health history of the following diseases, conditions, or occurrences: respiratory disease; unstable coronary heart disease (e.g. chest pain or heart failure); inflammatory bowel disease; cancer; neurological or hematological disorders; antibiotic use within the past 3 months; pregnancy or intention to become pregnant; and recent surgery; Lastly, individuals that were prescribed medications that may influence whole body metabolism (e.g. beta-blockers or anti-hypertensives) or body weight (e.g. thyroid stimulating hormone regulators or antidepressants) had to provide a medical record showing a stable dosage for a minimum of 6 months prior to enrollment. This pilot RCT was registered on clinicaltrials.gov (Identifier: NCT02346838), approved by the institutional review board of Virginia Tech, and all participants provided verbal and written informed consent prior to enrollment.

*Procedures*

Upon recruitment, all participants underwent a baseline screening visit. After determining eligibility at the screening visit, qualifying participants then underwent three baseline testing visits (Figure 5). Participants were randomized in a double blind design to six weeks of supplementation (Figure 4)[191] with inulin (10g/day [Frutafit® IQ, Sensus American, Inc., Lawrenceville, NJ; 100% chicory root inulin, DP 9-12]) or maltodextrin (10g/day) after completion of baseline testing visits. Each supplement was assigned with either letter A or B and the supplement code was kept by one researcher (BD) in order to keep key study personnel blinded to participants' group assignment. Block randomization was used to assign participants to a supplementation group and the laboratory coordinator randomized all participants. Energy needs were calculated for each participant based on body weight and previously reported

habitual activity levels,[166,170] and participants then entered the controlled diet and supplementation phase of the protocol. Testing visits one, two, and three were repeated at week 6, and all participants continued to consume the controlled diet and supplements until follow-up testing was completed.




Inulin [Linkage: β(2,1)] Maltodextrin [Linkage: α(1,4)]

**Figure 4.** Structural differences between inulin and maltodextrin.

*Measurements*

At baseline screening all participants arrived to the lab fasted, and completed an OGT test and blood draws to assess blood chemistries. A health history questionnaire and the Godin Leisure-Time Questionnaire were also completed.[170] Lastly, all participants were provided a four-day food intake record with instructions for recording. All food intake records (FIRs) were returned at testing visit one, reviewed with participants, entered into Nutrition Data System for Research software (NDS-R v. 2014; University of Minnesota) and analyzed by a single research technician (CM).

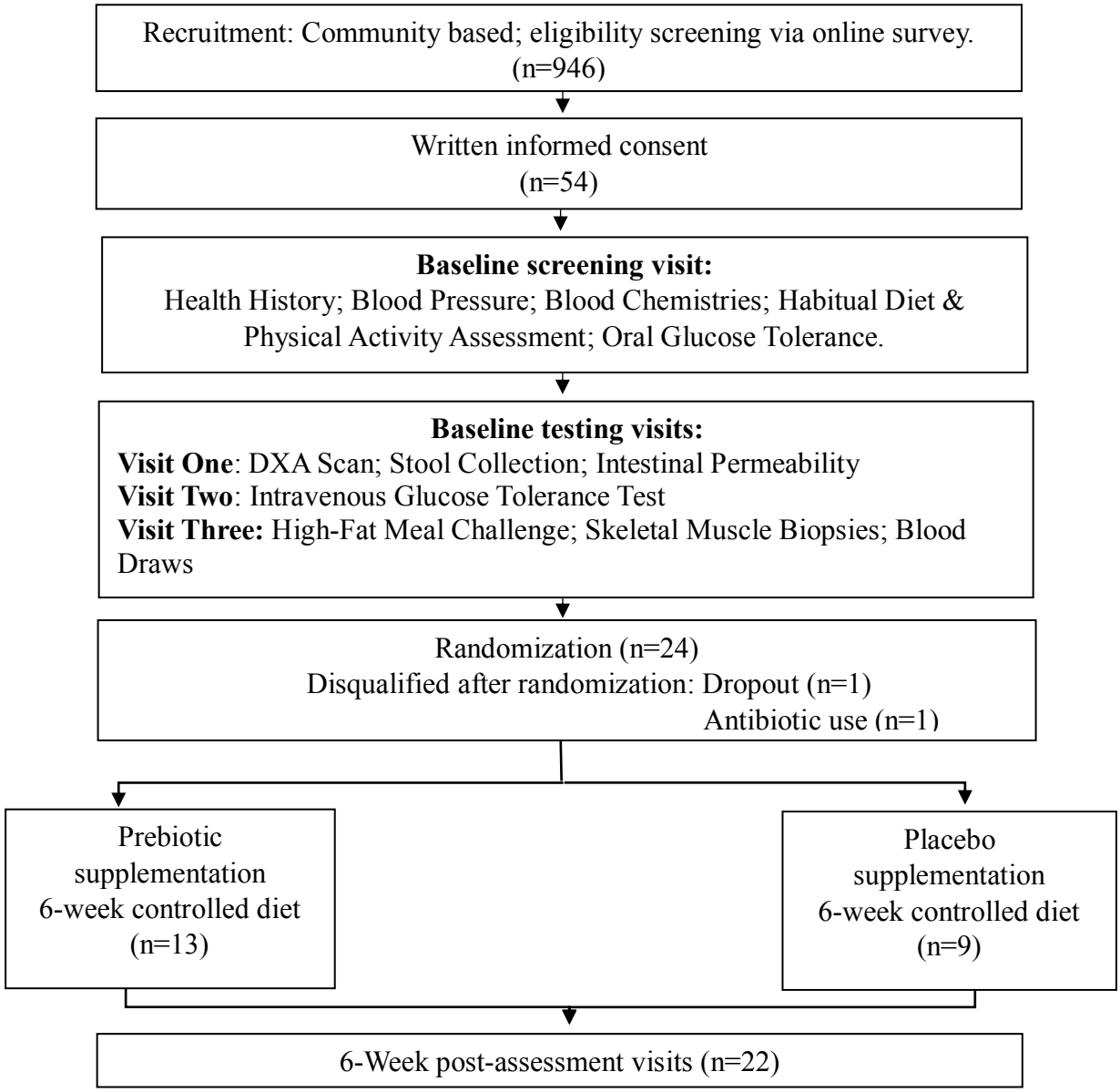



**Figure 5**. Study design, enrollment, and completion schematic.

Participants arrived to all baseline and follow-up testing visits fasted. Visit one included a dual-energy x-ray absorptiometry (DEXA [Prodigy Advance, GE Healthcare]) scan and provision of a stool collection kit (Omnigene gut for microbiome, Owatonna, ON, Canada) with instructions to return the kit by the end of baseline (or follow-up) testing visits. The v4 region of the bacterial 16S rRNA gene region from participant stool samples was amplified and all

samples were sequenced using the Illumina MiSeq platform.[188] The Shannon and Chao1 indices were used to evaluate α-diversity (e.g. evenness and richness).[192-194]

After the DEXA scan, participants consumed of a four-sugar beverage, a standardized sucrose-free breakfast [Jimmy Dean® ham, egg and cheese muffin sandwiches], and were provided with 2 urine containers for 24-hour collection to measure intestinal permeability.[173-175] Urinary sugars (e.g. lactulose, mannitol, sucralose, and sucrose) from intestinal permeability measures were analyzed using tandem quadrupole ultra-performance liquid chromatography mass spectrometry.[195] Intestinal permeability for the upper and lower gastrointestinal tract were calculated and analyzed as % urinary excretion and excretion ratios of urinary sugars.[173-175,195,196] Furthermore, intestinal permeability was divided into gastroduodenal (expressed as: 0-5h % sucrose excretion and sucrose-mannitol ratio), small intestinal (lactulose-mannitol ratio 0-5h and 6-24h), and colonic permeability (expressed as: 0-5h and 6-24h % sucralose excretion and sucralose-mannitol ratio).

Participants returned urine containers the following morning and completed an intravenous glucose tolerance test (IVGTT) for testing visit two. The IVGTT consisted of a baseline glucose injection (0.3g/kg; 50% solution), and insulin (0.025U/kg) was injected 20 minutes after the initial glucose injection. In total, 29 blood draws were collected over a three-hour testing period.[156] Plasma glucose samples were analyzed on a YSI Glucose Analyzer (Yellow Springs, OH), and serum insulin was analyzed via ELISA (ALPCO Diagnostics, Salem, NH). Whole body insulin sensitivity for each participant was approximated with Bergman's minimal model (MINMOD Millenium).[178] IVGTT variable assessed included insulin sensitivity index (SI), glucose effectiveness (Sg), acute insulin response to glucose (AIRg), and disposition index (DI).

Testing visit three consisted of a high-fat breakfast meal challenge [Jimmy Dean® sausage, egg & cheese biscuit sandwiches: 850 kcal; 65% kcals from fat; 21% saturated fat] and two skeletal muscle biopsies. Briefly, participants underwent one biopsy in the vastus lateralis, then consumed the high-fat breakfast meal, and underwent a second biopsy on the remaining leg four-hours later. Glucose and pyruvate oxidation were evaluated by measuring $^{14}C$-$C0_2$ production. Complete and incomplete fatty acid oxidation were assessed by measuring $^{14}C$-$C0_2$ and acid soluble metabolites. Metabolic flexibility was determined by $^{14}C$-$C0_2$ production from the oxidation of [1-$^{14}C$]-pyruvate with and without the availability of non-labeled palmitic acid. All substrate metabolism measures assessed via skeletal muscle homogenates from biopsied tissue has been previously described.[77,156,178-180]

Upon completion of all baseline-testing visits, participants began the controlled diet (55% carbohydrate; 30% fat [8% saturated fat]; 15% protein) and supplementation phase. At each lab visit, breakfast and supplementation were supervised and participants were provided with a cooler of their remaining prepared food and beverage items for that day. During week one of the controlled diet participants arrived at the laboratory at the same time every day. Bodyweight (Scale-Tronix Inc,; White Planes, New York) was measured and the returned cooler was checked at each visit prior to consumption of the supplement and breakfast. To determine a bodyweight stability range, the first week of body weights were averaged and a range (+/- 2 lbs.) was set for each participant. Accelerometers and a four-day wear log (3 weekdays + 1 weekend day) were also provided (ActiGraph LLC, Pensacola, Florida USA) to objectively quantify habitual PA during the first week of the diet. After the first week of daily visits, participants were then provided with a Monday-Wednesday-Friday laboratory visit schedule.

Body weight was measured and supervised supplementation and breakfast occurred at each remaining visit. Compliance to the controlled diet was also assessed at each laboratory visit, wherein all food containers were weighed back and residual volume of food was calculated (prepared food + container weight) – (returned food + container weight). Participant calorie level was adjusted based on weekly body weight trends when necessary (e.g. 2 or more days outside of the predetermined weight stability range). Accelerometers were provided again on the sixth week of the controlled diet to ensure no changes in habitual PA occurred.[172] Following the sixth-week participants underwent testing visits 1-3 again.

*Adverse events and side effect monitoring*

During the controlled diet phase of the investigation, participants were instructed to alert study personnel of any atypical gastrointestinal symptoms or side effects (e.g. gas, bloating, or diarrhea), which were reported on a standardized questionnaire.[157,197]

*Calculations and Statistical analyses*

Compliance to the controlled diet was calculated for each food provided to each participant for all 6 weeks (calculated as: provided food weight – consumed weight = compliance). Following the completion of all 22 participants, habitual diet, PA, and blood chemistry results were analyzed with independent samples t-tests to evaluate baseline group differences. Two-way repeated measures analyses of variance (RMANOVA) were used to test for group x time effects from baseline to week 6. When significance was detected, t-tests were used to determine whether there were differences between treatment groups at a single time point or within a treatment group over time. Statistical analyses were performed in GraphPad Prism (version 7; GraphPad Software).

## RESULTS

Retention rate was 92% (Figure 5) for randomized participants. There were no group differences in any variables at baseline (all $p>0.05$). Participants were primarily white females (65%), had an average ADA risk screener score of 5, and mean BMI and body fat were in the obese range (Table 3). Prediabetes indicators (means) were in the normal range except for HbA1c in the placebo group. Mean blood pressure was elevated and total and low-density lipoprotein cholesterol values exceeded optimal levels. Accelerometry measurements indicated a combined average of 9 waking hours spent in sedentary time, approximately 4.5 hours engaged in light physical activities, and 7,368 steps daily. Participants reported consuming 2,107 calories (17% protein; 46% carbohydrate; and 38% fat), 20 grams of dietary fiber, and 3,433 milligrams of sodium per day on average. Baseline data are summarized in Table 3.

Compliance to the controlled diet was 97.5%. As a secondary measure of compliance, body weight was tracked at each visit. There were no significant changes in body weight or PA between groups over time (data not shown [all $p>0.05$]). Two participants reported mild gastrointestinal side effects that included bloating and loose stool. However, these side effects resolved within 48 hours; therefore, the daily supplementation dose was not lowered.

**Table 3.** Baseline participant characteristics

| **Descriptives** | **Placebo (n=9)** | **Inulin (n=13)** |
|---|---|---|
| Sex | Men=3<br>Women = 6 | Men=5<br>Women=8 |
| Race | Caucasian = 9 | Caucasian=12<br>African=1 |
| ADA Risk Score | 5 ± 0 | 5 ± 0 |
| Age (years) | 54.2 ± 3.2 | 54.5 ± 2.1 |
| **Anthropometrics** | | |
| Height (in.) | 66.4 ± 1.2 | 66.6 ± 1.2 |
| Weight (lb.) | 197.3 ± 6.6 | 197.8 ± 8.7 |
| BMI ($kg/m^2$) | 31.2 ± 0.83 | 31.4 ± 0.9 |
| Body fat (%) | 42.3 ± 9.9 | 40.1 ± 6.7 |

| **Blood chemistries and biometrics** | | |
|---|---|---|
| FBG (mg/dl) | 90 ± 4 | 96 ± 4 |
| 2-hr OGT (mg/dl) | 118 ± 17 | 121 ± 12 |
| HbA1c (%) | 5.7 ± 0.11 | 5.4 ± 0.1 |
| TC (mg/dl) | 209 ± 10 | 215 ± 8 |
| HDL (mg/dl) | 57 ± 6 | 50 ± 3 |
| LDL (mg/dl) | 123 ± 14 | 138 ± 9 |
| VLDL (mg/dl | 29 ± 7 | 27 ± 3 |
| TG (mg/dl) | 147 ± 33 | 134 ± 14 |
| SBP/DBP (mmHg) | 128 ± 3<br>79 ± 3 | 130 ± 3<br>77 ± 2 |
| **Dietary intake** | | |
| Kcals | 2,119 ± 191 | 2,094 ± 165 |
| Protein (grams)<br>(% kcals) | 79 ± 8<br>15 ± 0 | 98 ± 6<br>19 ± 0 |
| Carbohydrates (grams)<br>( % kcals) | 258 ± 24<br>49 ± 1 | 227 ± 18<br>43 ± 0 |
| Fats (grams)<br>(% kcals) | 85 ± 11<br>36 ± 1 | 92 ± 9<br>40 ± 0 |
| Dietary fiber (g) | 22 ± 2 | 17 ± 2 |
| Soluble fiber (g) | 7 ± 1 | 7 ± 1 |
| Pectins (g) | 2 ± 0 | 2 ± 0 |
| Sodium (mg) | 3166 ± 279 | 3699 ± 270 |
| **Habitual physical activity** | | |
| Sedentary time (min/d) | 534 ± 42 | 566 ± 41 |
| Light PA (min/d) | 304 ± 34 | 244 ± 35 |
| MPA (min/d) | 36 ± 10 | 21 ± 5 |
| VPA (min/d) | 3 ± 3 | 0 ± 0 |
| Step count (total/d) | 8,126 ± 1177 | 6,610 ± 748 |

All variables expressed as mean ± SEM. All group comparisons were non-significant ($p>0.05$). Abbreviations used: SBP= systolic blood pressure; DBP= diastolic blood pressure; TC= total cholesterol; HDL= high-density lipoproteins; LDL= low-density lipoproteins; VLDL= very low-density lipoproteins; TG=triglycerides; kcals=kilocalories; PA= physical activity;

There were no group differences (n=21) for the Shannon and Chao1 indices (Table 4. [all $p>0.05$]). However *Bifidobacteria* significantly changed over time within the inulin group ($p=0.01$). A visual presentation of the data demonstrated that the inulin group had an increased number of *Bifidobacteria* when compared to the placebo group, but diversity was similar between groups. Figure 6 displays the change over time for both supplementation groups.

| **Table 4.** Gut microbiome outcomes for α-diversity, *Bifidobacteria* and change over time | | | | |
|---|---|---|---|---|
| **Variable** | **Placebo** | | **Inulin** | |
| | **Baseline** | **Week 6** | **Baseline** | **Week 6** |
| **Chao1 index** | 282.3 ± 6.3 | 276.1 ± 5.8 | 257 ± 6.8 | 252.2 ± 9.9 |
| **Shannon index** | 8.778 ± 0.2 | 9.0 ± 0.1 | 8.7 ± 0.2 | 8.8 ± 0.1 |
| ***Bifidobacteria*†** | 45.1 ± 23.8 | 54.6 ± 25.7 | 15.6 ± 8.4 | 111.9 ± 38.2 |
| OTUs between each group are expressed as mean ± SEM.<br>*Bifidobacteria*= †Time effect (p=0.02) | | | | |

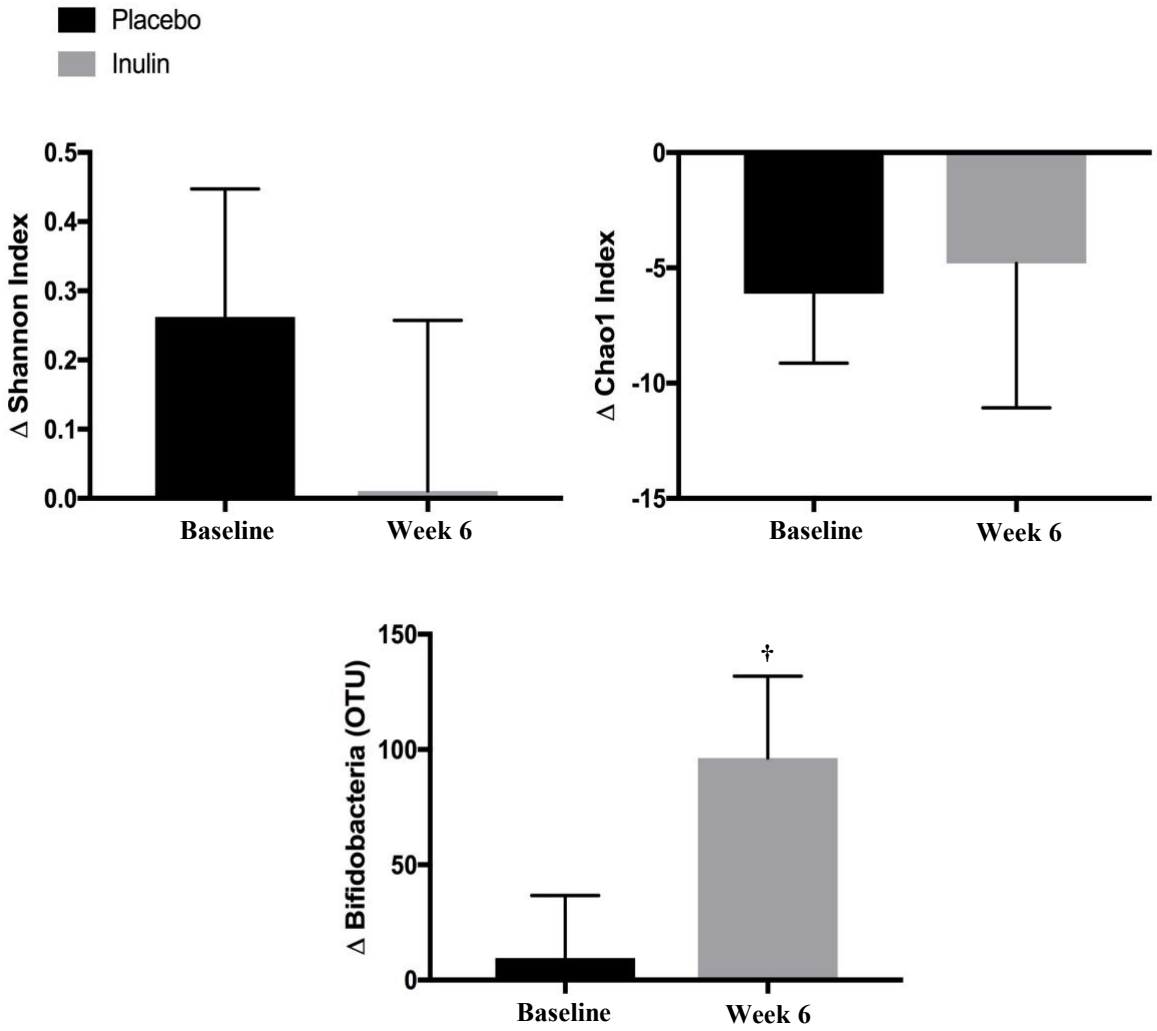



**Figure 6.** Direction and magnitude of change for α-diversity and *Bifidobacteria* counts over time. Data are presented as mean ± SEM, and changes over time were non-significant (all p>0.05) for the Shannon and Chao1 indices (no units). Bifidobacteria showed a significant time effect for the inulin group (†time effect [p=0.02].

There were no group differences (n=18) over time for any measures of intestinal permeability; SI; DI; or any other IVGTT-related variable (n=17 [all p>0.05]). Data are presented in Tables 5 and 6. Fasted and meal response for skeletal muscle metabolism and enzyme variables (n=17) are presented in Table 7 and Figure 7, respectively. Meal response was defined as the change in fasted-to-fed at a single time point. At week 6 there was a difference in meal response between groups over time for metabolic flexibility (p=0.01), and the placebo group significantly changed over time (p=0.02). Meal response change over time are defined and presented visually in Figure 7 visually presents the changes over time for substrate oxidation and metabolic flexibility.

**Table 5.** Intestinal permeability outcomes and change over time

| Variable | Placebo | | | Inulin | | |
|---|---|---|---|---|---|---|
| | **Baseline** | **Week 6** | **Δ-score** | **Baseline** | **Week 6** | **Δ-score** |
| **Small intestine permeability** | 0.0117 ± 0.001 | 0.0096 ± 0.0013 | -0.0021 ± 0.0018 | 0.0109 ± 0.0014 | 0.0090 ± 0.0010 | -0.0018 ± 0.0011 |
| • 0-5h (ratio) | 0.0342 ± 0.0065 | 0.0266 ± 0.0043 | -0.0076 ± 0.0058 | 0.0323 ± 0.0053 | 0.0218 ± 0.0032† | -0.0105 ± 0.0040 |
| • 6-24h (ratio) | | | | | | |
| **Gastroduodenal permeability** | 0.0241 ± 0.0060 | 0.0289 ± 0.0160 | 0.0048 ± 0.0170 | 0.0226 ± 0.0041 | 0.0326 ± 0.0114 | 0.0010 ± 0.0010 |
| • 0-5h (%) | 0.0014 ± 0.0004 | 0.0017 ± 0.0010 | 0.0004 ± 0.0010 | 0.0013 ± 0.0002 | 0.0013 ± 0.0010 | 0.0001 ± 0.0010 |
| • 0-5h (ratio) | | | | | | |
| **Colonic permeability** | | | | | | |
| • 0-5h (%) | 1.391 ± 0.2642 | 1.262 ± 0.3510 | -0.1292 ± 0.5634 | 1.444 ± 0.4707 | 1.052 ± 0.2723 | -0.3920 ± 0.4739 |
| • 0-5h (ratio) | 0.0863 ± 0.0208 | 0.0833 ± 0.0238 | -0.0030 ± 0.0333 | 0.0817 ± 0.0706 | 0.0490 ± 0.0083 | -0.0327 ± 0.0197 |
| • 6-24h (%) | 6.416 ± 0.8898 | 6.355 ± 2.718 | -0.0608 ± 2.889 | 5.493 ± 1.045 | 3.186 ± 1.227 | -2.307 ± 0.9152 |
| • 6-24h (ratio) | 0.5011 ± 0.0905 | 0.4764 ± 0.1703 | -0.0248 ± 0.2078 | 0.3616 ± 0.0694 | 0.2121 ± 0.0910 | -0.1496 ± 0.0826 |

All values are expressed as mean ± SEM for urinary excretion ratios or % urinary excretion.
Δ calculation = (week 6 excretion ratio % excretion – baseline excretion ratio % excretion= Δ-score)
% excretion: (total excretion/provided dose)*100
Permeability ratios: Small intestinal - lactulose:mannitol; Gastroduodenal- sucrose:mannitol; Colonic- sucralose:mannitol

**Table 6**. Insulin sensitivity and direction and magnitude of change over time

| Variable | Placebo | | | Inulin | | |
|---|---|---|---|---|---|---|
| | **Baseline** | **Week 6** | **Δ-score** | **Baseline** | **Week 6** | **Δ-score** |
| **AIRg** | 574.5 ± 173.2 | 598 ± 167.2 | -1450 ± 396.5 | 495.4 ± 96.0 | 463.8 ± 78.7 | -1041 ± 529.4 |
| **DI** | 1748 ± 440.1 | 2025 ± 430.4 | -276.3 ± 250.3 | 1472 ± 380 | 1537 ± 569.9 | -64.3 ± 251.9 |
| **SI** | 4.2 ± 1.0 | 4.8 ± 1.2 | -0.6 ± 1.2 | 2.9 ± 0.5 | 3.1 ± 0.9 | -0.2 ± 0.5 |
| **Sg** | 0.2 ± 0.0 | 0.0 ± 0.0 | -0.0 ± 0.0 | 0.0 ± 0.0 | 0.02 ± 0.0 | -0.0 ± 0.0 |

All values are expressed as mean ± SEM. All interactions were non-significant ($p>0.05$). Units for SI and AIRg variables are expressed as: $((\mu/liter)^{-1}min^{-1})$. Sg units are expressed as: $(min^{-1})$. DI values are unit-less (formula= $SI^{\alpha} \cdot \Phi$). IVGTT Δ calculations: Week 6 variable – baseline variable = Δ-score

**Table 7.** Fasted participant skeletal muscle metabolism and enzyme variables before and after 6-weeks of supplementation with placebo or inulin

| Variable | Placebo | | Inulin | |
|---|---|---|---|---|
| | Baseline | Week 6 | Baseline | Week 6 |
| **Glucose Oxidation** | 5.8 ± 1.0 | 5.5 ± 1.0 | 5.3 ± 1.0 | 5.9 ± 1.1 |
| **Fatty Acid Oxidation** | 6.9 ± 1.1 | 7.4 ± 0.7 | 7.0 ± 1.0 | 7.6 ± 1.0 |
| **Pyruvate Oxidation** | 354.5 ± 36.5 | 339.9 ± 45.9 | 248.9 ± 23.4 | 285 ± 29.6 |
| **Metabolic Flexibility** | 32.4 ± 3.8 | 23.1 ± 4.0 | 22.5 ± 4.4 | 31.5 ± 3.8 |
| **Citrate synthase** | 52.5 ± 8.2 | 53.3 ± 10.0 | 41.6 ± 7.6 | 39.8 ± 5.0 |
| **Cytochrome-c Oxidase** | 139.1 ± 25.0 | 171.8 ± 40.2 | 102.7 ± 20.7 | 132.8 ± 15.9 |

All values are expressed as mean ± SEM. All interactions were non-significant ($p>0.05$). Units for skeletal muscle metabolism are expressed as: (μmol/mg protein/hour). Metabolic flexibility is expressed as ratio of: pyruvate oxidation ± free fatty acids.

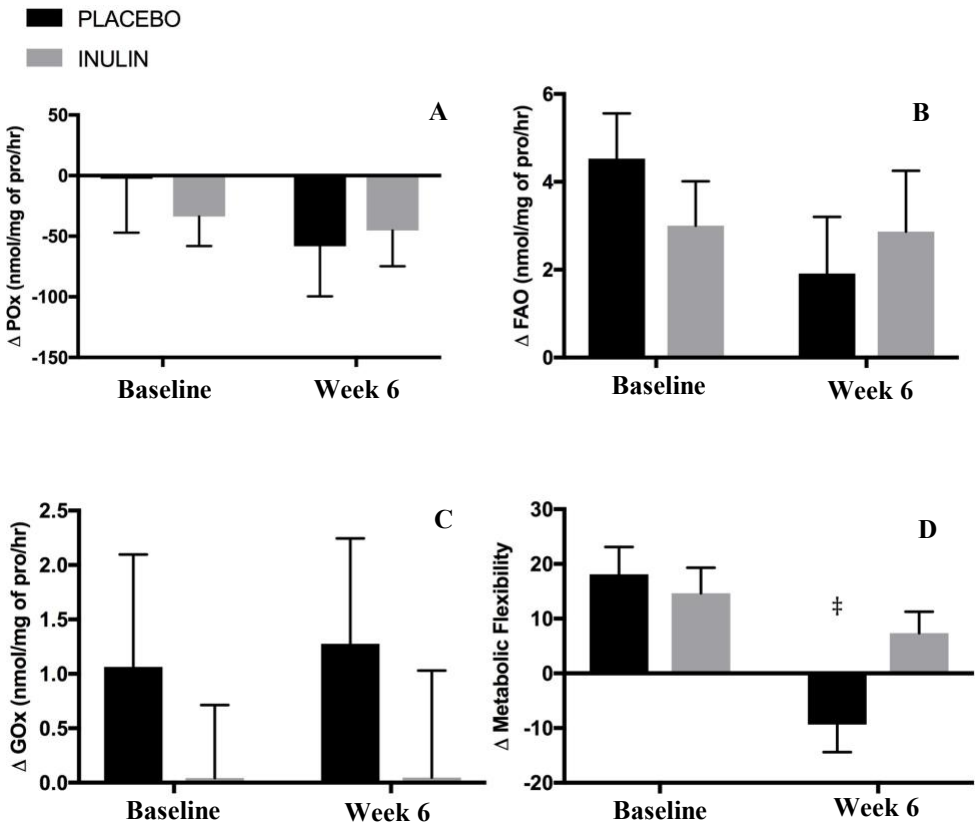



**Figure 7.** Meal response magnitude and direction of change for (A) pyruvate oxidation (POx), (B) fatty acid oxidation (FAO) (C) glucose oxidation (GOx), and (D) metabolic flexibility, at baseline and week 6 of supplementation. Data are presented as mean ± SEM. ‡ = Treatment (p= 0.01) and time (p=0.03) effects.

## DISCUSSION

This pilot RCT was the first to address the relationship of prebiotic supplementation with inulin on whole body insulin sensitivity and metabolic flexibility while also accounting for changes in the gut microbiome and intestinal permeability. The major objective of this study was to evaluate the effect of inulin on metabolic health in adults at-risk for T2D. It was hypothesized that prebiotic supplementation with inulin would improve whole body insulin sensitivity,[178] skeletal muscle metabolic flexibility,[78,79,179] and that increases in *Bifidobacteria* [46,127] and

improved gut barrier function[19,21-23] may provide insights into changes in insulin sensitivity and metabolic flexibility. However, supplementation with inulin did not improve gut barrier function, whole body insulin sensitivity, or skeletal muscle metabolism (e.g. substrate oxidation of fatty acids and glucose). There were no apparent trends for pyruvate, fatty acid or glucose oxidation, to provide insight into why these changes occurred. Findings from this investigation demonstrated the well-known *Bifidogenic* effect of inulin supplementation on the gut microbiome.[46] Overall, inulin supplementation was well tolerated and resulted in positive changes in the gut microbiome, but the effect of this change has on whole body metabolism and T2D risk remain unknown and was not demonstrated in findings from this pilot RCT.

Inflammation, obesity, and dysbiosis of the gut microbiome are strongly associated, although a causal and directional mechanism to explain this association has not yet been elucidated. [198] Modulation of the gut microbiome through prebiotic supplementation appears to be one strategy to influence host health and possibly diminish disease risk.[199] The number of investigations evaluating prebiotics and their role in mitigating disease risk are increasing.[200,201] To date, there are mixed findings on whether or not prebiotics promote satiety, reduce energy intake, and improve glucose control, lipid metabolism, and gut barrier function.[202,203] However, new insights into the gut microbiome may also provide insights into how prebiotics exert beneficial effects on host health and metabolism (e.g. short chain fatty acid biosynthesis and regulation).[201]

Much of the available evidence that has specifically assessed the interactions between inulin supplementation, inflammation and glucose control has utilized a combination of inulin and other prebiotics.[119,120,130,204,205] To date, only a small number of studies have studied the effects of daily supplementation with inulin on glucose control and metabolism.[117,118,121-123,206]

Of these investigations, three found that FBG, HbA1c decreased,[117,122,123] one found pro-inflammatory markers decreased (e.g. TNF-α, lipopolysaccharide, IL-4, etc.), [122] and two reported improved insulin sensitivity.[118,122] Additionally the majority of the studies also reported a decrease in body weight and/or BMI. [117,118,122,123] Only two trials have evaluated inulin in adults with prediabetes, and only one found that inulin was effective at improving FBG but not glucose tolerance. [117,118] Notably, dosage in adults with prediabetes was 30g/d and there were no diet, exercise or bodyweight controls which may explain the positive outcomes from these trials. [117,118] Furthermore, none of these studies have included gut microbiome outcomes. Taken together these studies indicate a need for more research in prebiotics, specifically inulin, to determine their respective influences and mechanisms of action independent of lifestyle.

Strengths of this investigation include high participant retention rate and compliance to the controlled diet. Participant PA levels, body weight, body fat also did not change over the course of the study, and there were no significant gastrointestinal side effects associated with an inulin dose of 10g/d. This study also implemented several lifestyle controls to account for confounding factors that have not been considered in previous inulin supplementation studies including body weight stability, a controlled diet, and objectively monitored habitual PA. However, the sample size was small, racially homogenous, and primarily female. Additionally, the majority of participants met inclusion criteria based upon ADA risk screener scores rather than from a clinical indicator of prediabetes (e.g. FBG, HbA1c, or OGT), which may account for the limited outcomes in this investigation. Currently, the metabolic impact of inulin on T2D risk is not well understood. Preliminary findings from this investigation indicate that inulin supplementation did increase *Bifidobacteria* in the gut microbiome, but this change did not

improve intestinal permeability, whole body insulin sensitivity, or skeletal muscle metabolic flexibility.

## CONCLUSIONS

Prediabetes is a major public health concern, as most adults with prediabetes progress to develop T2D.[1] Simple and effective dietary strategies are needed to reduce the risk and prevalence of chronic diseases in the U.S. Findings from this pilot study suggest that inulin does not mitigate T2D risk in adults. Taken together, these findings along with the limited available evidence in adults with prediabetes indicate that the efficacy of inulin to prevent or delay T2D in adults is unclear. Future investigations will need to consider the effectiveness of inulin supplementation to improve metabolic health and reduce chronic disease risk in other populations.

## Chapter 4. Does exercise alter gut microbial composition? A systematic review.


### Abstract

The objectives of this systematic review of literature were to evaluate and summarize research in mammals that have investigated the effects of exercise or physical activity (PA) on gut microbial composition. This review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines. Databases for this review included: PubMed; PubMed Central; Medline; Cumulative Index of Nursing and Allied Health Literature (CINAHL); Web of Science; Commonwealth Agricultural Bureaux (CAB) Direct; Health Source: Nursing Academic Edition; Clinicaltrials.gov; PROSPERO; and The Cochrane Library. Database searches yielded 85 articles. Of those, 25 met inclusion criteria: 17 rodent, 1 canine, 2 equine, and 5 human investigations. All rodent and equine studies included control groups; whereas, only one human study included a control group. Remaining investigations were cross-sectional or longitudinal. All rodent investigations controlled for dietary intake, and one human investigation implemented 3-d dietary control measures. Thirteen studies utilized forced exercise, and 11 assessed voluntary or habitual exercise or PA patterns. Diversification within the Firmicutes phylum was consistently observed in exercise groups across studies. There were no consistent trends within Bacteroidetes, Actinobacteria, or Proteobacteria. The potential interactions between dietary composition and exercise and their respective influences on the intestinal microbiome were not well characterized. Exercise appeared to influence gut microbiome composition in rodent models independent of diet, and was associated with an increased butyrate-producing bacteria in the gut microbiome of humans. Overall quality of evidence in humans was low, and risk of bias was unclear. Future investigations should

standardize reporting outcomes and control for diet, exercise duration, mode, and intensity, to further determine the relationship between exercise, gut microbiome composition, and health.

## INTRODUCTION

Chronic diseases are largely preventable by adoption of healthy lifestyle behaviors, such as regular physical activity (PA) [207] and healthy dietary intake patterns.[208] In contrast, sedentary behaviors [209], physical inactivity, [209,210] and consumption of a western diet (e.g. high-fat, high-sugar) have been associated with aging and the development chronic diseases.[209,211,212] Exercise[213] and PA[213] confer a wide range of health benefits, such as improved cardiorespiratory fitness, lower body mass index (BMI), and lower risk for chronic disease.[214] Many mechanisms by which physical inactivity and/or consumption of a western diet contribute to disease development and progression have been identified, including the dysbiosis of the gut microbiome.

The gut microbiome is a broad term that refers to colonization of bacteria, fungi, archaea, protozoa, and viruses within the gastrointestinal tract[37] Furthermore, an estimated 100 trillion microorganisms, and 1000 species of yeast, bacteria, and parasites inhabit the human gut microbiome.[36,38,39] The influences of dietary intake on the gut microbiome have been extensively studied.[215] For example, the microbiome assists in: fermentation, extraction, and facilitation of phytonutrient delivery to host organ systems, among its other functions.[41,42] The ability of the microbiome to accomplish this in a way that facilitates health largely depends on lifestyle, diet, environment, and genetic factors.[216,217] However, little information is available on other lifestyle behaviors (e.g. exercise and PA) that may shape the gut microbial composition. To date, there have been no *systematic* reviews evaluating the independent effects exercise and/or PA on the gut microbiome composition, although multiple narrative reviews have been written on the independent effects of exercise on the gut microbiome. Therefore, the objectives of this

systematic review were to identify research articles that evaluated the effect of exercise or PA on the gut microbiome, and to summarize translational findings to inform future research.

## METHODS

### *Study Inclusion and Exclusion Criteria*

The population, intervention, comparison, outcome, and study (PICOS) model was utilized to develop eligibility criteria for studies returned via search terms entered into online research databases, and criteria are presented in Table 8. The review protocol described herein has been registered with PROSPERO (registration number: CRD42018075833). Briefly, this review included experimental trials, randomized controlled trials (RCTs) and quasi-experimental designs (QEDs), including observational and cohort studies. Systematic reviews, nonsystematic reviews, meta-analyses, narrative reviews, and textbook publications were not included. However, reviews and meta-analyses were retained and reference lists were reviewed as an added measure to ensure search comprehensiveness. Studies whose primary objectives focused on quantitative or qualitative changes to gut microbial composition associated with exercise or habitual PA were included. The results of included studies were screened for qualitative or quantitative differences in α- and β-diversity, abundance/richness, evenness, the ratio of Bacteriodetes:Firmicutes, and shifts in bacterial taxa. These characteristics of gut microbiome changes are defined in Supplemental Digital Content 1 ([SC1] [Appendix D]).

We included all animal studies in mammals of all ages with the exception of those which focused on congenital conditions and those involving nutritional supplementation to enhance exercise performance. There were no further inclusion/exclusion criteria set.

**Table 8.** PICOS objectives

| | |
|---|---|
| **Population** | Mammalian models with or without chronic disease |
| **Intervention** | How does exercise/PA alter the intestinal microbiome composition in the presence of health or chronic disease and various habitual dietary conditions? |
| **Comparison** | What changes occur within the intestinal microbiome after acute or chronic exercise/PA interventions when compared to sedentary controls? |
| **Outcomes** | Microbial diversity, abundance/richness, evenness, and changes in gram +/- bacteria |
| **Study Design** | Randomized controlled trials, quasi experimental designs, observational, and cross-sectional studies |

*Database search strategies*

Search strategies were conducted based upon the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.[218] Databases utilized for this systematic review were: PubMed; PubMed Central; Medline via ProQuest; Web of Science; Cumulative Index to Nursing and Allied Health Literature (CINAHL) via EbscoHost; Science Direct via Elseveir; CAB Direct via CABI; and Health Source: Nursing/Academic Edition via EbscoHost. Search terms were also entered into ClinicalTrials.gov, Cochrane Library, and PROSPERO to ensure that trials with recently published data were included, and that no systematic reviews on this topic were previously published or registered. Databases were selected based upon both their coverage in biological and health sciences, and their coverage of national and international research. The search included articles published on or before January 5, 2018. In total, there were 36 search combinations, and Boolean search operators were used for each database. Search terms combinations are listed in Table 9, and refinement criteria across databases are presented in Table 10. An example of a complete search strategy for one database is presented in Table 11. After each search term combination was refined by title and abstract within a database, that search was downloaded and imported to EndNote x8.0.1.[219] The title and

abstract of each imported article were appraised by a single reviewer against the eligibility criteria outlined above. All articles that had a title or abstract, which appeared to meet eligibility criteria, were retained for full text review.

| **Table 9.** Search term combinations | |
|---|---|
| **Exercise and:** | |
| **Physical activity and:** | |
| **Fitness and:** | |
| **Sports and:** | · Microbiome<br>· Gut Microbiome<br>· Intestinal Microbiome<br>· Microbiota<br>· Gut Microbiota<br>· Intestinal Microbiota<br>· Microflora<br>· Gut Microflora<br>· Intestinal Microflora |

*Data Extraction, Data Synthesis, and Risk of Bias*

Data from articles retained for full-text review were independently extracted by a single reviewer (CM). Data extraction guidelines and recommendations published by The Centre for Reviews and Dissemination were used to prevent selection bias and to provide objective framework for inclusion/exclusion decisions.[220] The information collected from retained articles included: author, year, aims, sample size, participant or subject characteristics, methods, reported gut microbiome composition measures, significant taxonomy findings, and declared funding source. Study results were classified primarily by species, and secondarily by health status. Risk of bias was evaluated independently by two reviewers (CM and BD) using the Cochrane Risk of Bias AUB Kq1 Tool for each article included.[221] Bias levels are reported based upon the number of articles categorized into each level: high, low, or unclear, which are presented in Table 13.

**Table 10.** Search limits by database

| **Database** | Search Limits | |
|---|---|---|
| **PubMed** | Advanced searches: | Title |
| | | Abstract |
| | Document type: | Case report |
| | | Clinical study |
| | | All clinical trials |
| | | All journal articles |
| | | Randomized control trials |
| | | Meta-analyses |
| | | Reviews |
| | | Validation studies |
| **PubMed Central** | Advanced searches: | Title |
| | | Abstract |
| | Document type: | Not applicable |
| **Medline** | Advanced searches: | Title |
| | | Abstract |
| | Document type: | Case report |
| | | Clinical study |
| | | All clinical trials |
| | | All journal articles |
| | | Randomized control trials |
| | | Meta-analyses |
| | | Reviews |
| | | Validation studies |
| **Web of Science** | Advanced searches: | Title |
| | | Topic * |
| | Document type: | Articles |
| | | Reviews |
| **CINAHL** | Advanced searches: | Title |
| | | Abstract |
| | Document type: | Academic journals |
| **Science Direct** | Advanced searches: | Title |
| | | Abstract |
| | Document Type: | Journal articles |
| **CAB Direct** | Advanced searches: | Article title |
| | | Abstract |
| | Document type: | Journal article |
| **Health Source** | Advanced searches: | Article title |
| | | Abstract |
| | Document type: | Journal article |
| **Clinicaltrials.gov** | Advanced search: | Studies with results |
| | Document type: | Not applicable |
| **Cochrane Library** | Advanced search: | Title, Abstract, Keywords Ɨ |
| | Document type: | Cochrane reviews |
| | | Trials |
| | | Other reviews |
| | | Methods studies |
| **Prospero** | Filtered search: | Published review status |

* "Topic" was used in place of "abstract," as this option was unavailable as a limit within the database.
Ɨ "Title, Abstract, Keywords" are one singular search option, unable to be separated.

**Table 11.** Detailed PubMed search with Boolean search operator

| Search Term Combination |
| --- |
| ((exercise[Title/Abstract] AND microbiome[Title/Abstract]) OR (exercise[Title/Abstract] AND intestinal microbiome[Title/Abstract]) OR (exercise[Title/Abstract] AND gut microbiome[Title/Abstract]) OR (exercise[Title/Abstract] AND microbiota[Title/Abstract]) OR (exercise[Title/Abstract] AND intestinal microbiota[Title/Abstract]) OR (exercise[Title/Abstract] AND gut microbiota[Title/Abstract]) OR (exercise[Title/Abstract] AND microflora[Title/Abstract]) OR (exercise[Title/Abstract] AND intestinal microflora[Title/Abstract]) OR (exercise[Title/Abstract] AND gut microflora[Title/Abstract]) OR (physical activity[Title/Abstract] AND microbiome[Title/Abstract]) OR (physical activity[Title/Abstract] AND intestinal microbiome[Title/Abstract]) OR (physical activity[Title/Abstract] AND gut microbiome[Title/Abstract]) OR (physical activity[Title/Abstract] AND microbiota[Title/Abstract]) OR (physical activity[Title/Abstract] AND intestinal microbiota[Title/Abstract]) OR (physical activity[Title/Abstract] AND gut microbiota[Title/Abstract]) OR (physical activity[Title/Abstract] AND microflora[Title/Abstract]) OR (physical activity[Title/Abstract] AND intestinal microflora[Title/Abstract]) OR (physical activity[Title/Abstract] AND gut microflora[Title/Abstract]) OR (fitness[Title/Abstract] AND microbiome[Title/Abstract]) OR (fitness[Title/Abstract] AND gut microbiome[Title/Abstract]) OR (fitness[Title/Abstract] AND intestinal microbiome[Title/Abstract]) OR (fitness[Title/Abstract] AND microbiota[Title/Abstract]) OR (fitness[Title/Abstract] AND gut microbiota[Title/Abstract]) OR (fitness[Title/Abstract] AND intestinal microbiota[Title/Abstract]) OR (fitness[Title/Abstract] AND microflora[Title/Abstract]) OR (fitness[Title/Abstract] AND gut microflora[Title/Abstract]) OR (fitness[Title/Abstract] AND intestinal microflora[Title/Abstract]) OR (sports[Title/Abstract] AND microbiome[Title/Abstract]) OR (sports[Title/Abstract] AND intestinal microbiome[Title/Abstract]) OR (sports[Title/Abstract] AND gut microbiome[Title/Abstract]) OR (sports[Title/Abstract] AND microbiota[Title/Abstract]) OR (sports[Title/Abstract] AND gut microbiota[Title/Abstract]) OR (sports[Title/Abstract] AND intestinal microbiota[Title/Abstract]) OR (sports[Title/Abstract] AND microflora[Title/Abstract]) OR (sports[Title/Abstract] AND gut microflora[Title/Abstract]) OR (sports[Title/Abstract] AND intestinal microflora[Title/Abstract])) |

## RESULTS

An overview of search results and study flow throughout the initial identification, screening, eligibility, and inclusion process of the initial search is depicted in Figure 8. In total, 17,342 citations were identified from 11 electronic databases (Table 10) and 36 search term

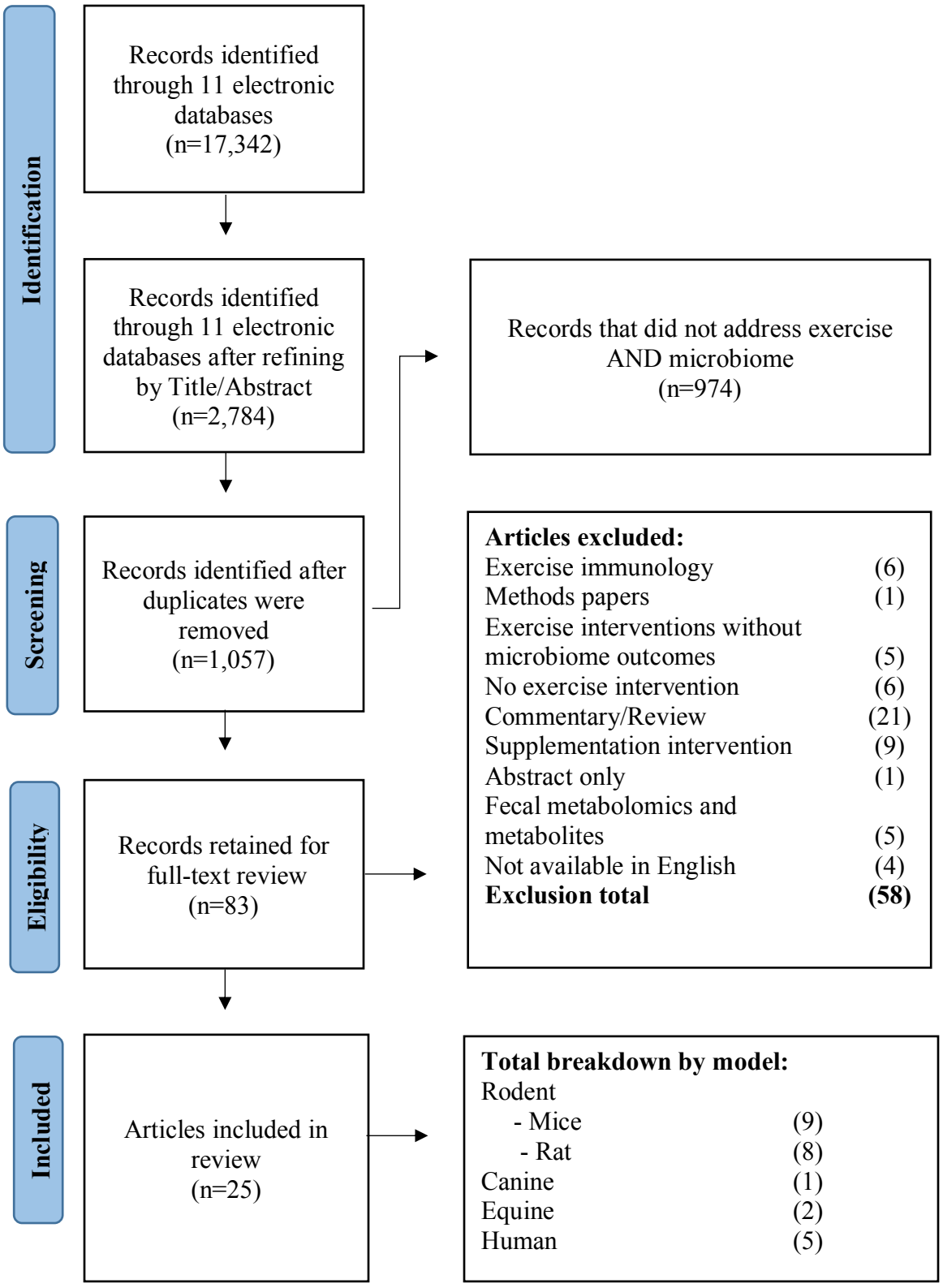



**Figure 8.** Flow diagram for systematic review search process.

combinations (Table 11). The search totals were further refined by title and abstract and totaled 2,784 articles. Duplicates were then removed and a total of 1,057 articles remained for screening. After screening and full text review, 25 articles were eligible for inclusion. A summary of finding table was generated and included author, year, aim, sample size, participant or subject characteristics, methods, study design, gut microbiome composition measures, significant taxonomy findings, and funding source for included articles, were categorized in descending order primarily by species and alphabetized secondarily publication year. Table data are provided in Supplemental Digital Content 2 ([SC2][Appendix E]).

**Table 12.** Risk of bias

| | High | Low | Unclear |
|---|---|---|---|
| | | | Evans<br>Kang<br>Allen, 2015<br>Lambert<br>Campbell<br>Denou<br>Houghton<br>Lamoureux<br>Liu, 2017<br>Matsumoto<br>Quiepo- Ortuño<br>Petriz<br>Liu, 2015<br>Mika<br>Welly<br>Batacan<br>Feng<br>Kieler<br>Janabi, 2016<br>Janabi, 2017<br>Clarke<br>Stewart<br>Allen, 2017<br>Bressa<br>Paulsen |

Each category represents that articles potential risk of bias according to the Cochrane AUB KQ1 Risk of Bias Assessment form.

*Bias assessment*

The risk of bias was unclear for all 25 articles. The primary reasons for unclear ratings for risk of bias included lack of sufficient details for random sequence generation, allocation concealment, blinding measures, and incomplete outcome data. Experimental design quality and reporting are issues that have been previously identified as problematic in animal research.[222] Appropriate tool selection for appraisal of translational research is challenging, as there no validated tools available to critically appraise clinical trials across all models used (e.g. cell vs. animal vs. human).[223]

*Rodent studies*

Nine studies included in this review focused on mouse models [224-232] and eight studies utilized rat models.[233-240] The rodent lines studied are detailed in SC2. Collectively, nine studies implemented dietary comparison groups that included high-fat diets (HFDs),[225-228,233,234,237] casein-sucrose blends,[238] and caloric restriction.[239] Of the seven remaining studies, seven fed a standard chow diet[224,229-232,235,240] and one did not report dietary composition.[236] The percentage of dietary fat (in 7 studies) ranged from 45%-60% fat, but the composition of dietary fat (e.g. % saturated fat) was not reported in any of the studies. The type of exercise was evaluated in two broad categories: voluntary and involuntary. Involuntary exercise was further categorized as either endurance training or interval training. Voluntary exercise (defined as unlimited access to a running wheel) was employed in nine studies, [224,225,227,231,234,235,237-239] and forced exercise was used in ten studies. [224,226,228-233,236,240] All studies used treadmills and walking or running as the mode of exercise or PA.

Two studies compared voluntary to forced exercise; [224,231] the remaining studies compared their respective exercise intervention group to sedentary controls. Thirteen

investigations focused on the effect of exercise in healthy rodent models[224-228,231,233-239] and six investigations focused on the effect of exercise in genetically- or environmentally-induced disease in rodents.[225,226,229,230,232,236] The models for disease conditions included high-fat diet-induced obesity (HFDIO),[225,226] genetically obese, [236] type 2 diabetes,[229] hypertension,[236] mitochondrial dysfunction,[230] and myocardial infarction.[232]

More robust diversification of sub-species within the Firmicutes phylum were reported in healthy exercise-trained animal models [224,225,227,228,233,235,237,238] and the results of one study indicated that rodents selectively bred for high aerobic capacity had greater diversification within the Firmicutes phylum when compared with their low aerobic capacity counterparts (See SC2).[234] In contrast, exercise was not consistently associated with robust diversification within Bacteroidetes, Actinobacteria, and Proteobacteria phyla in models of chronic disease compared with healthy controls.[229,230,236] There were no trends in taxonomy findings between exercise types (e.g. voluntary, endurance, and interval training). In addition, exercise appeared to exert independent shifts on various taxonomy within the gut microbiome, and may have attenuated the effects of unfavorable dietary conditions (e.g. mediating effects of a HFD or aging on gut microbiome composition and function).[225,227,228,237]

The association between exercise and community evenness was only reported in three studies.[224,233,235] The findings varied based upon index used for analysis.[224] Community evenness was not modified by training intensity.[233] Community richness was reported in five studies.[224,231,235-237] The findings varied based upon selected indices for analysis,[224] with no significant differences between forced versus voluntary exercise,[231] or forced exercise in obese rodents when compared to sedentary weight-matched controls.[237] However, increases in community richness in obese and hypertensive rats were not observed following forced

exercise,[236] and richness was reduced in juvenile rats following exercise when compared to their adult exercising counterparts.[235]

Significant shifts in relative abundance following exercise were reported for several investigations.[224,226-230,232-237,239,240] The results of many, [224,226,230,236,239,240] but not all[231,233,237] studies reported significant shifts in in α-diversity; the remaining did not address α-diversity.[225,227-229,238] β-diversity was reported for eight studies,[227,231-235,237,240] with two studies citing no significant changes[231,232] following exercise, and the remaining varying in direction of change. Lastly, shifts in the ratio of Bacteroidetes:Firmicutes were variably reported across studies with some reporting decreases,[228,240] increases,[226,227,230,235,239] and no change following exercise.[224,234,240] In the remaining studies changes in the ratio were either unclearly reported,[236] or not reported at all.[225,226,229,231-233,238,239]

*Large animal studies*

One investigation evaluated the influence of exercise on the gut microbiome during a weight loss protocol in canines.[241] Two companion studies assessed the acute and long-term effects of forced exercise in equines.[242,243] One study utilized dietary controls,[241] and two studies did not.[242,243] All studies used forced treadmill exercise.[241-243] Evenness and richness were not reported for canine or equine investigations. However, in canines relative abundance appeared to peak midway through the exercise intervention.[241] There were no significant trends for α-diversity in canines, and β-diversity was not reported.[241] Relative abundance, α-diversity, and significant β-diversity shifts in equines were noted at several time points across 12-weeks of exercise,[242] but there were no shifts attributed to acute exercise.[243] Phylogenetic changes associated with exercise were observed for Bacteroidetes, Firmicutes, Proteobacteria, and Spirochaetes,[242] but did not result in greater diversification.

*Human studies*

Five human studies included for review assessed the association of exercise or PA and the gut microbiome composition.[244-248] Four studies included healthy adults.[244-246,248] Three studies included adults with obesity,[244] adults with a diagnosis of breast cancer that were post-primary treatment,[247] or well-managed type-1 diabetes.[248] Only one study in lean and obese adults implemented dietary controls for a three day period leading up to stool sample collection.[244] The remaining investigations utilized 24-hour recalls,[248] food frequency questionnaires,[245,246] and three day food intake records to account for dietary intake.[247] Methods for exercise and/or physical activity quantification included: physical activity questionnaires;[246] self-reported physical activity levels;[247] accelerometry;[245] one did not specify how engagement in regular exercise or PA was determined;[248] and, only one trial utilized an exercise intervention and measured changes after a six-week intervention.[244]

Evenness was not reported for any human investigations. Only one study addressed richness, which did not appear to be associated with PA level.[245] Relative abundance for a variety of bacteria were reported, and abundance was consistently higher in exercise-trained and physically active adults when compared to sedentary adults.[244-246,248] Alpha-diversity was greater in athletes when compared to controls,[246] but no other investigations found differences among their respective groups. Cardiorespiratory fitness was associated with higher β-diversity in one investigation[247] but was not significantly different in physically active adults compared to their sedentary counterparts in another study.[245] However, the observable changes in β-diversity associated with exercise training appeared to be dependent on obesity status and became more similar in obese compared with lean following the intervention,[244] and were similar between normoglycemic adults when compared to adults with well-managed type 1 diabetes.[248] There

were no significant shifts in the ratio of Bacteroidetes:Firmicutes in adults who habitually exercised when compared to sedentary individuals.[245] The remaining studies in humans did not report on this ratio.[244,246-248] Overall, generalizability across studies is limited, given the wide variety of study methods, outcomes reported, and populations studied.

**DISCUSSION**

To our knowledge, this is the first systematic review of the translational evidence addressing whether exercise is associated with altered gut microbial community structure. Our review was limited to studies in healthy mammals and models of chronic disease. In rodents, there was no consistent association between exercise and the direction of change in specific phyla comprising the gut microbiome. However, exercise was associated with an increase in butyrate producing bacteria in multiple studies. There was no consistent impact of exercise on shaping the gut microbiota in the very small number of studies in large animals. The evidence supporting an association between exercise and altered gut microbial communities in humans was inconsistent and low quality. The inconsistency in reporting precluded an objective risk of bias categorization.

In general, there was a lack of consistent evidence supporting a role for exercise in modifying specific taxonomic groups or indices of richness, diversity, or evenness among studies in rodents. Differences in study design, choice of animal strain, mode, intensity, and duration of exercise, dietary factors, and inconsistency in reporting may have contributed to the discrepant findings among studies. However, there was a tendency for exercise to be associated with an increase butyrate producing bacteria which may be important given the role of these taxa in contributing to intestinal barrier function and colon health.[249-251]

There were only three studies in large animals that were identified for inclusion in our review. In dogs, the addition of exercise to diet-induced weight loss had no discernable impact on gut microbial community structure.[241] In contrast, exercise training in horses was associated with phyla level changes in Bacteriodetes, Proteobacteria, and Spirochaetes.[242,243] Perhaps not surprisingly, an acute bout of exercise did not appear to exert an obvious influence on gut microbial community composition in horses.

The evidence linking exercise or physical activity with alterations in gut microbial community structure in humans was low quality and inconsistent. Furthermore, it was difficult to disentangle the relative influence of exercise and diet on gut microbial communities in most studies.[244-248] For example, Clarke et al.[246] reported that α-diversity and the proportion of numerous taxa were higher in professional rugby athletes compared with weight-matched sedentary control participants. However, the differences in gut microbial community structure in between groups could not be attributed solely to differences in exercise, i.e., habitual dietary intake was also different between the groups. Only one randomized controlled trial was identified and included in our review. Allen et al.[244] reported that six weeks of endurance exercise training was associated with an increase in β-diversity that was dependent on obesity status. β-diversity was lower in obese individuals at baseline but not different from lean individuals following exercise training. Several bacterial taxa and fecal short chain fatty acid concentrations were also increased following exercise training and returned toward baseline with return to a sedentary state. Importantly, dietary intake was controlled for 3 days prior to stool collection at baseline and follow-up in attempt to minimize the potential confound of differences in habitual dietary intake on gut microbial composition. However, to our knowledge there are no

published studies that have controlled dietary intake during the entire course of an exercise training intervention. This would be an important element of future studies on this issue.

There are several aspects of our systematic review that should be emphasized. Our review is the first to be registered with PROSPERO to address this body of literature and utilizing a comprehensive search strategy with a broad selection of national and international databases. The implementation of PRISMA standards assured that relevant articles were not missed and review methods were standardized and reproducible. The inconsistency in reporting in animal studies was variable and precluded objective categorization and interpretation for risk of bias.[222] There were five studies in humans that were included in our review and only one was a randomized controlled trial. In general, the risk of bias was unclear using the Cochrane AUB Kq1 Risk of Bias Assessment tool;[221] insufficient detail was provided regarding random sequence generation, allocation concealment, and blinding related to performance and detection bias. Taken together, the quality of evidence for this body of literature was considered low.

Recent advances in DNA sequencing and computational technology has resulted in rapid growth in the number of studies exploring the role of exercise in shaping gut microbial community structure. Methods to improve taxonomic resolution have rapidly evolved and as a consequence the volume and detail of data generated in a single study has increased. While these biotechnology advances are necessary for this field to move forward comparisons across a decade of microbiome research is challenging given the inherently limited taxonomy data reported in earlier studies. The mouse microbiome has only been extensively characterized recently.[252] The limited information available from earlier mouse studies reporting only cecal contents [229,234,237,238] may be a source of discrepancy among studies. In addition, there was little

overlap between the first reference database for the mouse gut microbiota with human gut microbial diversity.[252]

There are several caveats that should be considered when attempting to translate and compare exercise and gut microbiome studies in mice and humans. First, there are significant difference in the anatomy and physiology of the mouse and human gastrointestinal tract.[253] Second, a large fraction of bacterial taxa found in the mouse gut are not present in humans.[253] Third, inconsistency in sample site in the mouse studies include our review further limit the translatability to humans. Future studies in "humanized" gnotobiotic mouse models may provide additional translational insight into determining whether an exercise trained phenotype is transmissible via microbiota transplant.

There are several opportunities that could be considered to advance our understanding of the role of exercise in shaping the gut microbiota. First, future research should use standardized reporting methods to improve the quality of evidence and reduce risk of bias, particularly in animal studies.[254,255] Second, energy intake and macronutrient composition (e.g. carbohydrate and fat composition) should be controlled and reported to minimize the potential confounding of habitual dietary intake on the gut microbial community composition. Third, the characteristics of exercise training (e.g. mode, frequency, intensity, and dose) need to be considered with translation in mind. Finally, future research is needed on the influence of exercise on the function of specific taxa while taking advancing of metabolomic, proteomic, and transcriptomic approaches. The field has moved beyond describing qualitative changes in gut microbiome composition.[256,257]

## CONCLUSIONS

The investigation of the gut microbiome in health and disease is a rapidly growing area of investigation. However, the impact of exercise on the gut microbiome is poorly understood due, at least in part, to the limited number and low quality of studies in this area. Although the available evidence would suggest that exercise appears to alter gut microbial composition, the metrics (e.g. abundance, evenness, richness, and diversity) were not consistently reported across studies.[225,238,245,246,248] Exercise does appear to be associated with an increase in butyrate-producing bacteria (e.g. *Rosueburia, hominis, Faecalibacterium pausnitzii,* and *Ruminococcaceae*) primarily in rodent models[224,225,227,234,235,239,245] and a limited number of studies in humans.[245,246] The latter may be an important mechanism for improving intestinal and metabolic health with exercise. Future research should focus on the practicality and feasibility of interventions in humans to identify the differential influences and relationship between exercise and PA on gut microbiome composition and their potential respective impact(s) on human health.

## Chapter 5. Conclusions and future directions.

The gut microbiome is a novel area of research that is continuously evolving. The potential impacts that modifiable lifestyle factors (e.g. diet and exercise) have on shaping and re-shaping the gut microbiome are still not well understood. Furthermore, the ways by which the gut microbiome may influences chronic disease risk and how disease risk may in-turn influence the gut microbiome are unknown. Evidence from this pilot RCT demonstrate that prebiotic supplementation with inulin was well tolerated and selectively targets *Bifidobacteria* in the gut microbiome. However, the lack of observable changes in remaining outcome variables suggest that the *Bifidogenic* effect of inulin does not mitigate risk for T2D in overweight or obese adults who are not prediabetic.

A systematic review of literature indicated that exercise does appear to influence the gut microbiome of animals and humans by increasing short-chain fatty acid biosynthesis (e.g. production of butyrate) in the gut microbiome. [224,225,227,234,235,239,245] Although the discrepancies across reporting outcomes, quality of evidence, and statistical approaches from these investigations limits the interpretations that can be made from reported findings. Only one study in humans has evaluated the relationship between exercise and the gut microbiome with implemented dietary controls.[237] Therefore the influence of diet on the relationship between exercise and the gut microbiome in humans is also not well understood.

Currently the mechanisms by which inulin may mitigate or diminish chronic disease risk in humans are not well established. Similarly the effects of exercise on gut microbiome composition and function is a novel area of research. The effects of exercise on the composition and function of the gut microbiome and how this relationship influences overall disease risk are unknown. Mechanistic studies including: evaluation of the impact of inulin supplementation on

disease risk in diverse populations; exploration of the relationship between exercise and the gut microbiome under varied controlled diet conditions; and the "–omic" impact (e.g. metabolomics, proteomic, transcriptomic, etc.) of these interventions should be considered. In summary, more investigations are needed to elucidate how the combined effects of prebiotics, dietary intake, and exercise influence the gut microbiome and subsequently alter chronic diseases risk in humans.

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**MEMORANDUM**

**DATE:** February 11, 2015

**TO:** Kevin Davy, Brenda Davy, Andrew P Neilson, Matthew Wade Hulver, Monica Anne Ponder, Jose Rivero M.D., Nabil Eskandar Boutagy, Kris Osterberg, Karen M Strat, Cassie Marie Mitchell, et. al.

**FROM:** Virginia Tech Institutional Review Board (FWA00000572, expires April 25, 2018)

**PROTOCOL TITLE:** Prebiotic Supplementation and Cardiometabolic Health in Prediabetic Adults

**IRB NUMBER:** **13-694**

Effective February 9, 2015, the Virginia Tech Institution Review Board (IRB), at a convened meeting, approved the Amendment request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at:

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(Please review responsibilities before the commencement of your research.)

**PROTOCOL INFORMATION:**

| | |
|---|---|
| Approved As: | **Full Review** |
| Protocol Approval Date: | **September 9, 2014** |
| Protocol Expiration Date: | **September 8, 2015** |
| Continuing Review Due Date*: | **July 27, 2015** |

*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

**FEDERALLY FUNDED RESEARCH REQUIREMENTS:**

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If this IRB protocol is to cover any other grant proposals, please contact the IRB office (irbadmin@vt.edu) immediately.

**Office of Research Compliance**
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Blacksburg, Virginia 24061
540/231-4606 Fax 540/231-0959
email irb@vt.edu
website http://www.irb.vt.edu

**MEMORANDUM**

**DATE:** January 14, 2016

**TO:** Kevin Davy, Brenda Davy, Andrew P Neilson, Matthew Wade Hulver, Monica Anne Ponder, Jose Rivero M.D., Karen M Strat, Cassie Marie Mitchell, Elaina Lynn Marinik, Madlyn Irene Frisard, et. al.

**FROM:** Virginia Tech Institutional Review Board (FWA00000572, expires July 29, 2020)

**PROTOCOL TITLE:** Prebiotic Supplementation and Cardiometabolic Health in Prediabetic Adults

**IRB NUMBER:** **13-694**

Effective January 14, 2016, the Virginia Tech Institution Review Board (IRB) Chair, David M Moore, approved the Amendment request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

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| Approved As: | **Full Review** |
| Protocol Approval Date: | **September 9, 2015** |
| Protocol Expiration Date: | **September 8, 2016** |
| Continuing Review Due Date*: | **July 25, 2016** |

*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

**FEDERALLY FUNDED RESEARCH REQUIREMENTS:**

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**MEMORANDUM**

**DATE:** July 21, 2016

**TO:** Kevin Davy, Brenda Davy, Andrew P Neilson, Matthew Wade Hulver, Monica Anne Ponder, Jose Rivero M.D., Karen M Strat, Cassie Marie Mitchell, Elaina Lynn Marinik, Madlyn Irene Frisard, et. al.

**FROM:** Virginia Tech Institutional Review Board (FWA00000572, expires January 29, 2021)

**PROTOCOL TITLE:** Prebiotic Supplementation and Cardiometabolic Health in Prediabetic Adults

**IRB NUMBER:** **13-694**

Effective July 21, 2016, the Virginia Tech Institution Review Board (IRB) Chair, David M Moore, approved the Amendment request for the above-mentioned research protocol.

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| Approved As: | **Full Review** |
| Protocol Approval Date: | **September 9, 2015** |
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| Continuing Review Due Date*: | **July 25, 2016** |

*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

**FEDERALLY FUNDED RESEARCH REQUIREMENTS:**

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**MEMORANDUM**

**DATE:** August 8, 2016

**TO:** Kevin Davy, Brenda Davy, Andrew P Neilson, Matthew Wade Hulver, Monica Anne Ponder, Jose Manuel Rivero, Karen M Strat, Cassie Marie Mitchell, Elaina Lynn Marinik, Madlyn Irene Frisard, et. al.

**FROM:** Virginia Tech Institutional Review Board (FWA00000572, expires January 29, 2021)

**PROTOCOL TITLE:** Prebiotic Supplementation and Cardiometabolic Health in Prediabetic Adults

**IRB NUMBER:** **13-694**

Effective August 8, 2016, the Virginia Tech Institution Review Board (IRB), at a convened meeting, approved the Continuing Review request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

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| Approved As: | **Full Review** |
| Protocol Approval Date: | **August 8, 2016** |
| Protocol Expiration Date: | **August 7, 2017** |
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**FEDERALLY FUNDED RESEARCH REQUIREMENTS:**

Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

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# The effect of prebiotic supplementation with inulin on cardiometabolic health: Rationale, design, and methods of a controlled feeding efficacy trial in adults at risk of type 2 diabetes

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ABSTRACT

Prediabetes is associated with low-grade chronic inflammation that increases the risk for developing type 2 diabetes (T2D) and cardiovascular disease (CVD). An elevated lipopolysaccharide concentration, associated with dysbiosis of the intestinal microbiota, has been implicated in the development of both T2D and CVD. Selective modulation of the intestinal microbiota with prebiotics reduces intestinal permeability and endotoxin concentrations, inflammation, and metabolic dysfunction in rodents. The effect of prebiotic supplementation on cardio-metabolic function in humans at risk for T2D is not known. The primary aim of this trial is to determine the influence of prebiotic supplementation with inulin on insulin sensitivity and skeletal muscle metabolic flexibility in adults at risk for T2D. We hypothesize that prebiotic supplementation with inulin will improve insulin sensitivity and skeletal muscle metabolic flexibility. We will randomize 48 adults (40–75 yrs) with prediabetes or a score ≥5 on the American Diabetes Association (ADA) risk screener to 6 weeks of prebiotic supplementation with inulin (10 g/day) or placebo. Subjects will be provided with all food for the duration of the study, to avoid potential confounding through differences in dietary intake between individuals. Intestinal permeability, serum endotoxin concentrations, insulin sensitivity, skeletal muscle metabolic flexibility, endothelial function, arterial stiffness, and fecal bacterial composition will be measured at baseline and following treatment. The identification of prebiotic supplementation with inulin as an efficacious strategy for reducing cardio-metabolic risk in individuals at risk of T2D could impact clinical practice by informing dietary recommendations and increasing acceptance of prebiotics by the scientific and medical community.



## 1. Introduction

In 2012, approximately 86 million U.S. adults aged 20 years and older had prediabetes [1]. Low-grade chronic inflammation plays an integral role in the pathogenesis of atherosclerosis [2–4], and may increase risk for development of type 2 diabetes (T2D) [5–8] and cardiovascular disease (CVD)-related events [4,5]. Numerous studies in animal models [9–13] and humans [14–18] have implicated the intestinal microbiota (i.e., bacteria residing in the gastrointestinal tract) in the pathophysiology of obesity and T2D.

Studies in both human and rodent models suggest that the consumption of a high fat/high sugar, westernized diet may lead to changes in the composition/activity of the gut microbiota (e.g., depletion of Bifidobacteria), increase intestinal permeability to luminal antigens, and cause metabolic endotoxemia, i.e., elevated endotoxin concentrations [9,11,19,20]. In turn, metabolic endotoxemia is associated with the development of a low-grade chronic inflammatory state, obesity and insulin resistance in rodents [9,11]. In humans, endotoxin concentrations are higher in prediabetes and T2D compared with normoglycemic individuals. [21,22] In addition, elevated endotoxin concentrations are associated with an increased risk of incident T2D [23]. Importantly, Frisard et al. [24] demonstrated that skeletal muscle, the primary site of insulin stimulated glucose disposal, is a target for circulating endotoxin. Furthermore, low dose endotoxin concentrations,

*Abbreviations:* T2D, Type 2 diabetes; CVD, Cardiovascular disease; ADA, American Diabetes Association; BP, Blood pressure; OGTT, Oral glucose tolerance test; IVGTT, Intravenous glucose tolerance test; FMD, Flow mediated dilation; EID, Endothelial independent vasodilation; C–F, Carotid–femoral; PWV, Pulse wave velocity; SSN, Suprasternal notch; ANCOVA, Analysis of covariance; CHD, Coronary heart disease; FBG, Fasting blood glucose.

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1551-7144/

consistent with metabolic endotoxemia, activate skeletal muscle TLR4 resulting in state of metabolic inflexibility consistent with that observed in insulin resistance and T2D [25,26].

Several lines of evidence implicate the gut microbiota in mediating CVD risk [27]. First, serum endotoxin increases progressively with the number of metabolic syndrome components [23] and with the 10-yr. CVD risk score [28]. Second, endothelial dysfunction, an early step in atherogenesis, occurs following endotoxin exposure in humans [29–31]. In addition, elevated serum endotoxin is independently associated with arterial stiffness [32]. Finally, chronic elevations in endotoxin have been associated with carotid intima media thickening and incident CVD [33,34]. These findings suggest that gut dysbiosis and the subsequent increases in circulating endotoxins may lead to adverse changes in vascular function and structure, and elevated CVD risk.

Consumption of non-digestible polysaccharides (i.e. prebiotics) is an effective way to improve overall gut health [35,36]. Prebiotics are "selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring health benefit(s) upon host health." [37] Commonly used prebiotics include inulin-type fructans, fructo-oligosaccharides, xylo-oligosaccharides and galacto-oligosaccharides [38]. In rodents, the proliferation of a targeted bacterial species (i.e. *Bifiodobacterium* spp. and *Lactobacillus* spp.) contributes to host cardio-metabolic health by reducing intestinal permeability, endotoxin concentration, and pro-inflammatory cytokines [38–41]. In humans, the prebiotic inulin appears to be particularly efficacious in increasing the abundance of Gram-positive Bifidobacteria, while decreasing the proportions of Gram negative bacteria (see reviews [42,43]). However, the potential benefits of prebiotic supplementation on cardio-metabolic dysfunction in humans are unclear.

The identification of inulin supplementation as a simple and efficacious strategy for reducing cardio-metabolic risk in individuals at risk for T2D could impact clinical practice by informing dietary recommendations and increasing acceptance of prebiotics by the scientific and medical community. In turn, our findings could lead to enhanced adoption and maintenance of inulin supplementation as a cardio-metabolic risk-reducing behavior in individuals at risk for T2D.

## 2. Aims and hypotheses

The general aim of this clinical efficacy trial is to determine the effect of prebiotic supplementation with inulin on cardio-metabolic function in those at risk for T2D. For reference, a conceptual framework figure has been provided (Fig. 1) along with the specific aims and hypotheses:

Aim 1: To determine whether prebiotic supplementation with inulin improves insulin sensitivity and skeletal muscle metabolic flexibility in individuals at elevated risk of developing T2D. We hypothesize that prebiotic supplementation with inulin will improve insulin sensitivity and skeletal muscle metabolic flexibility in these individuals.

Aim 2: To determine whether prebiotic supplementation with inulin will reduce arterial stiffness and improve endothelial function in individuals at elevated risk of developing T2D. We hypothesize that prebiotic supplementation with inulin will reduce arterial stiffness and improve endothelial function in these individuals.

Aim 3: To determine the relationship between changes in the gut microbiota (i.e., the abundance of important groups of bacteria such as Bifidobacteria), intestinal permeability, and endotoxin concentration with prebiotic supplementation. We hypothesize that the magnitude of change in Gram-positive gut microbiota, intestinal permeability, and endotoxin concentrations with prebiotic supplementation will be correlated with the magnitude of change observed in the aforementioned metabolic and cardiovascular variables. The primary outcome is change in insulin sensitivity following treatment. Secondary outcomes include skeletal muscle metabolic flexibility, arterial stiffness, and endothelial function.

## 3. Study design

### 3.1. Overview

The Virginia Tech Institutional Review Board has approved the study protocol. The nature, purpose, risks and benefits of the study will be explained to each potential participant before obtaining written and verbal informed consent. We will include 48 adults with prediabetes or at increased risk for T2D, as determined by the American Diabetes

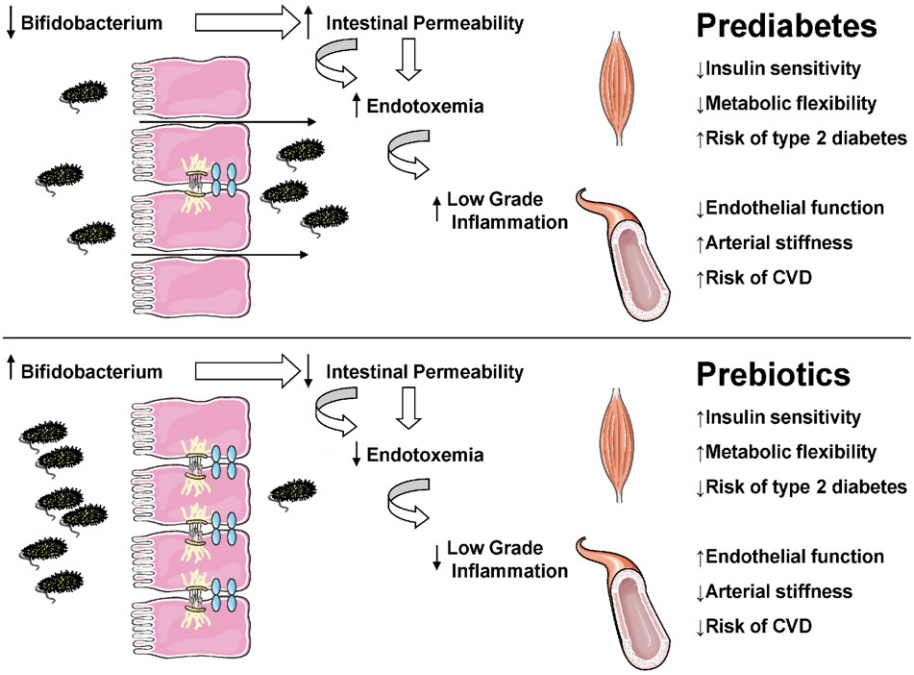


**Fig. 1.** Conceptual framework for hypothesis testing.

**Table 1**
Prebiotic supplementation and cardiometabolic health: participant eligibility criteria.

| |
|---|
| Inclusion criteria: |
| • Population: men and women 40–75 years of age |
| • Sedentary to recreationally active (i.e., no program of regular aerobic exercise (<2 days/wk and <20 min/d) for the previous 12 months) |
| • BMI: 25–40 kg/m$^2$ |
| • Prediabetic or at increased risk: ADA screener (score ≥ 5), HbA1c (5.7–6.4 mg/dl), FBG (100–125 mg/dl) or OGTT (140–200 mg/dl) |
| • Doctor's note for patients with CHD |
| • Blood pressure: ≤160/100 mm/Hg |
| • Total cholesterol and triglycerides: ≤300 mg/dl and ≤450 mg/dl |
| Exclusion criteria: |
| • Current smokers |
| • Diabetic or use of diabetes medications (i.e., metformin or insulin) |
| • Health history of respiratory disease, unstable CHD (i.e., chest pain or heart failure), inflammatory bowel disease, cancer, neurological or hematological disorders, or substance abuse |
| • Antibiotic use in the past 3 months |
| • Current enrollment in other research studies |
| • Currently pregnant or intent to become pregnant |
| • Currently taking prescribed NSAIDs, antioxidants, or fiber supplements |
| • Recent surgery |
| • Food allergies, intolerances, and/or religious, cultural, or other dietary restrictions |

Note: BMI = body mass index; ADA = American Diabetes Association; FBG = fasting blood glucose; OGT = oral glucose tolerance; CHD = coronary heart disease. NSAID = non-steroidal anti-inflammatory drugs.

Association (ADA) Diabetes Risk Screener [44]. Eligibility and exclusion criteria are presented in Table 1. Participants will be randomized to 6 weeks of prebiotic supplementation (inulin) or a placebo (maltodextrin), and will be provided with all of their food and beverages for the 6-week intervention period to avoid the potential confounding effects of differences in dietary intake on the gut microbiome. Measurements of outcome variables will be performed at baseline and following 6 weeks of treatment (Fig. 2).

A double-blind, placebo-controlled, parallel group design will be utilized for the present study. All participants who successfully complete the screening process will be enrolled in the study. Following baseline measurements, individuals will be randomized to one of two groups: supplementation with 10 g/d of inulin, or with 10 g/d of maltodextrin (placebo) for 6 weeks. Product details are provided in the following section. The supplements will be mixed into water and provided with the supervised breakfast meal consumed in the laboratory dining facility. An inulin dose of 10 g/d was selected as the prebiotic type and dose because it is well-tolerated [45] and increases fecal Bifidobacteria within as few as 2 weeks [42,43]. Importantly, Bifidobacteria are the most common prebiotic target [42,43] and have been shown to reduce endotoxin concentrations and improve gut barrier function [46–49]. The 6-week feeding period duration was selected to allow adequate time for changes in the gut microbiota to exert its hypothesized effects and to improve the feasibility of diet adherence while considering the participant burden associated with a controlled feeding study. Randomization will be stratified by gender under the supervision of an individual not involved in the collection or analysis of the study data. Separate randomization schemes will be developed for males and females to ensure equal numbers within each gender strata. Individuals performing outcome measurements will be appropriately blinded.

### 3.2. Product standardization and palatability testing

Participants will be supplemented with 10 g/d of either inulin (Frutafit® IQ, Sensus American, Inc., Lawrenceville, NJ; 2 kcal/g) or placebo (maltodextrin; 4 kcal/g) for 6 weeks. All participants will begin taking inulin or the placebo at a 5 g loading dose the first 7 days prior to taking to full 10 g amount. Frutafit® IQ is composed of 100% inulin from chicory root. The nutrient composition of the supplements will be accounted for in the standardized diets (Section 3.3) by using an average of 30 kcal and 10 g carbohydrate per day. By comparison, usual intake of inulin in the US population is 2.6 g/d, primarily from wheat products and onions [50]. Fruitafit IQ was obtained from a single lot for the entire study along with a certificate of analysis documenting the composition and purity of the product.

Prior to the onset of the trial, the palatability and solubility of both supplements (10 g dose) in water and in orange juice (one cup) were assessed by the research staff to determine the optimal supplement delivery mode. When mixed into either water or orange juice, both

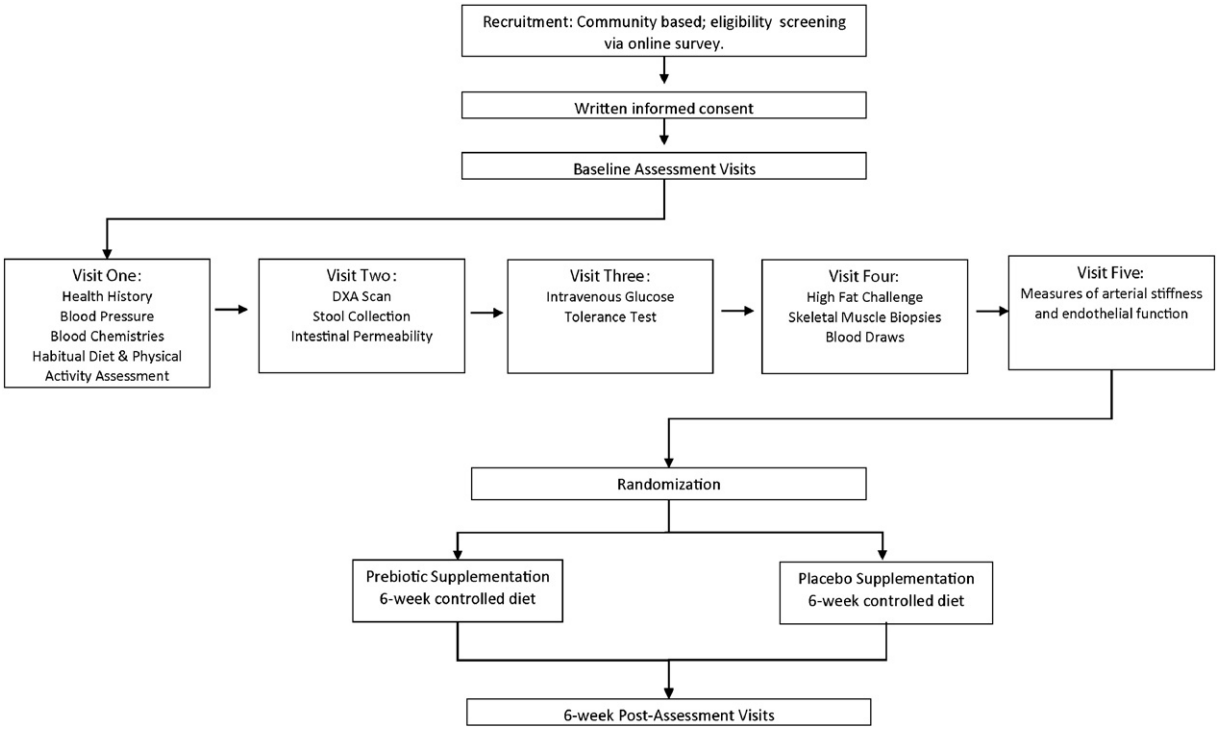


**Fig. 2.** Study design schematic.

supplements were tasteless and acceptable, and both dissolved well in either beverage. Due to the calorie-controlled nature of the study diets, water was selected as the beverage for supplement delivery.

### 3.3. Diet design and standardization

All participants will be fed a standardized diet (55% carbohydrate, 30% fat [8% saturated fat], 15% protein) isocaloric to their individual energy requirements for 6 weeks to avoid the potential confounding effect of individual differences in dietary intake on the gut microbiota. The standardized diets will be prepared in the Metabolic Kitchen and Dining Laboratory at Virginia Tech, by ServSafe®-certified research assistants, and consist of a 7-day cycle of menus with 3 meals and a snack. Menus will be developed for each day for each of the following 5 cal levels: 1500 kcal, 2000 kcal, 2500 kcal, 3000 kcal, 3500 kcal. An example of a one-day menu for the 2000 kcal level with nutrient information is provided in Table 2. Probiotic foods (e.g., yogurt) will be excluded from the diet. Daily soluble fiber intake will be maintained at ≤2 g/1000 kcal and total dietary fiber will be maintained at or below the US daily average intake of 8 g/1000 kcal [51–54]. Sodium intake will be maintained at less than 3000 mg/d, except for the 3500 kcal level, which will be under 3500 mg/d [55,56].

The process used for menu development was as follows. First, a list of readily available food items (i.e., from local grocery stores, commercial food suppliers) was selected for inclusion in the controlled diets by a research dietitian. The label information for each food item selected was then matched to a comparable item in the nutritional analysis software's database (Nutrition Data Systems for Research; NDS-R, Nutrition Coordinating Center, University of Minnesota), that provided detailed nutrient composition information on each food item used in the controlled diet. The research dietitian then developed 7 days of menus to meet the daily calorie and nutrient targets for each of the 5 kcal levels. Individual macronutrients were considered acceptable if they were ± 5 g of the daily targeted amount, with the exception of soluble fiber, that was ± 1 g of the daily targeted amount. The menus were then reviewed by a second research dietitian to verify that the daily energy and nutrient target levels were achieved, and that the proposed food portions were reasonable. After verification that daily energy and nutrient target levels were achieved, daily food preparation forms were developed for each day of the controlled diet that provided gram amounts for each food item and preparation instructions for the metabolic kitchen research assistants.

Energy requirements for each participant will be determined using estimated resting energy expenditure based upon age, weight, height, and sex multiplied by an activity factor based on self-reported physical activity levels [57]. Participants will consume breakfast in our dining facility and be given their daily dose of inulin or placebo (mixed into water) at this supervised meal for each day of the controlled diet. The remainder of their meals for the day will be taken with them in a large portable cooler bag. The menu and instructions (i.e., for food preparation; to consume all foods provided, etc.) for the day will be included. Any uneaten items will be returned to the metabolic kitchen the following day, weighed by the research assistants, and recorded on the food preparation sheet for that participant. Participants will be blinded from their weight and weighed at each visit during the controlled feeding period, and any trend of >1.0 kg weight loss or gain over a 3-day period will be countered by the addition or subtraction of 250 kcal food modules (e.g., 45 g low-sodium saltines, 20 g Swiss cheese) with the same macronutrient composition as the overall diet. Participants will be permitted to consume no more than three 6 fl oz. caffeinated beverages daily [58,59]. Caffeinated beverages allowed during the 6-week controlled feeding period will consist of unsweetened tea and coffee, which will be brewed in the metabolic kitchen. At each visit, participants will be asked to report if any non-study food or beverage were consumed since the preceding laboratory visit. Participants were instructed not to consume food and beverages (excluding water) outside the study diet. Participants who repeatedly (>3 d/week) fail to consume 100% of the prescribed diet will be excluded.

### 3.4. Participant recruitment and screening

Recruitment will take place over a 2-year period. Direct mailers, advertisements in local newspapers, campus email listservs, and posted flyers will be utilized as recruitment methods. Individuals who contact the research coordinator will be emailed a link to an online screening survey to determine if they meet basic eligibility criteria (age, body mass index and medical/supplement use). Those who meet these basic eligibility criteria will be sent the informed consent form that provides details about the study requirements and an initial in-person visit will be scheduled. During this visit verbal and written consent and,

**Table 2**
Sample 2000 kcal menu.

| Food item (approximate volumetric quantity) | Gravimetric quantity (g) | Energy (kcal) | Protein (g) | Carbohydrate (g) | Fat (g) | Saturated fat (g) | Fiber (g) | Sodium (mg) |
|---|---|---|---|---|---|---|---|---|
| *Breakfast [bagel with cream cheese, and apple juice]* | | | | | | | | |
| Plain, white bagel (1 bagel) | 81 | 207.7 | 8.1 | 40.9 | 1.3 | 0.3 | 1.8 | 418.8 |
| Strawberry cream cheese (1.8 tbs) | 28 | 78.8 | 0.9 | 4.4 | 6.4 | 4.0 | 0.04 | 97.4 |
| 100% apple juice, from concentrate (1 cup) | 240 | 137.1 | 0.2 | 33.4 | 0.3 | 0.1 | 0.2 | 10 |
| *Lunch [sandwich with cookies, and lemonade]* | | | | | | | | |
| White bread (2 slices) | 86 | 231.4 | 6.6 | 43.6 | 3.4 | 0 | 2 | 586 |
| Low sodium roast beef, deli slices (4 slices) | 100 | 189.4 | 36.1 | 0 | 5 | 1.7 | 0 | 44.6 |
| Low sodium Swiss cheese, deli slices (1 slice) | 28 | 106.2 | 7.5 | 1.5 | 7.8 | 5.0 | 0 | 4 |
| Mayonnaise (2 tbs) | 25 | 182.2 | 0.2 | 0.8 | 19.8 | 3.0 | 0 | 142.3 |
| Vanilla wafer cookies, mini (19 cookies) | 33 | 149 | 1.2 | 20.3 | 7.0 | 2.0 | 0.6 | 82 |
| Lemonade (2 cups) | 480 | 213 | 0.4 | 52.4 | 0.2 | 0 | 0 | 20 |
| *Dinner [pasta with Alfredo sauce]* | | | | | | | | |
| Penne pasta, cooked in unsalted water (1 cup) | 139 | 215.7 | 8.1 | 42.9 | 1.3 | 0.2 | 2.5 | 1 |
| Alfredo sauce (0.75 cup) | 61 | 94.5 | 3.9 | 5.1 | 6.5 | 4.0 | 0.8 | 282 |
| *Snack* | | | | | | | | |
| Graham crackers, honey-flavored (2 crackers) | 30 | 129 | 2.3 | 23.2 | 3.0 | 0.5 | 1.2 | 183.8 |
| *Supplement* | | | | | | | | |
| Inulin/placebo | 10 | 30 | 0 | 10 | 0 | 0 | 0 | 0 |
| Total | 1341 | 1944 | 75.5 | 278.5 | 62.0 | 20.8 | 9.1 | 1872 |
| Controlled diet targets (% total energy) | – | 2000 | 75 (15%) | 275 (55%) | 67 (30%) | 18 (8%) | 16 (<8 g/1000 kcal) | <3000 |

subsequently, a detailed health history will be obtained from each eligible volunteer. If a participant meets all eligibility criteria (Table 1), subsequent baseline testing visits will be scheduled (Fig. 2).

### 3.5. Procedures

Participants will be instructed to arrive for laboratory testing between 7:00 am and 10:00 am after a 12 h overnight fast (including abstinence from caffeine containing foods/beverages) and having performed no vigorous physical activity for the previous 48 h. In addition, participants will report being free of acute illness/infection for the prior 2 weeks and a supplementary infection and inflammation questionnaire will be answered. Participants will undergo 5 laboratory visits at baseline and again following the 6-week intervention (Fig. 2).

#### 3.5.1. Body mass and composition

Participants will be weighed on a digital scale accurate to ±0.1 kg, and height will be determined using a scale-mounted stadiometer (Scale-Tronix Inc.; White Planes, New York). Body composition will be assessed using dual energy X-ray absorptiometry (Prodigy Advance, GE Healthcare) by a limited licensed radiologic technician as required by the Virginia Department of Health.

#### 3.5.2. Plasma lipids and lipoproteins concentrations

Plasma lipid and lipoprotein concentrations (i.e., total cholesterol, high- and low-density lipoprotein cholesterol, and triglycerides) will be measured in a Clinical Laboratory Improvement Amendments-certified laboratory (Solstas Lab Partners) (Table 1).

#### 3.5.3. Resting blood pressure

Mercury sphygmomanometry will be used to measure blood pressure (BP) according to American Heart Association guidelines [60]. Participants will be instructed to remain seated and resting for 10 min prior to the first BP measurement with a minimum of 1 min between each BP measurement. Participants will be instructed to keep both feet on the floor without crossing legs, and the right arm will be supported at heart level. Blood pressure will be measured twice on participants who display a normal or pre-hypertensive value on the first measurement. The average of two additional measurements will be used for participants with an initial blood pressure in the hypertensive range.

#### 3.5.4. Habitual physical activity and dietary intake

Physical activity levels will be assessed during screening via the Godin Leisure Time Questionnaire to insure participants are sedentary to minimally active [61]. Upon study enrollment, habitual physical activity level will be assessed at baseline and follow-up using the ActiGraph GT3x accelerometer (ActiGraph LLC, Pensacola, Florida, USA). The ActiGraph GT3x is a triaxial accelerometer designed to measure physical activity for extended periods of time and will be utilized in this study to monitor activity over four consecutive days (3 weekdays and 1 weekend day) to capture 60% of the workweek and 50% of the weekend. The ActiGraph will be initialized to record continuously at 15-s epochs (i.e. time intervals) and will be analyzed using the Freedson cut-point equations [62]. In addition to wearing the accelerometer, participants will be sent home with a form and instructions to note the wear time and non-wear time and any pertinent notes regarding why the accelerometer was removed (i.e. sleeping, showering, etc.). All non-wear times will be excluded from the analyses conducted in the accompanying manufacturer software. Participants will wear the accelerometer for 4 consecutive days on the right hip at each assessment point to ensure compliance with our instructions to maintain baseline levels of habitual physical activity [63]. Assessment will occur concurrently with habitual dietary intake assessment.

Baseline dietary intake, including total energy and macronutrient intake, will be assessed using detailed 4-day food intake records. Participants will be instructed on procedures for measuring and recording food intake for 4 consecutive days. Participants will be provided with detailed recording forms, and a booklet of 2-dimensional food models to assist with accurate portion size determination. Returned records will be reviewed with the participant by research staff to ensure clarity and completeness of the food intake record. Records will be analyzed by trained research assistants using the NDS-R software (v. 2014) to determine participant's habitual dietary energy and macronutrient intake. Participants with who are taking prebiotic supplementation will be asked to discontinue for a minimum of 3-months prior to participating in the study.

#### 3.5.5. Intestinal permeability

Intestinal permeability will be assessed using the four-sugar probe procedure [64–66]. Following an overnight fast, participants will ingest 40 g sucrose (Spectrum Chemical MFG. Corp., Gardena CA and New Brunswick, NJ), 5 g lactulose (Qualitest Pharmaceuticals, Huntsville, AL), 1 g mannitol (Spectrum), and 1 g sucralose (Spectrum) in 500 ml water [64–66]. Participants will be instructed to consume an additional 500 ml of water within a 2-h timeframe following consumption of the sugar probe. In addition, participants will be provided a standardized sucrose-free meal to consume within the first 5 h of the urine collection period. All of the food consumed that day will be free of artificial sweeteners. Urine will be collected from 0 to 5 and 6–24 h in containers with 5 ml 10% thymol (VWR, Radnor, PA) to inhibit bacterial growth. Total urine volume will be recorded, and aliquots frozen at −80 °C for later analysis. Urinary sugars will be measured on a Waters Acquity UPLC-TQD [67]. Permeability will be defined as % urinary excretion and excretion ratios for sugars from hours 0–5 (upper GI) and hours 6–24 (lower GI) [64–67]. Fig. 3 shows LC–MS chromatogram of an internal standard and each of the 4 sugars recovered from urine of 1 of the Co-I's (AN) 5 h after ingestion.

#### 3.5.6. Plasma endotoxin and inflammatory cytokine concentrations

Plasma endotoxin concentration will be determined using the PyroGeneTM Recombinant Factor C Endotoxin Detection Assay (Lonza International). Pro-inflammatory cytokines (TNFα, IL-6, and MCP-1) in plasma will be measured by ELISA (American Diagnostica Inc., New York, NY).

#### 3.5.7. Glucose tolerance and insulin sensitivity

Oral glucose tolerance tests (OGTT) will be used to determine eligibility during baseline screening. Following baseline blood sampling, participants will consume a single 10 fluid ounce beverage containing 75 g glucose (Sun-Dex, Fisherbrand, Fisher Scientific, Hanover Park, IL). A second blood sample will be obtained 120 min following consumption of the beverage. Thresholds for normal, prediabetic and diabetic at the 2 h time point are: <140 mg/dl, 140–200 mg/dl, and >200 mg/dl, respectively [68].

Whole body insulin sensitivity will be estimated using Bergman's minimal model (MINMOD Millennium) during a modified frequently-sampled Intravenous Glucose Tolerance Test (IVGTT) [69]. Intravenous catheters will be inserted in an antecubital vein of each arm for blood collection or glucose and insulin injection. Two baseline plasma blood samples (($t$) = −10 and −1 min) will be drawn. Glucose (0.3 g/kg; 50% solution) will be injected at time 0 and insulin (0.025 U/kg) will be injected at ($t$) = 20 min. Additional blood samples (3 ml) will collected at ($t$) = 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 22, 23, 24, 25, 27, 30, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 min during the 3-h protocol. Plasma glucose concentration (mg/dl) will be analyzed immediately using a YSI Glucose Analyzer (Yellow Springs, OH). Insulin (μU/ml) will be determined later via an ELISA (ALPCO Diagnostics, Salem, NH). Samples will analyzed in duplicate.

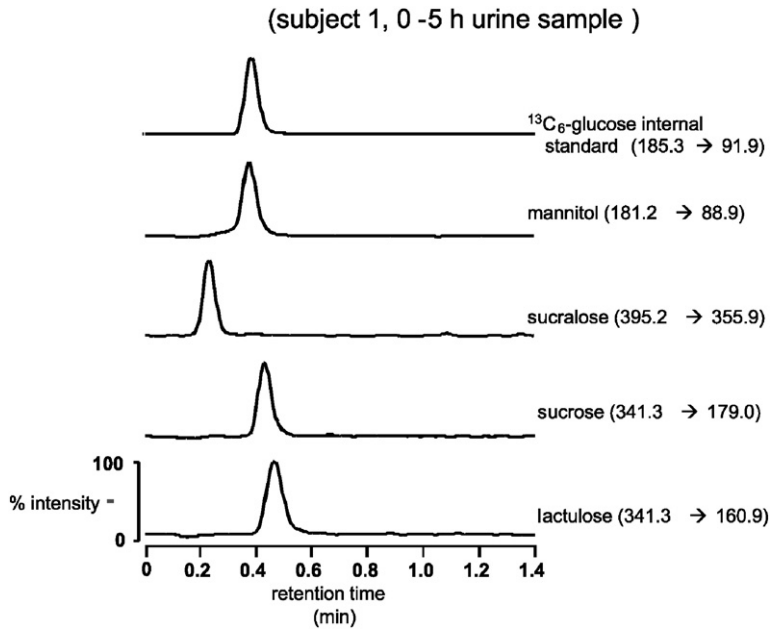


**fig. 3.** Liquid chromatography–mass spectrometry chromatogram of internal standard and 4 sugars recovered from urine of Co-I (AN) 5 h after ingestion.

*3.5.8. Skeletal muscle biopsies, substrate metabolism, and metabolic flexibility*

Participants will arrive for testing after a 12-h overnight fast. Muscle biopsies in the vastus lateralis will be obtained using a modified Bergstrom needle technique. Following local anesthesia (2% lidocaine), a 1.0 cm incision will be made through the skin and fascia. Biopsy tissue obtained will be obtained using a multi-pass approach. The incision will be closed with a steri-strip.

A catheter will be inserted in an antecubital vein for a baseline blood sample following the first biopsy. Participants will then be provided with a high-fat test meal (Breakfast-style sausage biscuits; 850 kcal; 63% of energy from fat; 21% saturated fat), which will be consumed within a 10-min period. The test meal will be followed by hourly blood sampling for 4 h, in addition to the initial baseline blood sample, and a second muscle biopsy will occur at the 4-h mark. After the second biopsy, participants will be given a snack and standardized discharge instructions.

Fatty acid, glucose, and pyruvate oxidation will be assessed in skeletal muscle homogenates, prepared from ~100 mg of biopsied tissue, using [1-$^{14}$C]-palmitic acid, [U–$^{14}$C]-glucose, and [1-$^{14}$C]-pyruvate, respectively, as previously described. [24,70,71] Briefly, complete and incomplete fatty acid oxidation will be assessed by measuring $^{14}C–CO_2$ and acid soluble metabolites, respectively. Glucose and pyruvate oxidation will be assessed by measuring $^{14}C–CO_2$ production. Metabolic flexibility will be assessed by measuring $^{14}C–CO_2$ production from the oxidation of [1-$^{14}$C]-pyruvate with/without the presence of non-labeled palmitic acid. Reductions in pyruvate oxidation in the presence of palmitic acid will be examined to assess metabolic flexibility.

*3.5.9. Cardiovascular outcomes*

Flow mediated dilation (FMD) of the brachial artery will be assessed using duplex ultrasonography (HP Sonos 7500) with a high resolution linear array transducer according to published guidelines [72] and recent recommendations [73]. Reactive hyperemia will be produced by inflation of a pediatric BP cuff around the forearm for 5 min. Off line analysis of baseline and post-reactive hyperemic diameters and velocities will be performed using edge detection software (Vascular Analysis Tools, Medical Imaging Applications, Inc). Endothelium independent vasodilation (EID) will be assessed by measuring brachial arterial dilation for 10 min following administration of 0.4 mg of sublingual nitroglycerine. Both FMD and EID will be expressed as mm and % change from baseline diameter.

Carotid–femoral (C–F) pulse wave velocity (PWV), our primary measure of arterial (aortic) stiffness, will be obtained by serially measuring carotid and femoral artery waveforms using a high fidelity, non-invasive applanation tonometer (NIHem, Cardiovascular Engineering, Inc.) as previously described [74,75]. Briefly, subjects will be studied in the supine position after ~10 min of rest. A semi-automated computed controlled device will be used to auscultate brachial arterial pressure between 3 and 5 times at 2-min intervals, in order to obtain BP stability (±5 mmHg difference for both systolic and diastolic blood pressure). Next, a high fidelity finger probe tonometer will be used to obtain carotid, brachial, radial, and, femoral artery waveforms over 10–20 cardiac cycles. Arterial waveforms will then be saved to a computer device for later analysis. Body surface measurements will be made from the suprasternal notch (SSN) to the carotid, brachial, and radial recording sited using a Gulik tape measure and to the femoral recording site using a large caliper.

Tonometry waveforms will be signal-averaged and the electrocardiogram R wave will serve as a fiducial point [75]. BP values will be read by an experienced reviewer and the average of the systolic and diastolic BP will be used to calibrate the peak and trough of the signal averaged brachial waveform. Brachial diastolic and mean arterial pressures will then be used to calibrate the other arterial waveforms. C–F PWV will be calculated by dividing the travel distance (SSN to carotid recording site − SSN to the femoral recording site) by the travel time obtained from the foot to foot of the signal-averaged carotid and femoral pulse waves β-Stiffness index, a relatively BP independent index of carotid artery stiffness, will be measured using an ultrasound unit (Sonos 7500, Phillips Medical Systems) equipped with a high-resolution linear array transducer (3–11 MHz) and applanation tonometry (NIHem, Cardiovascular Engineering, Inc.) as previously described [76]. Briefly, after resting in the supine position for ~20 min, longitudinal B mode images of the cephalic portion of left common carotid artery, 1–2 cm proximal to the carotid bulb, will be obtained over 15 cardiac cycles by placing the transducer at a 90° angle over the artery. When clear visibility of the near and fall walls are obtained, the images will be stored on an optical disk for offline quantification. The maximal and minimal carotid artery diameters of 3 consecutive cardiac cycles will be acquired with commercially available software (Vascular Research Tools 5, Medical Imaging Applications,

LLC). Carotid artery BP will be acquired from applanation tonometry of the carotid artery and brachial artery auscultation as described above. β-Stiffness index will be calculated as: $\beta = \ln(\frac{P1}{P0})/((\frac{D1}{D0}/D0)$, where P1 represents carotid artery systolic pressure, P0 represents carotid artery diastolic pressure, D1 represents the maximal diameter recorded during systole, and D0 represents the minimal diameter recorded during diastole.

*3.5.10. Assessment of gut microbiota*

Stool samples will be collected daily for 3 days at baseline and the final 3 days of supplementation during the intervention. Participants will be provided with sterile plastic containers intended for stool sampling (Omnigene gut for microbiome, Owatonna, ON, Canada). Samples will be kept in a portable refrigerated cooler, and delivered to the Human Integrative Physiology Laboratory within 24 h of collection. The sample will be immediately frozen at −80 °C until final processing and analysis. Total bacterial DNA will be extracted from fecal samples using QIAamp DNA Stool Mini kit (QIAGEN California). Total fecal bacterial copies will be assessed using real-time quantitative polymerase chain reaction (qPCR) of the housekeeping gene rpoD and aliquots from the 3 composite days mixed equally. Abundance of select bacteria (*Bifidobacterium*, *Lactobacillus*), previously shown to be modulated by inulin will also be quantified by qPCR with previously published primers. Fecal bacterial community composition will be assessed using Tag-Encoded Pyrosequencing. The V4 region of the bacterial 16S rRNA gene will be amplified from fecal microbial DNA using barcoded PCR primers [77]. Additionally a specific barcoded primer for *Bifidobacterium* will be used to amplify this group of bacteria, which has been shown to be under-represented by standard primer sets [78]. The amplicons from each reaction will be mixed in equal amounts based on concentration and will be subjected to sequencing using the Illumina MiSeq platform. Baseline fecal bacterial compositions will be compared to fecal samples obtained after 6 weeks supplementation with inulin and a detailed characterization of the gut microbiota performed via bioinformatics pipelines including MG-RAST [79].

*3.5.11. Side effect monitoring*

Participants will be asked to report any side effects they experience during the intervention period. Gastrointestinal side effects related to the diet and/or treatment will be recorded using a standardized questionnaire, and addressed by study personnel. The side effect questionnaire will rate gas/bloating, nausea, flatulence, cramping, diarrhea, constipation, and GI rumbling from none (rating of 0) to severe (rating of 3) [45].

## 4. Data analysis

### 4.1. Power analysis

Sample size/power calculations were based on the number of participants needed to detect statistically ($P < 0.05$) and physiologically/clinically significant differences in the magnitude of change in insulin sensitivity and aortic PWV with prebiotic supplementation compared with placebo. With 2 groups, 2 repeated measures, and $\alpha = 0.05$ we will have greater than 80% power to detect significant group by time interactions for an effect size as small as 0.50 with minimum sample of $n = 20$ participants per group. Our conservative estimate is for 20% attrition and hence we plan for $n = 24$ per group or a total $n = 48$ participants. Using our prior published and unpublished data, we estimated an effect size (prebiotic-placebo/S.D.) of 0.61 for insulin sensitivity ($+20 \pm 32\%$) and 0.54 for aortic PWV ($-20 \pm 37\%$ difference in the reduction). As such, we will have greater than 90% power to detect significant group by time interactions. If dropouts exceed the 20% level, we will recruit additional participants to achieve our desired sample size.

### 4.2. Statistical analysis

We will conduct descriptive univariate analyses on all study variables. Data will be examined for the presence of outliers, violations of normality and missing data. Major violations of normality will be corrected with an appropriate transformation procedure. In case of an outlier, rather than transform the data, the outlier will be "Winsorized," that is, replaced by the most extreme value in the tail of the distribution.

To test our hypothesis that prebiotic supplementation will improve insulin sensitivity (primary outcome) and skeletal muscle metabolic flexibility in prediabetic individuals, we will use a multiple-sample repeated measure analysis of variance with between-subject factors approach. This is a common design in randomized controlled trials, where subjects are randomized to different treatment and control groups and followed across time. Because the data are repeated, we will treat the multiple observations as nested within individuals. This will allow us to make a direct comparison between the time points while accounting for the correlation in the data to make the correct inference regarding group differences. For our analysis, the group by time interaction will be of primary interest. A compound symmetry error structure will be chosen for this model. We will use an identical approach for testing our hypothesis associated with specific aim 2. Our study is not designed nor is it powered to detect significant differences in intervention efficacy across the stratification variables (i.e., age, gender, etc.). However, these subsamples will be compared on the main dependent variables at baseline. If the groups are found to be different from each other then they will be entered in the model as a covariate.

Aim 3 is exploratory in nature so we will begin with correlational analyses. We will use analysis of covariance (ANCOVA) with insulin sensitivity (or arterial stiffness) as the dependent variable, the treatment as the independent variable, and endotoxin concentration, serving as the covariate. If changes in endotoxin concentration with inulin supplementation are: 1) directly correlated with changes in insulin sensitivity among the individual subjects, and 2) the treatment is no longer significant in the ANCOVA, this will be interpreted as support for the concept that changes in insulin sensitivity (or arterial stiffness) with inulin supplementation are mediated, at least in part, by reductions in endotoxin concentration. We will use the same approach using percent abundance of bacterial taxonomic groups and intestinal permeability as covariates. Similarly, we will explore whether total fecal bacteria or changes in abundance of other bacterial members are significant covariates. We will also explore this using other approaches, such as mediation analysis or structural equation modeling.

### 4.3. Missing data

We will use an intention-to-treat analysis as our primary analytic approach. We will examine the missing data patterns and utilize maximum likelihood algorithms with the mixed linear model ANOVAs to longitudinally compare our outcomes across the three groups. Maximum likelihood algorithm estimations use all available data to construct weighted averages across the different patterns of missing data to provide valid point estimates and confidence intervals for population parameters [80]. As a secondary analysis, we will conduct a completers-only analysis and restrict the analysis to only those individuals who complete the interventions. In our experience, the two approaches yield similar results. However, if the results differ we will interpret the findings based on the intention-to-treat analysis, but report both so that the readers can reach their own conclusion.

### 4.4. Data management and quality control

The Principal Investigator (PI) will ultimately be responsible for the quality of the data. The project coordinator will be responsible for handling all data, entering data on the study computer, performing data

editing, and maintaining a secure filing system for the study data forms that will serve as an ultimate backup (and the source for data random re-entry). Before a form is entered, the data entry staff and PI will inspect the form for completeness and legibility. Each form will be logged into a microcomputer-based system for tracking, validation of assignment, and checked against duplication of visits or forms. This will identify problems in subject records and enable clarification. The data will then be entered into a data entry system that will be constructed to check (1) the validity of the subject identification, (2) each field entered for allowable response (range checks), and (3) validity of examination dates. Data will be duplicate keyed, verified for accuracy, and accumulated and managed using MS Access. Once all data are received, entered, and completeness verified, analysis will proceed with a listing of all data for each subject, summary statistics for all variables at each measurement period, a listing of subjects who are in noncompliance with the study protocol, and statistical analysis.

The PI will ultimately be responsible for quality control of study procedures and measurements. He will supervise performance of all of the various study protocols, questionnaires, forms, and measurements. An operations manual will be developed and the procedures strictly followed. Training sessions will cover all study procedures, including recruitment, informed consent, measurements, and specimen handling. Adherence to the procedures in the operations manual will be assured by periodic assessment and retraining.

## 5. Discussion

There is currently little information regarding if or how prebiotics improve cardio-metabolic function in humans, particularly in prediabetic individuals who are at high risk for developing T2D and experiencing cardiovascular events. Although the concept that dysbiosis of the gut microbiota leads to metabolic endotoxemia and increased risk of cardio-metabolic disease is not novel, very little information is available in humans. The significance of this trial includes providing proof of concept efficacy of prebiotic supplementation with inulin on cardio-metabolic dysfunction and assessing its relation with changes in gut bacterial communities, intestinal permeability and metabolic endotoxemia in individuals at increased risk for T2D. Our findings could lead to identification of inulin supplementation as a simple and efficacious adjunctive therapy for reducing cardio-metabolic risk in prediabetes, which could change clinical practice by informing dietary recommendations and increasing acceptance of prebiotics by the scientific and medical community.

There are several innovative aspects of this clinical trial. First, we are testing hypotheses involving a novel concept for which there are little data in humans. Second, we have linked our ideas to the important physiological problem of metabolic endotoxemia that has been implicated in T2D and CVD. Third, we are focusing on individuals at high risk for T2D, including those with prediabetes, who are a growing segment of the population at high risk for adverse CVD-related events that may precede T2D onset. Fourth, hundreds of prebiotic products are available, yet little is known about their cardio-metabolic health benefits. Finally, we may identify a simple and efficacious adjunctive lifestyle approach to reduce T2D and CVD risk. Importantly, if our hypotheses are supported, our findings could have a significant impact on clinical practice and public health.

### *5.1. Potential challenges and limitations*

A primary challenge of our study will be participant recruitment, enrollment, and retention. Recruitment will be ongoing and challenges will be addressed and managed via weekly research team meetings. Participant enrollment and retention will be managed by study personnel and the project coordinator. All participants will visit with study personnel a minimum of 3 times/week and any concerns or issues will be immediately addressed by the project coordinator.

Strict adherence to the controlled diet may also pose a challenge to enrollment and retention. To overcome barriers related to the controlled diet, daily menus are designed to reflect the "average U.S. diet." Therefore, the foods consumed during this study will be similar in composition and volume to the participant's habitual intake. All participants will be given menus to review prior to initiating the controlled diet, in order to familiarize them with what they will be expected to consume. Any questions or concerns related to the diet will be addressed at that time by the project coordinator. All participants will be informed of the expectations of the controlled diet component prior during the consenting process. For example, all food must be consumed each day and difficulty consuming any of the food must be reported to study personnel. Strategies such as re-portioning the pre-designated amounts may be utilized. Participants who cannot or will not comply with the controlled diet will be excluded from participating.

Blinding subjects to inulin may be difficult if there are gastrointestinal side effects. However, the nature of the outcomes (biochemical and physiological) and the utilization of a controlled feeding paradigm will minimize any potential for this as a limitation. It is possible that any observed improvement in certain outcomes (e.g., blood lipids) could be attributed to the low saturated fat content of the diet provided. However, we are employing a randomized controlled trial design, and all of the participants will be receiving the same diet. Thus, the impact of the treatment on our outcomes should be preserved.

This investigation should provide vital preliminary data for a larger, more comprehensive trial that would serve to establish the efficacy on the effects of inulin supplementation on cardio-metabolic function in prediabetic individuals. One of the hallmarks of science is replication of study findings. As such, it will be important to replicate the findings of the proposed small clinical trial that may have limited generalizability to a broader context.

## 6. Conclusions

To date, the potential benefits of prebiotic supplementation on cardio-metabolic dysfunction in humans has received little attention. This trial will address this important research gap, by exploring the role of the prebiotic inulin in modifying cardio-metabolic risk among adults at increased risk for T2D. The results of this trial could impact clinical treatment approaches, and contribute to the evidence base for developing dietary guidelines that address the amount and types of dietary fiber to consume to maximize health benefits.

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**Appendix C**

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100 Lenox Drive, Ste 104
Lawrenceville, NJ 08648
Tel: 646 452-6140 Fax: 646452-6150

# CERTIFICATE OF ANALYSIS

**PRODUCT: Frutafit® IQ**

**BATCH NUMBER: 246A802526**

| Parameter | Specification | Actual values[1] | Unit | Method |
|---|---|---|---|---|
| ***Physical Aspects*** | | | | |
| **Dry matter content** | 95-99 | 96.3 | % | ICUMSA GS2/1/3/9-15(2007) |
| | | | | |
| ***Composition on dry matter*** | | | | |
| **Carbohydrates** | >99.5 | >99.5 | % | |
| **Inulin/Fiber (DP2-DP60)** | ≥90 | 93.1 | % | AV_029a |
| **Fructose,glucose,disaccharides** | ≤10 | 6.9 | % | ICUMSA GS7/4/8-23(2002) |
| **Ash** | ≤0.2 | <0.2 | % | ICUMSA GS8-7(1998) |
| | | | | |
| ***Microbiology*** | | | | |
| **Aerobic plate count (30°C)** | ≤1000 | 100 | CFU/gram | ISO 4833:2003 |
| **Aerobic plate count (55°C)** | ≤1000 | <100 | CFU/gram | ISO 4833:2003 (at 55°C) |
| **Yeast** | ≤20 | <10 | CFU/gram | ISO 6611:2004 |
| **Molds** | ≤20 | <10 | CFU/gram | ISO 6611:2004 |
| ***Bacillus cereus*** | ≤100 | <100 | CFU/gram | ISO 7932:2004 |
| **Enterobacteriaceae** | absent | absent | /gram | ISO 21528-1:2004 |
| ***Staphylococcus aureus*** | absent | absent | /gram | ISO 6888-3:2003 |
| ***Salmonella*** | absent | absent | /400grams | ISO 6785:2007 |
| | | | | |
| ***Other Information*** | | | | |
| **Production Date** | | 11/15/2013 | mm/dd/yyyy | |
| **Use Before** | | 11/15/2018 | mm/dd/yyyy | |

*All batches comply with EU Regulations for foodstuffs regarding microbial criteria, pesticides and heavy metals*

Vera Damen
Quality Assurance Manager

**Appendix D**

Supplemental Digital Content 1. Summary of terminology and operational definitions

α-diversity: a measurement to determine evenness and richness.

β-diversity: a measurement of distance or dissimilarity between samples.

Evenness: the distribution and proportion of bacterial populations

Relative abundance: the proportion of a species of taxa

Richness: the number of species or taxa presented within a sample; diversity of taxa within a sample.

Dysbiosis: disruption of symbiotic bacterial communities within the gut microbiome.

**Appendix E**

**Supplemental Digital Content 1.** Characteristics and outcomes of included studies sorted by mammal type and publication year.

| Study (first author, publication year) | Control group? (Y/N) | Model: (mammal, age/life stage, health status, sample size) | Primary study aim | Intervention and group design | Dietary controls | Methods | Gut microbiome composition measures | Significant taxonomy findings | Declared Funding Sources § |
|---|---|---|---|---|---|---|---|---|---|
| **Murine (Mouse)** | | | | | | | | | |
| Evans, 2014 | Y | Adult male C57BL/6 mice, healthy, n=48 | To determine effect of voluntary exercise vs. no access to exercise on the intestinal microbiome. | 1. Low-fat diet (LFD), 2. HFD 3. LFD + wheel access (WA) 4. HFD + WA | Yes, LFD and HFD groups. LFD: 10% fat HFD: 60% fat (62% saturated; 35.9% mono-; 32% poly-unsat-urated). | Terminal restriction fragment length polymorphi sm (TRFLP), PCR assay, 16S rRNA sequencing of fecal contents. | β-diversity, abundance, bacterial family and phyla, bacteroidetes:firmicutes | **HFD:**<br>Bacteroidetes<br>⬇S24-7 (f)<br>Firmicutes<br>⬆Clostridiaceae (f)<br>⬆Lachnospiraceae (f)<br>⬆Ruminococcaceae (f)<br>⬇Turicibacteraceae (f)<br>⬇Erysipelotrichaceae (f)<br>Actinobacteria<br>⬇Bifidobacteriaceae (f)<br>**HFD + WA:**<br>Bacteroidetes<br>⬆S24-7 (f)<br>Firmicutes<br>⬆Clostridiaceae (f)<br>⬆Lachnospiraceae (f)<br>⬆Ruminococcaceae (f)<br>⬆Lactobacillaceae (f)<br>⬇Turicibacteraceae (f)<br>⬇Erysipelotrichaceae (f)<br>**LFD + WA:**<br>Bacteroidetes<br>⬆S24-7<br>Firmicutes<br>⬆Lactobacillaceae (f)<br>⬆Lachnospiraceae (f)<br>⬆Ruminococcaceae (f)<br>⬇Turicibacteraceae (f)<br>⬇Erysipelotrichaceae (f) | G,O |

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| | | | | | | | | Actinobacteria<br>⬇Bifidobacteriaceae (f) | |
| Kang, 2014 | Y | Adult male C57BL/6 J mice, healthy, n=40 | To determine interaction between habitual dietary conditions and exercise level on intestinal microbiome and behavior | 1. Normal diet (ND), 2. ND + forced exercise (F.Ex.), 3. High-fat diet (HFD) 4. HFD + F.Ex. | Yes, HFD and ND groups. ND: 10% fat HFD: 60% fat | PCR, 16S rRNA sequencing of fecal contents. | Operational taxonomic units (OTUs), relative abundance, bacteroidetes:firmicutes and bacterial phyla, genus, and family | **HFD:**<br>Firmicutes<br>⬆Streptococcus (g)<br>⬇Tenericutes<br>**HFD + F.Ex:**<br>⬇Bacteroidetes<br>⬆Firmicutes<br>⬇Streptococcus (g)<br>⬇Tenericutes<br>**ND + F.Ex:**<br>⬇Bacteroidetes<br>⬆Firmicutes<br>⬇Tenericutes | G,O |
| Allen, 2015 | Y | Adult male C57BL/6J, healthy, n=29 | To compare effects of voluntary exercise via wheel access (WA.) vs. F.Ex. on the intestinal microbiome. | 1. F.Ex.<br>2. WA<br>3. Sedentary (sed) | No, all were fed a standard chow diet. | PCR, 16S rRNA gene sequencing of fecal contents. | OTUs, α-diversity, richness & evenness, relative abundance, bacteroidetes:firmicutes bacterial genera and phyla | **F.Ex:**<br>Firmicutes<br>⬆Dorea (g)<br>⬆Coprococcus (g)<br>⬆Oscillospira (g)<br>⬆Ruminococcus (g)<br>⬆Butyrivibrio (g)<br>⬆Tenericutes<br>Proteobacteria<br>⬆Nautilia (g)<br>**WA**<br>⬇Bacteroidetes<br>Prevotella (g)<br>Firmicutes<br>⬇Turicibacter (g)<br>⬆Anaerotruncus (g) | O |
| Lambert, 2015 | Y | Adult male C57BL mice, healthy (db/+), and Type 2 Diabetic [T2D (*db/db*)], | To compare the effect of F.Ex. on the intestinal microbiome in T2D mice when compared to their healthy | *1. Db/db*,<br>2. F.Ex + *db/db*,<br>*3. db/+*<br>4. F.Ex + *db/+* | No, all were fed a standard chow diet. | PCR, 16S rRNA gene sequencing of cecal contents. | Total bacteria, bacterial phyla and species, bacteroidetes:firmicutes relative abundance | **Db/db:***<br>Firmicutes<br>⬇Clostridium cluster I<br>⬆Clostridium Cluster XI<br>**Db/db +F.Ex:**<br>Bacteroidetes<br>⬇Bacteroides (g)<br>⬇Prevotella (g)<br>Actinobacteria | G,O |

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| | | n= ~36 | and sedentary counterparts. | | | | | ⬇Bifidobacterium (s)<br>Proteobacteria<br>⬇Enterobacteriaceae (f)<br>Euryarchaeota<br>⬇Methanobrevibacter (g)<br>**Db/+ + F.Ex:**<br>Bacteroidetes<br>⬇Bacteroides (g)<br>⬇Prevotella (g)<br>Firmicutes<br>⬆Clostridium cluster I<br>Actinobacteria<br>⬆Bifidobacterium (s)<br>Euryarchaeota<br>⬇Methanobrevibacter (g) | |
| Campbell, 2016 | Y | Adult male C57BL/6NTac, healthy, n=36 | To compare the effects of voluntary exercise vs. sedentary time on intestinal integrity and intestinal microbiome in lean and obese mice. | 1. Lean sed.,<br>2. HFDIO + sed.<br>3. Lean + WA<br>4. HFDIO + WA. | Yes, HFD: 45% fat, and ND: 10% | Terminal restriction fragment length poly-morphism (TRFLP) and pyro-sequencing of 16S rRNA fecal contents. | OTUs, bacterial genus, phyla, family, species | **HFDIO:**<br>Firmicutes<br>⬆Clostridiales (o)<br>**HFDIO + WA:**<br>Firmicutes<br>⬆Clostridales (o)<br>⬆Faecalibacterium prausnitzii (s)<br>⬆Peptococcus (s)<br>⬆Allobaculum (s)<br>**Lean + WA:**<br>⬆ Firmicutes<br>⬆Clostridales (o)<br>⬆Faecalibacterium prausnitzii (s)<br>⬆Lachnospiraceae (f)<br>⬆Allobaculum (s)<br>⬆Clostridium (g) | G |
| Denou, 2016 | Y | Adult male C57BL/6 mice, healthy, n=32 | To determine the effect of HIIT training on the intestinal microbiome | 1. HFDIO + HIIT<br>2. HFDIO + untrained<br>3. ND + sed.<br>4. HFD + | Yes, HFD: 45% fat and standard chow diet. | PCR, 16S rRNA sequencing of fecal contents. | OTUs, α-diversity, bacterial genus, phyla, relative abundance, bacteroidetes:firmicutes | **HFD:**<br>⬇Bacteroidetes<br>⬆Firmicutes<br>**HFDIO + HIIT**:<br>⬆Bacteroidetes<br>⬆Bacteroidales (o)<br>⬇Firmicutes | G,O |

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| | | | after HFD-induced obesity (HFDIO). | sed. | | | | | |
| Houghton, 2017 | Y | PolgA $^{mut/mut}$ and wild-type PolgA $^{+/+}$, mitochondrial dysfunction, n=19 | To determine how changes in mitochondrial function of aging colonic epithelium influences gut microbiome composition, and whether or not exercise can modulate observed changes in composition in an accelerated aging model. | 1. Sed.<br>2. Age + F.Ex.<br>3. Age + sed. | No, all were fed a standard chow diet. | PCR, 16S rRNA of fecal contents. | OTUs, α-diversity, bacterial genus, relative abundance, bacteroidetes:firmicutes | **Age + F.Ex:**<br>⬆ Bacteroidetes<br>Proteobacteria<br>⬆ Desulfovibrio (g)<br>⬇ Firmicutes<br>Deferribacteraceae<br>⬆ Mucispirillum (g) | G, O |
| Lamoureux, 2017 | Y | Adult C57BL/6 mice, healthy, n=42 | To measure effect of exercise on intestinal microbiome in voluntary and forced moderate intensity exercise. | 1. Sed.<br>2. WA<br>3. F.Ex. | No, all were fed a standard chow diet. | PCR, 16S rRNA sequencing of fecal samples. | OTUs, α-diversity, β-diversity, species richness, relative abundance, bacterial phyla. | No significant taxonomy findings between any groups. | G |
| Liu, 2017 | Y | Adult C57BL/6 | To investigate | 1. Sed.<br>2. Sed. + MI | No, all were fed a | 16S rRNA sequencing | OTUs, α-diversity, β-diversity, bacterial | **F.Ex.**<br>Bacteroidetes | O |

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| | | mice, healthy n=* *Sample size per group not reported. | effects of exercise on intestinal microbiome after myocardial infarction (MI). | 3. Sed. + Sham surgery (S.sx), 4. F.Ex, 5. F.Ex + MI 6. F.Ex + S.sx | standard chow diet. | of fecal samples. | phyla. | ⬆Butyricimonas (g)<br>⬆Prevotella (g)<br>Verrucomicrobia<br>⬆Akkermansia (g)<br>**F.Ex + S.Sx**<br>Bacteroidetes<br>⬆Sphingobacteriales<br>Firmicutes<br>⬆Erysipelotrichaceae<br>Verrucomicrobia<br>⬆Akkermansia<br>**F.Ex + MI**<br>Proteobacteria<br>⬆Phenylobacterium<br>⬆Roseateles | |
| **Murine (Rat)** | | | | | | | | | |
| Matsumoto, 2008 | Y | Adult male Wistar rats, healthy, n=14 | To determine the effect of WA on the intestinal microbiome. | 1.Sed. 2.WA | Yes, all were fed a 25% casein-sucrose diet. | PCR, 16S rRNA sequencing of cecal contents. | Bacterial species, and n-butyrate producing bacteria. | **WA:***<br>Firmicutes<br>⬆Ruminococcus hydro-genotroplicus (sp.)<br>⬆Clostridium (sp)<br>⬆SM7/11 (f)<br>⬆Lactobacillus gasseri (s)<br>⬆T2-87 (f)<br>Proteobacteria<br>⬆Escherichia coli (sp) | Not declared |
| Queipo-Ortuño, 2013 | Y | Adult male Dawley rats, healthy, n=40 | To determine the effects of physical activity and nutrition status on the intestinal microbiome. | 1. Activity-based anorexia (ABA) + WA, 2. ABA-control, 3. WA + ad libitum (ad lib) feeding, 4. Sed + ad lib feeding. | Yes, ABA groups had food access restricted to 1 hour/day. | PCR, 16S sequencing of fecal contents. | Diversity, abundance, phyla and genera-level bacteria, bacteroidetes:firmicutes | **ABA + WA:**<br>⬇Bacteroidetes<br>⬆Bacteroides (g)<br>⬆Prevotella (g)<br>⬇Firmicutes<br>⬆Clostridium (g)<br>⬆Enterococcus (g)<br>⬇B.Coccoides-E rectale (sp)<br>⬇Lactobacillus (g)<br>⬇Actinobacteria<br>⬇Bifidobacterium (g)<br>⬆Proteobacteria<br>Euryarchaeota | G,O |

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| | | | | | | | ⬆M. smithii (s)<br>**ABA-control:**<br>Firmicutes<br>⬆Enterococcus (g)<br>⬇B. Coccoides-E rectale (sp)<br>⬇Lactobacillus (g)<br>Actinobacteria<br>⬇Bifidobacterium (g)<br>Euryarchaeota<br>⬆M. smithii (s)<br>**WA. + ad lib:**<br>Bacteroidetes<br>⬆Bacteroides (g)<br>⬇Prevotella (g)<br>⬇Firmicutes<br>⬇Clostridium(g)<br>⬇Enterococcus (g)<br>⬆B. Coccoides-E rectale (sp)<br>⬆Lactobacillus (g)<br>⬆Actinobacteria<br>⬇Bifidobacterium (g)<br>**Sedentary+ ad lib:**<br>Actinobacteria<br>⬇Bifidobacteria (g) | |
| Petriz, 2014 | Y | Zucker and Wistar Rats, obese, non-obese, or hyper-tensive, n=9, sex not specified. | To determine the effect of F. Ex. on the intestinal microbiome in the presence of known disease genotypes. | 1. F.Ex + Non-obese, 2. F.Ex + Obese, 3. F. Ex + Hyper-tensive | None, and diet compo-sition not reported. | Pyro-sequencing, PCR, 16S rRNA of fecal contents. | OTUs, α-diversity, bacterial species richness, bacterial phyla, relative abundance | **F.Ex Non-obese**:<br>Firmicutes<br>⬇Streptococcus (g)<br>**F.Ex Obese**:<br>Firmicutes<br>⬆Streptococcus alactolyticus (s)<br>⬆Lactobacillus (g)<br>⬆Ruminococcus gnavus (s)<br>⬇Ruminococcus flavefaciens (s)<br>Actinobacteria | G,O |

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| | | | | | | | | ⬆Bifidobacterium animalis (s)<br>⬆Bifidobacterium pseudolongum (s)<br><u>Proteobacteria</u><br>⬆Pseudomonas (g)<br>⬆Aggregatibacter pnemotropica (s)<br>**F. Ex Hypertensive**:<br><u>Firmicutes</u><br>⬇Allobaculum (g)<br><u>Proteobacteria</u><br>⬇Aggregatibacter (g)<br>⬇Sutturella (g) | |
| Liu, 2015 | Y | Female high cardio-respiratory capcity (HCR) and low cardio-respiratory (LCR) rats, healthy, n=30 | To determine how voluntary exercise alters the intestinal microbiome in ovari-ectomized rats with intrinsically HCR and LCR fed a HFD. | 1. LCR + WA<br>2. LCR + Sed.<br>3. HCR + WA.,<br>4. HCR + Sed., | Yes, 45% HFD after OVX. | PCR, 16S rRNA of cecal contents. | OTUs, α-diversity, β-diversity, bacterial phyla, species richness, bacteroidetes:firmicutes | **LCR + WA**:*<br>⬇<u>Firmicutes</u><br>⬇Ruminococcus (f)<br>⬇Christensenellaceae (f)<br>⬆<u>Proteobacteria</u><br>⬆Heliobacteraceae (f)<br>⬆Desulfovibrionaceae (f)<br>⬆<u>Cyanobacteria</u><br>**HCR + WA:**<br>⬆<u>Firmicutes</u><br>⬆Ruminococcus (f)<br>⬆Christensenellaceae (f)<br>⬆Clostridium (g)<br>⬇<u>Proteobacteria</u><br>⬇Heliobacteraceae (f)<br>⬇Desulfovibrionaceae (f)<br>⬇<u>Cyanobacteria</u><br><br>**HCR + Sed. Vs LCR + Sed:**<br><u>Bacteroidetes</u><br>⬆Porphyromonadaceae (f)<br><u>Firmicutes</u><br>⬆Lachnospiraceae (f)<br>⬆Peptococcaceae (f) | G |

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| | | | | | | | | Proteobacteria<br>⬆Heliobacter (g) | |
| Mika, 2015 | Y | Male juvenile and adult F344 rats, healthy, n=40 | To determine whether juvenile rats would promote a leaner intestinal microbiome phenotype with from 6-weeks of voluntary wheel running compared to adult counterparts. | 1. Juvenile + WA<br>2. Juvenile + Sed.<br>3. Adult + WA<br>4. Adult + Sed. | No, standard chow diet. Full compo-sition not provided. | PCR, 16S rRNA of fecal samples. | OTUs, α-diversity, β-diversity, evenness, richness, relative abundance, bacterial phyla and genus, bacteroidetes:firmicutes | **Juvenile + WA:**<br>⬆Bacteroidetes<br>⬇Rikenellaceae g_AF12 (g)<br>⬇Rikenellaceae g_ (g)<br>⬇Firmicutes<br>⬆Blautia (s)<br>⬆Anaerostipes (s)<br>⬇Proteobacteria<br>⬆Desulfovibrio (s)<br>⬆Euryarchaeota<br>⬆Methanosphaera (s)<br>**Adult + WA:**<br>Bacteroidetes<br>⬆Rikenellaceae g_AF12 (g)<br>⬆Rikenellaceae g_ (g)<br>Firmicutes<br>⬆Turicibacter (s) | G |
| Welly, 2016 | Y | Juvenile male obesity-prone CD rats (OP-CD), healthy, n=30 | To determine how the intestinal microbiome is altered in the presence of energy deficits created from exercise versus diet in an obesity-prone phenotype. | 1. HFD + WA<br>2. HFD + Sed<br>3. HFD weight matched (WM) | Yes, 45% HFD for all groups. | PCR, 16S rRNA of cecal contents | OTUs, bacterial phyla, family, genera, and relative abundance, bacteroidetes:firmicutes. | **HFD + WA**:*<br>Bacteroidetes<br>⬇Rikenellaceae (f)<br>⬇S24-7 (f)<br>Firmicutes<br>⬆Streptooccaceae (f)<br>**HFD WM:**<br>⬇Streptococcus (g) | G,O |
| Batacan, 2017 | Y | Adult male Wistar Rats, healthy, | To compare the effects of diet (HFD | 1. Control + ND<br>2. Control + | Yes, ND and 25% HFD | PCR, 16S rRNA of fecal | OTUs, α-diversity, β-diversity, richness, evenness, bacterial | **LIT + ND**:<br>Firmicutes<br>⬆Lactobacillus johnsonii | O |

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| | | n=57 | vs ND) and two exercise intensities on the intestinal microbiome over a 12-week period. | HFD, 3. Sed. + ND, 4. Sed. + HFD, 5. Light-intensity training (LIT) + ND, 6. LIT + HFD, 7. HIIT + ND 8. HIIT + HFD | were used in all activity groups. | contents | species, family, and class. | (s)<br>⬆Clostridium geopufricans (s)<br>⬆Actinobacteria<br>⬆Bifidobacteriaceae (f)<br>⬆Coriobacteriaceae (f)<br>⬆Proteobacteria<br>⬆Parasutterella excrementihominis (s)<br>⬆Tenericutes<br>⬆Erysipelotrichaceae (f)<br>**HIIT + ND**:<br>Firmicutes<br>⬆Clostridium saccharolyticum (s)<br>⬆Clostridium geopufricans (s)<br>Proteobacteria<br>⬆Parasutterella exrementihominis (s)<br>**LIT + HFD**:<br>Firmicutes<br>⬆Clostridium (c)<br>**HIIT + HFD**:<br>Firmicutes<br>⬆Clostridium (c) |
| Feng, 2017 | Y | Adult male HCR and LCR rats. Exact sample size not specified. | To determine whether exercise can prevent post-operative cognitive decline, neuroinfla-mmation and associated | 1. HCR + Sx<br>2. HCR + S.Sx<br>3. LCR + Sx<br>4. LCR+ S.Sx | No, standard chow diet. Full compo-sition not provided. | PCR, 16SRNA gene and DNA sequencing of fecal contents. | OTUs, α-diversity, β-diversity, bacteroidetes:firmicutes | **HCR + Ex:***<br>⬇Bacteroidetes<br>⬆Firmicutes<br><br>*Surgical condition not specified |

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| | | | shifts in intestinal microbiome. | | | | | | |
| **Canine** | | | | | | | | | |
| Kieler, 2017 | N | Adult large- and medium-breed dogs who were sedentary and overweight, healthy, n=18 | To determine if exercise influences shifts on intestinal microbiome during weight loss protocol. | 1. Diet + F.Ex.<br>2. Diet | Yes, all animals were fed same dry diet food. | PCR, 16S rRNA sequencing of fecal samples. | OTUs, α-diversity, bacterial phyla and genus. | No significant changes to intestinal microbiome as a result of exercise. | O |
| **Equine** | | | | | | | | | |
| Janabi, 2016 | Y | Adult male and female horses (mares and geldings [n=8]), healthy, and older seasonal controls (n=4), healthy | To determine the effects of exercise on the equine intestinal microbiome over 12-weeks. | 1. Seasonal control (SC)<br>2. F.Ex. | No. All animals were fed standard rations of grian and alfalfa, and grass ad-libitum. | PCR, 16S rRNA sequencing of fecal contents. | OTUs, α-diversity, β-diversity, relative abundance of bacterial phyla, genera, and species | **F. Ex. °**<br>⬆ Bacteroidetes<br>⬆ Dysgonomonas (g)<br>⬆ Firmicutes<br>⬆ Clostridium (g)<br>⬆ Proteobacteria<br>⬆ Spirochaetes<br>⬆ Treponema (g) | O |
| Janabi, 2017 | Y | Adult male and female horses (mares and geldings), healthy, n=8. | To determine the acute effect of exercise on the intestinal microbiome before and after a 12- | 1. SC 1<br>2. SC 2<br>3. F. Ex 1<br>4. F.Ex 2 | No-all animals were fed standard ration of grain and alfalfa/ grass hay ad lib. | PCR, 16S rRNA sequencing of fecal contents. | OTUs, α-diversity, β-diversity, αrelative abundance of bacterial phyla, genera, and species | **SC 1 °**<br>Firmicutes<br>⬇ Clostridium (g)<br>⬇ Fusicatenibacter saccharivorans (s)<br>Spirochaetes<br>⬇ Treponema zioleckii (s) | O |

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| | | | week exercise training protocol. | | | | | | |
| **Human** | | | | | | | | | |
| Clarke, 2014 | Y | Adult male rugby players, and weight matched high and low BMI controls, healthy, n=83 | To determine the relationship between exercise or sedentary time and the intestinal microbiota. | 1. Rugby, 2. Low BMI, 3. High BMI, | No-diets were assessed via food frequen-cy question-naires (FFQ) | PCR, 16S rRNA sequencing of fecal contents. | OTUs, α-diversity, bacterial phyla, family, and genera. | **Rugby vs. High BMI**<br>⬇ Bacteroidetes<br>⬆ RC9 gut group (g)<br>⬆ S24-7 (f)<br>⬆ Firmicutes<br>⬆ Succinivibrio (g)<br>⬆ Succinivibrionaceae (f)<br>Verrucomicrobia<br>⬆ Akkermansiaceae (f)<br>⬆ Akkermansia (g)<br>**Rugby vs. Low BMI:**<br>⬇ Bacteroidetes<br>⬆ Prevotellaceae (f)<br>⬆ Prevotella (g)<br>⬇ Bacteroides (g)<br>⬆ S24-7 (f)<br>Firmicutes<br>⬇ Lactobacillaceae (f)<br>⬇ Lactobacillus (g)<br>⬆ Erysipelotrichaceae (c)<br>⬆ Succinivibrionaceae (f)<br>⬆ Succinivibrio (g) | G,O |
| Stewart, 2016 | Y | Adults with Type 1 Diabetes (T1D), and Adult non-diabetic controls, otherwise healthy, n=20 | To determine if the intestinal microbiome of physically fit adults with managed T1D resembles the intestinal microbiome of physically | 1. T1D<br>2. Non-T1D | No, diets were assessed with 24-hour dietary recalls. | PCR, 16S rRNA sequencing of fecal samples. | OTUs, α-diversity, abundance, bacterial phyla and species | **T1D†**<br>Firmicutes<br>Lachno-spiraceae (f)<br>Dialister (g)<br>Actinobacteria<br>Actinomyces (g)<br>Collinsella (g)<br>**Non-T1D†**<br>Firmicutes<br>Lachno-spiraceae (f)<br>Anoxybacillus (g)<br>Clostridium _sensu_stricto (g) | O |

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| | | | fit adults without T1D. | | | | | Coprococcus (g)<br>Actinobacteria<br>Coriobacteri-aceae (f)<br>Proteobacteria<br>Aurantimonas (g)<br>Burkholderales (o)<br>Zoogloea (g)<br>Schlegelella (g)<br>Comamona-daceae (f) | |
| Allen, 2017 | Y | Normal weight and obese sedentary adults, otherwise healthy, n=32. | To determine influence of longitudinal exercise intervention on the intestinal microbiome where subjects served as their own controls. | 1. Sed. + lean<br>2. Sed. + obese<br>3. Ex. + lean<br>4. Ex + obese | Yes, 3-day control diet prior to fecal samples. | PCR, 16S rRNA sequencing | OTUs, α-diversity , β-diversity, bacterial genera, species | No significant findings between groups after 6 weeks of exercise or 6 weeks of washout. | O |
| Bressa, 2017 | N | Females, premeno-pausal, healthy, n=40 | To obser-vationally compare the intestinal microbiome differences between sedentary and physically active females. | 1. Sed<br>2. Active | No, diets were charac-terized using FFQs. | PCR, 16S rRNA sequencing | OTUs, α-diversity, β-diversity, bacterial phyla, family, genus, and species, bacteroidetes:firmicutes | **Sed.**<br>⬆ Bacteroidetes<br>⬆ Barnesiellaceae (f)<br>⬆ Barnesiellaceae unclassified (g)<br>⬆ Odoribacteraceae (f)<br>⬆ Odoribacter (g)<br>Firmicutes<br>⬆ Turicibacter (g)<br>⬆ Ruminococcaceae unclassified (g)<br>⬆ Ruminococcus (g)<br>Proteobacteria<br>⬆ Desulfovibrionaceae (g)<br>**Active**<br>Bacteroidetes<br>⬆ Paraprevotella (g) | G, O |

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| | | | | | | | | Firmicutes<br>⬆ Faecalibacterium prautznnii (s)<br>⬆ Roseburia hominis (s)<br>⬆ Coprococcus (g)<br>Actinobacteria<br>⬆ Bifidobacterium (g)<br>Proteobacteria<br>⬆ Haemophilus (g)<br>Verrucomicrobia<br>⬆ Akkermansia muciniphila (s) | |
| Paulsen, 2017 | N | Female breast cancer survivors (BCS), otherwise healthy, n=12 | To evaluate the influence of CRF and psychosocial factors on the intestinal microbiome in BCS. | 1. BCS | No, diets were assessed with 3-day food intake records. | PCR, 16S rRNA sequencing | OTUs, α-diversity, β-diversity, bacterial phyla, family, order, and genus | BCS:<br>No quantitatively significant differences. | G, O |

Significant results are presented by study within the "Significant bacterial taxonomy findings" column. All significant findings are denoted with an arrow indicating the direction of change, and are categorized within their respective phyla, and further characterized where: (c)=Class; (o)= Order; (f)= Family; (g)=Genus; and (s)=Species. *Represents results with cecal contents only. †Direction of results not reported. ° Direction of change varied based on bi-weekly timepoint over 12-week intervention