Exercise, Genistein, and Their Combined Effect on Gut Microbiota and Mitochondrial Oxidative Capacity After 12-Week of a Western Diet on C57BL/6 Adult Mice

by

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ABSTRACT

Obesity is one of the most challenging health conditions of our time, characterized by complex interactions between behavioral, environmental, and genetic factors. These interactions lead to a distinctive obese phenotype. Twenty years ago, the gut microbiota (GM) was postulated as a significant factor contributing to the obese phenotype and associated metabolic disturbances. Exercise had shown to improve and revert the metabolic abnormalities in obese individuals. Also, genistein has a suggested potential anti-obesogenic effect. Studying the dynamic interaction of the GM with relevant organs in metabolic homeostasis is crucial for the design of new long-term therapies to treat obesity. The purpose of this experimental study is to examine exercise (Exe), genistein (Gen), and their combined intervention (Exe + Gen) effects on GM composition and musculoskeletal mitochondrial oxidative function in diet-induced obese mice. Also, this study aims to explore the association between gut microbial diversity and mitochondrial oxidative capacity. 132 adult male (n=63) and female (n= 69) C57BL/6 mice were randomized to one of five interventions for twelve weeks: control (n= 27), high fat diet (HFD; n=26), HFD + Exe (n=28), HFD + Gen (n=27), or HFD + Exe + Gen (n=24). All HFD drinking water was supplemented with 42g sugar/L. Fecal pellets were collected, DNA extracted, and measured the microbial composition by sequencing the V4 of the 16S rRNA gene with Illumina. The mitochondrial oxidative capacity was assessed by measuring the enzymatic kinetic activity of the citrate synthase (CS) of forty-nine mice. This study found that Exe groups had a significantly higher bacterial richness compared to HFD + Gen or HFD group. Exe + Gen showed the synergistic effect to drive the GM

towards the control group´s GM composition as we found *Ruminococcus* significantly more abundant in the HFD + Exe + Gen than the rest of the HFD groups. The study did not find preventive capacity in either of the interventions on the CS activity. Therefore, further research is needed to confirm the synergistic effect of Exe, Exe, and Gen on the gut bacterial richness and the capacity to prevent HFD-induced deleterious effect on GM and mitochondrial oxidative capacity.

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DEFINITION OF TERMS

- Genistein: The most abundant soy isoflavone with therapeutic effects when taken as a dietary supplement or as a part of soy-based food consumption.
- Microbiota: A collection of bacteria, archaea, lower and higher eukaryotes, and viruses in a defined environment (e.g., gut)
- Microbiome: The collection of microbial genes in a defined environment.
- Dysbiosis: The imbalance in beneficial-to-pathogenic species diversity and abundance within the microbial community.
- Prebiotic: A substrate that is selectively utilized by host microorganisms conferring a health benefit (as defined by the International Scientific Association for Probiotics and Prebiotics, ISAPP).
- Probiotic: Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (as defined by the ISAPP).
- Isoflavone: soy phytonutrients (genistein, daidzein)
- Alpha-diversity: Within-individual species diversity.
- Beta-diversity: Inter-individual species diversity.
- Mitochondria: A two-membrane organelle involve in the ATP regeneration, calcium-ion control, cell signaling, and apoptosis.
- Fatty acids β-oxidation: A catabolic process where fatty acids are broken down into Acetyl CoA for ATP production in the mitochondria.

- Tricarboxylic acid cycle (TCA) or Krebs cycle: The cycle that occurs in the matrix of the mitochondria to synthesize ATP following the complete oxidation of acetyl groups formed from carbohydrates, fat, or protein.
- Adenosine Triphosphate (ATP): A molecule compromised of an adenosine ring and three phosphate groups which provides energy to all living cells.
- Citrate Synthase: The enzyme catalyzing the first reaction of the TCA conversion of oxaloacetate and acetyl-CoA to citrate.
- β-Hydroxyacyl-CoA Dehydrogenase: The third enzyme in the fatty acid ß-oxidation pathway catalyzing the reaction from ß-hydroaxyl-CoA to ß-ketoacyl-CoA.

CHAPTER 1

INTRODUCTION

Obesity is one of the most challenging health conditions of our time. Its prevalence has almost tripled since 1975 and the number of people classifying as obese continues to rise.[1] Body weight status is classified by the World Health Organization (WHO) using body mass index (BMI, weight in kilograms divided by height squared in meters) in the following categories: underweight BMI lower or equal to 18.9 kg/m^2, normal weight BMI 19 – 24.99 kg/m^2, overweight BMI 25 – 29.99 kg/m^2, and obese BMI equal or greater to 30 kg/m^2. In addition, obesity is associated with the increased risk of developing type 2 diabetes,[2] cardiovascular diseases,[3] various types of cancer,[3,4] hypertension,[5] infertility,[6,7] liver disease,[8] and mortality.[9] While the prevalence of obesity continues to rise, treatments to address this disease remains a matter of debate.[10] Obesity is a multifactorial disease characterized by complex interactions between behavioral, environmental, and genetic factors.[11] These interactions lead to a distinctive obese phenotype[12] characterized by increased body fat composition,[13] low-grade chronic inflammation,[14] metabolic abnormalities (e.g., increased energy harvest from gut microbes),[15] and impaired physiological processes (e.g., decreased insulin sensitivity).[16]

Crucial physiological abnormalities experienced during the onset and development of obesity are the impairment of skeletal muscle function and the reduction in number of musculoskeletal mitochondria.[17] This is of relevance for the overall health of the body and energetic metabolism, as the muscle acts as an endocrine organ releasing anti-inflammatory myokines (e.g., IL-6 and IL-10) into the bloodstream.[18,19] Excessive

caloric intake and obesity-associated high-fat diet (HFD)[20] enhance fatty acid ß-oxidation activity in an attempt to overcome the extra lipid substrate. However, the excess lipids do not get completely oxidized, increasing the concentration of toxic lipid metabolism intermediates (e.g., ceramides) in the cytosol of myocytes and excess of Acetyl-CoA in the mitochondria. The accumulation of lipid intermediates and the excess Acetyl-CoA overwhelms the oxidative capacity of the tricarboxylic acid cycle (TCA) leading to mitochondrial dysfunction.[21,22]

Mitochondrial dysfunction has been observed in obese individuals. Dysfunction varies among individuals and has been described as a result of decreased oxidative phoshorylation[23] and oxidative capacity, a reduced number of mitochondria, excess accumulation of intramyocellular lipids (lipotoxicity), lower basal ATP synthesis, and increased cellular damage from reactive oxygen species (ROS).[17,24] As described by Koves and colleagues,[25] male rats fed a HFD experienced increased rates of fatty acid ß-oxidation in the mitochondria. Despite the higher activity of ß-oxidation, lipids were not entirely oxidized, and consequently toxic lipid metabolism intermediates accumulated (e.g., ceramides) in the myocytes. The imbalanced environment created by the accumulation of toxic fat metabolites suppressed the production of TCA cycle intermediates citrate, fumarate, and malate, and impeded the phosphorylation of protein-kinase B (ATK) necessary for the translocation of glucose transporters (GLUT4) to the outer cell membrane. This resulted in insulin resistance and poor glucose uptake into cells.[25]

Exercise enhances the oxidative capacity of musculoskeletal mitochondria in states of health and disease.[26] Importantly, a lack of physical activity and exercise has been described as one of the major behavioral aspects contributing to the onset of obesity.[27,28] According to the American College of Sports Medicine (ACSM)[29] physical activity is the voluntary movement produced by the contraction of skeletal muscle that results in a substantial increase in resting energy expenditure. Whereas, exercise is defined as a type of planned, structured, and repetitive physical activity that is done to improve and/or maintain physical fitness.[29] Different modalities of exercise including endurance (moderate- and high-intensity interval training),[30] and resistance training[31,32] have been shown to be effective in protecting and reducing the physiological abnormalities and comorbidities associated with obesity.[31] Exercise improves the health of obese individuals independent of weight loss.[33] In particular, endurance exercise increases cardiorespiratory fitness,[34,35] enhance muscle function,[36–39] and stimulates lipolysis in the adipose tissue[40] of obese individuals. Endurance exercise stimulates the activity of carbohydrate metabolism,[38] TCA cycle,[41] and ß-oxidation[39] enzymes, thereby improving the oxidative capacity of musculoskeletal mitochondria.

Dietary composition and diet quality (how the food intake aligns with the national food guidelines for macro and micronutrients recommended daily intake for a healthy life; the higher the score, the higher the quality of the diet a measuring score of food intake)[42] are considered one of the leading behavioral factors, in combination with the absence exercise, that contribute to the onset and progression of obesity and associated comorbidities.[10] The contribution of HFD or Western-style diets[43] to the obesity epidemic

has been of particular interest in the last two decades. Diet composition contributes to increases in body weight and modifications of adipose tissue that result in increased adipogenesis and impairment of metabolically active organs including the liver and skeletal muscle.[22,44]

Functional food ingredients, a diverse group of nutrients with health benefits attributed to their supplementation to a food product or consumption as a supplement,[45] have been studied as potential nutraceutical interventions to recuperate HFD-induced obesity and related metabolic abnormalities in animal and human models.[46–49] Among the different functional ingredients, the soy-derived isoflavone genistein has recently been highlighted for its potential role as an anti-obesogenic ingredient.[48,50–52] Genistein requires a conversion of glycosides into the bioactive aglycones by the gut microbiota to apply any systemic nutraceutical action.[53] Some gut bacteria had been identified to be responsible for the conversion of genistein to its active metabolites, such as *Lactobacillus spp. Bifidobacterium spp.* and *Slackia isoflavoniconvertens*.[53,54]

Over the last twenty years, the gut microbiota has been postulated as a novel significant factor contributing to the obese phenotype and associated metabolic abnormalities.[55–57] The human gut microbiota includes up to 100 trillion microbes (bacteria, archaea, virus, and fungi) and has 150 times more genes than the human genome.[58] Bacteria are the most abundant microbes in the gut, representing 97% of the gut microbiota population.[59] At the phylum-level, the gut microbiome is dominated by *Bacteroidetes* and *Firmicutes*, and to a lesser degree by *Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia.*[60] The gut microbial community executes numerous

relevant physiological functions such as extraction of energy, protection and maintenance of gut permeability, and regulation of the immune system.[61,62] However, when the microbial homeostasis (i.e., the balance of good and bad microbes) is disrupted, a reduction in diversity and stability of core gut microbiota occurs. This allows for an increase in opportunistic pathogenic microbes, also known as dysbiosis. Ultimately, this phenomenon is thought to leads to gastrointestinal disorders (e.g., inflammatory bowel disease) and systemic diseases (e.g., metabolic abnormalities).[63]

Obesity is characterized by a dysbiotic gut microbial community with an increased capacity to harvest and store energy.[64,65] The exact mechanism behind the relationship between dysbiosis and etiology of obesity is complex and remains understudied. What has been under investigation is the impact that behavioral factors, including diet and physical activity/exercise,[66–69] have on shaping the gut microbiota composition, and consequently its function. Rodent and human-based evidence suggests that HFD disrupts gut microbial homeostasis,[70–75] thereby reducing the content of Gram-positive bacteria[57] and increasing bacteria that play a significant role in the onset and development of obesity.[76] In contrast to HFD, an increase in dietary carbohydrate and fiber intake,[75,77] consumption of functional foods[78–83] (e.g., genistein), and physical activity/exercise[79,84,85] have been shown to exert a protective and therapeutic effect on the gut microbiota.

Genistein is one of the most abundant isoflavones in soy.[86] Genistein and derived metabolites have been shown to have nutraceutical capacities in different diseases (e.g., breast cancer).[87] The mechanism by which genistein exerts therapeutic effects on tumor-

growth and bone health is an area of rising study.[88,89] However, the mechanism behind the anti-obesogenic therapeutic effect still remains poorly understood.[48] Most studies hypothesize about the mediating role of the gut microbiota in genistein bioactivity, and consequently its therapeutic effects.[83,90,91] Lu et al. recently tested genistein, soybean polysaccharides (SP), and their combined effects on C57BL/6J mice fed an HFD to induce obesity for 12 weeks.[52] They found that genistein, SP, and combined treatment inhibited the high peak in blood glucose after an infusion of a glucose bolus. Also, genistein and SP showed lipolytic effects on adipose tissue and hepatocytes. Their use reduced the fat content in adipose tissue by regulating the expression of lipogenesis-related proteins (e.g., adipose differentiation-related protein, ADRP). Genistein and SP stimulated lipid oxidation enzymes (Acetyl-CoA carboxylase and fatty acid synthetase) in the liver, which reduced fat content. Genistein, SP, and the combined group also exerted significant reductions on the *Firmicutes/Bacteroidetes* ratio when compared to the HFD group. The same impact on *Firmicutes/Bacteroidetes* ratio and increased relative abundance of *Prevotella*, a health-associated microbe, was found in ovariectomized rats fed a isoflavone (daidzein and genistein) enriched diet for 27 weeks.[82]

Dietary and exercise interventions are temporary solutions for obesity (e.g., the weight loss in a lifestyle program is often recovered after a year) and the dysregulation of complex interactions among different organs (e.g., skeletal muscle and gut).[92] Studying the dynamic interaction of the gut microbiota with relevant host organs in metabolic homeostasis (e.g., skeletal muscle) is crucial for building different models for diagnosis and treatment of obesity. Many interactions are currently under investigation such as that

of the gut–brain axis,[93] but the gut–muscle axis has only recently been introduced.[94,95] Scheiman et al. showed that these two organs interact in the maintenance of energy metabolism.[94] The lactate produced during exercise as a consequence of anaerobic metabolism in the musculoskeletal cells was also recently found to reach the gut lumen where it was metabolized to propionate by *Veillonella atypica,* a bacteria from a genus increased in endurance athletes.[96] Less is known about the gut–muscle axis in disease. Houghton et al. suggest that gut microbiota metabolites derived from dietary phenolic components increase GLUT4 expression, increase phosphorylation of Protein Kinase B (Akt) and Phosphatidylinositol-3-Kinase (PI3K).[95] Consequently, enhancing the glucose uptake and metabolism in musculoskeletal tissues under normal and insulin resistant conditions (high-glucose, high-insulin).[95] The gut-muscle axis (figure 1) requires further investigation as a potential target for improving metabolic abnormalities in the obese population.

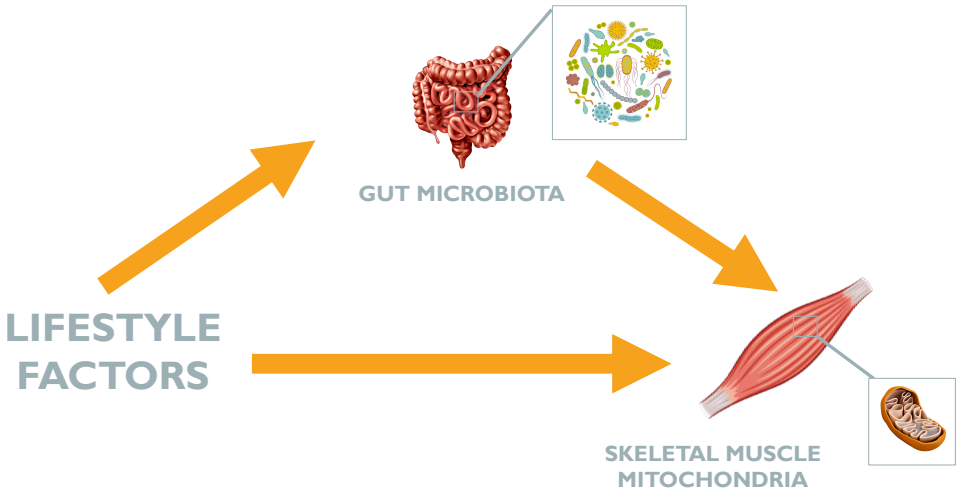

Figure 1. Behavioral factors – gut microbiota – muscle axis.

Purpose of Study

The purpose of this experimental study was to examine exercise, genistein, and their combined (exercise + genistein) intervention effects on gut microbiota composition and musculoskeletal mitochondrial function in diet-induced obese mice.

Specific Aims and Hypotheses

Aim 1: To determine the impact of exercise, genistein, or combined (exercise + genistein) treatment on gut microbial diversity and bacterial community structure in the presence of a 12-week high-fat, high-sucrose diet (HFD) in C57BL/6 mice.

H1: We hypothesized that the gut microbiota of exercise + genistein and control groups will not differ for alpha-diversity (Observed Operational Taxonomic Units (OTUs), Faith PD, Pielous' evenness, Shannon´s Diversity Index, and Chao1 Richness index) in the presence of a 12-week HFD and that the alpha-diversity for both groups will diverge significantly from the exercise and genistein-only intervention groups. Further, we expected that the alpha diversity following HFD will be significantly reduced compared to all other intervention groups.

H2: We hypothesized that the gut microbiota of exercise + genistein and control groups will not differ for beta-diversity (Jaccard dissimilarity index, Bray-Curtis dissimilarity distance, weighted/unweighted Unifrac dissimilarity distances) in the presence of a 12-week HFD and that the diversity in these two groups will differ significantly from the exercise and genistein-only intervention groups. Further we hypothesized that the community structure following an HFD will significantly differ from all other intervention groups.

H3: We hypothesized that the exercise + genistein and control groups will present with the same differentially abundant taxa in the presence of a 12-week HFD and that differentially abundant taxa in these two groups will differ from those of the exercise and genistein-only intervention groups. Further, we expect the HFD group significantly abundant taxa will differed from all intervention groups.

Aim 2: To assess the effect of exercise, genistein, or combined (exercise + genistein) intervention on mitochondrial oxidative capacity in skeletal muscle in the presence of 12 weeks of HFD in C57BL/6 mice.

H1: We hypothesized that the exercise + genistein and control groups would have similar TCA cycle activity (citrate synthase activity, CS) in the presence of a 12-week HFD and that CS activity in these groups would be greater than the exercise and genistein-only intervention groups. Further, we expected the HFD to have significantly lower CS activity compare to all other intervention groups.

Aim 3: To explore the mediation of gut microbiota on the Exe, Gen, and Exe + Gen on the mitochondrial oxidative capacity in the skeletal muscle of C57BL/6 mice fed an HFD for 12 weeks.

H1: We hypothesize that alpha diversity (Shannon diversity index) and CS activity will positively associate in all groups after adjusting for sex.

H2: We hypothesize that the gut microbiota alpha-diversity (bacterial richness) mediates the relationship between lifestyle behaviors (genistein and exercise) and the mitochondrial oxidative capacity.

CHAPTER 2

LITERATURE REVIEW

Public Health Problem: Obesity

Obesity has tripled its prevalence since 1975 and become one of the most complex preventable diseases to defeat in modern society.[1] According to the World Health Organization (WHO), obesity affects 650 million people in the world.[1] The National Center for Health Statistics (NCHS) in 2015-2016 reported that 39.8% of the adults in the United States (US) were obese, with no significant differences between men and women.[97] By 2030, obesity will have the largest impact on the global economy and in public health, as it will become the main non-communicable disease.[9] It is expected to affect 86% of adults in the US.[9]

The cost of obesity is divided into direct and indirect costs. Direct costs include spending on diagnosis and treatment of the disease, as well as comorbidities (e.g., type 2 diabetes mellitus), while indirect costs include the productivity, mortality rates, and disabilities that occur as consequences of obesity. As concluded by Hammond in 2010,[98] the collective economic burden of obesity exceeded $300 billion per year in the US, with $142 billion accounting for direct costs and $208 billion for indirect costs.

Obesity is a multifactorial disease with complex physiological interactions that lead to an increased risk for type 2 diabetes,[2] cardiovascular diseases,[3] various types of cancer,[3,4] hypertension,[5] infertility,[6,7] liver diseases,[8] and mortality.[9] Obesity is defined by excessive fat accumulation as a result of a chronic imbalance between energy intake and energy expenditure.[99–101] Interestingly, the literature has described two different

metabolic states of obesity: (1) metabolically healthy obese (MHO) and (2) metabolically unhealthy obese (MUO). Although both MHO and MUO have elevated fat mass, MUO individuals display an adverse metabolic profile characterized by disrupted insulin sensitivity, hypertension, chronic low-grade inflammation, elevated visceral ectopic adipose tissue, and dyslipidemia.[102] MHO individuals are still at higher risk of cardiovascular disease events in comparison to normal weight metabolically healthy individuals,[103] but MUO individuals have an increased risk of developing obesity-associated comorbidities, such as type 2 diabetes, when compared to MHO people.[99] Besides the knowledge of the heterogenicity of obese phenotypes, the lack of universally accepted criteria to diagnose MHO continues to be a matter of debate. Body Mass Index (BMI; body weight in kilograms, divided by height in meters squared) continues to be internationally used to diagnose obesity, despite its limitations for identifying whether person is metabolically healthy or not.[101]

Animal Models of Obesity

Animal models have been used since the 1950s to analyze the pathophysiology of diet-induced obesity.[104] Due to the multifactorial nature of obesity, different animal models have been used to study genetic and environmental factors (e.g., diet-induced obesity, DIO) that contribute to obesity.[105,106] Despite rodent models being the most abundant animal models used to assess obesity, other animal models (e.g., zebrafish) are used to study pathophysiological mechanisms of the disease.[106] As summarized by Zang and colleagues, zebrafish are an animal model of interest to study obesity and diabetes because of the conservation of metabolic mechanisms that are impaired during obesity

and diabetes, such as glucose homeostasis and lipid metabolism, when compared to humans.[107] Whereas, other bigger animals such as pigs, dogs, and non-human primates are used for the investigation of obesity because of their closer similarities to humans with regard to genetics, physical size, anatomy, and physiology.[106]

Rodent models are predominantly used because of the variety of strains (monogenic vs. polygenic) available to study, as well as their rapid development of metabolic abnormalities such as insulin resistance or disrupted glucose homeostasis, their valid representation of human tissues and physiological processes, and their cost-effective nature.[105,106] The most commonly used rodent models to assess the effects of diet-induced obesity (DIO, fed with diets containing 30-80% of energy from fats) are Sprague Dawley Rats or C57BL/6 mice.[108] The C57BL/6 mouse model is used because these animals are prone to developing severe obesity, insulin resistance, abnormalities in glucose homeostasis, and increased adipose tissue in a relatively short period of time (e.g., weeks).[109] Therefore, mouse models have relevance for understanding impairments in physiological mechanisms that occur during obesity. They also allow for the identification and development of effective and safe interventions and treatments that may easily translate to humans.

Pathophysiology of Obesity

The primary organs associated with disrupted metabolism during obesity are adipose tissue, liver, and skeletal muscle.[110–113] Under normal physiological conditions, adipose tissue serves as a depot of stored glycerol and fatty acids while also signaling as an endocrine organ; these actions play a pivotal role in the metabolic homeostasis of the

body.[110] Adipose tissue releases molecules (adipokines) to control body weight, satiety, and inflammation.[114] In obese individuals, adipose tissue content is increased. Ultimately, excess fat stores negatively influence the structure and function of adipocytes.[115,116] Pathophysiologically, the adipose tissue releases pro-inflammatory adipokines (e.g., Tumor necrosis factor alpha, TNFα), increases the release of free fatty acids into the bloodstream,[100,117] and enhances the trafficking and storage of lipids into non-adipose tissues (e.g., liver and skeletal muscle), a phenomenon known as lipotoxicity.[118] In the liver, increased lipid storage induces inflammation of the hepatocytes and the accumulation of toxic lipids, which ultimately leads to non-alcoholic fatty liver disease (NAFLD).[8,119,120] Lipotoxicity in the skeletal muscle leads to major disruptions in energy homeostasis, increased low-grade inflammation by reactive oxygen species (ROS), and insulin resistance.[113,121]

Skeletal Muscle

The skeletal muscle is the largest metabolic organ in the body and is a major contributor to energy metabolism.[122] Regulation of glucose and fatty acid oxidation is central for skeletal muscle cell (myocyte) function.[123] Myocyte membranes have insulin signaling receptors (ISR) that are stimulated by elevated insulin concentrations in the blood, previously released by the pancreatic ß-cells in response to increases in circulating blood glucose. Once the insulin binds to ISR, myocytes undergo a series of phosphorylation reactions to stimulate the translocation of glucose transporters (GLUT4) to the cell membrane. Glucose is then shuttled through GLUT4 into the cells so that it can be used as a fuel substrate or for storage as glycogen. In contrast, fatty acids are

transported to the inside of the myocytes by simple diffusion. Once glucose reaches the sarcoplasm (cytoplasm in the myocytes), it is catabolized via glycolysis. This results in the production of pyruvate and cytosolic-reducing equivalents (e.g., NADH). Whereas, fatty acids are transformed to fatty-acid acyl CoA in the outer mitochondrial membrane. These metabolic products are then transformed into Acetyl-CoA, an important mitochondrial intermediate that can be used in the tricarboxylic acid (TCA) cycle.

Mitochondria are dynamic organelles that regulate cellular activities including adenosine triphosphate (ATP) production via oxidative phosphorylation (OXPHOS) in the electron transport chain; programmed cellular death known as apoptosis; and regulation of reactive oxygen species (ROS) as a part of cellular redox balance reactions.[124] The main function of the mitochondria is the oxidation of substrates like glucose and fatty acids. Each of these substrates undergoes different oxidative processes that result in the production of Acetyl CoA. Glucose-derived acetyl CoA comes from glycolysis, while fatty acid-derived acyl CoA is oxidized via the ß-fatty acid oxidation cycle before entering the TCA cycle. Reactions in the TCA cycle and ß-oxidation pathway result in the synthesis of reduced intermediates, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), all of which are necessary for the transfer of electrons to the electron transport chain (ETC). The ETC consists of four protein-lipid complexes plus an ATP synthase complex. The four complexes shuttle electrons from NADH and $FADH_2$ to a series of electron acceptors that ultimately end up being passed to oxygen, the most electronegative electron acceptor in the chain. This series of redox reactions leads to free energy release, which is used to

create a proton gradient via proton transfer to the mitochondrial intermembrane space. The last complex, ATP synthase, couples energy released during proton flow down the previously generated gradient into the mitochondrial matrix, resulting in the phosphorylation of adenosine diphosphate (ADP) to form adenosine triphosphate (ATP).

In obese individuals, energy homeostasis is impaired as a result of dysfunctional mitochondria.[125] This dysfunction results from fewer mitochondria and reduced biogenesis, attenuated oxidative capacity, inefficient bioenergetics (amount of ATP produced per nutrient molecule), and imbalances between the fusion and fission of mitochondria (relevant processes for recycling of this organelle).[21,125–127] High-fat high-sugar diets (HFD), which have been shown to induce obesity, are associated with excessive adipose tissue deposition and increased lipid accumulation in the skeletal muscle and other organs, thereby exceeding the metabolic demands of cells in these tissues.[121,128] As a result of increased substrate, myocytes increase fatty acid catabolism via fatty acid ß-oxidation enzyme activity. However, lipids are only partially oxidized compared to physiological conditions, leading to an intra-mitochondrial accumulation of Acetyl-CoA and toxic lipid intermediates (e.g., ceramides and diacylglycerol) in the cytosol of the myocytes.[118] The TCA cycle cannot cope with the excess Acetyl-CoA, which leads to a disconnect between ß-oxidation and TCA cycle activity, thereby causing mitochondrial oxidative capacity dysfunction.[121] The reduced number of mitochondria in obese individuals also contributes to an inability to overcome the overload of lipids in the myocyte.[129]

Mitochondrial Oxidative Capacity

Decreased OXPHOS capacity in the muscle mitochondria can be characterized by a decrease in the activity of citrate synthase (CS) and increased activity of ß-hydroxyacyl-coenzyme A dehydrogenase (ß-HAD) in the TCA cycle and fatty acid ß-oxidation pathways, respectively. Alhindin et al.,[130] tested the association between mitochondrial oxidative capacity (CS and ß-HAD activities) and an HFD-induced (45% of the energy from fat) state of lipotoxicity in CS-deficient (congenital) C57BL/6J mice compared to normal controls. The data indicated that the absence of CS enzyme was correlated with greater lipotoxicity in muscle cells, and no significant differences were found in the ß-HAD activity between groups. However, there was no comparison between the baseline CS activity and 12-week after HFD in any of the groups, which makes it difficult to assess how the HFD directly impacted CS activity in control C57BL/6J mice.

CS activity has been studied to assess the impact of HFD in mitochondrial oxidative capacity. A consistent decline was found in CS activity and 3% reduction of the mitochondrial density (mitochondrial DNA/Nuclear DNA) in the skeletal muscle has been observed in male C57BL/6J mice after 4 and 16 weeks of following an HFD (36% from fat).[131] Others have observed no change in CS activity after 8-week of HFD (60% of the energy from fat) in the same strain of mouse.[132] Wistar rats fed for a more extended period (e.g., 12 weeks), resulted in reduced CS activity when compared to chow-fed rats.[133] Contrary to these results, G-Y Song et al.,[134] found that 16-weeks of HFD or high-fructose diet resulted in significantly increased CS activity among C57BL/6J mice. The

literature remains unclear on the impact of HFD-induced impairments on skeletal muscle CS activity. Further research on this TCA cycle enzyme is required to understand the extent to which an HFD induces impairments in the oxidative capacity of the skeletal muscle mitochondria.

Increased dietary fat intake has also been suggested to affect the lipolysis in the skeletal muscle mitochondria by enhancing ß-oxidation in an attempt to oxidize the excess flow of lipids. ß-HAD, the enzyme catalyzing the last step in the ß-oxidation of fatty acids, has been used as a marker of fatty acid oxidation. ß-HAD activity has been observed to increase significantly in the soleus muscle ($p<0.05$) in C57BL/6J mice fed an HFD (60% of the energy from fat) for 8 weeks. HFD exposure for 16-weeks showed the same trend with a significant increase in ß-HAD among C57BL/6J mice fed an HFD (%).[134] Still, other studies have indicated no significant changes in ß-HAD, or the ß-HAD/CS activity ratio in Wistar rats fed an HFD (39.7-45% of energy from fat) for 5 weeks.[135–139] It seems that HFD may have greater negative effects over the ß-HAD activity when the myocyte is exposed to a longer periods (5 weeks vs. 16-weeks) of elevated dietary fat.

Factors that Influence Metabolism in Obesity

A variety of treatments have been implemented to prevent the rise in obesity without successful long-term sustainable results.[140] The choice of initial therapy is a lifestyle intervention that includes weight loss through behavior change, including nutrition and exercise approaches.[141] If behavioral strategies are unsuccessful or individuals have reached advanced stages of obesity (e.g., BMI > 40 kg/m^2),

pharmacological and surgical treatment are often prescribed for weight management.[141] However, obesity goes beyond an excess of body weight and abnormal adipose tissue distribution and storage. The multifactorial nature (genetic, behavior, and environmental factors) of this disease makes it a challenging condition to prevent and treat.

Genetic factors

Genetics play a role in the current obesity epidemic. Loss-of-function mutations have been observed in specific genes (e.g., melanocortin 4 receptor, MC4R)[142] but appear to be rare, affecting ~5% of the global population. These mutations cause deficiencies in satiety hormones (e.g., leptin) (energy intake > energy expenditure), thereby impairing the energy homeostasis, and consequently increasing in total body weight.[143] Single nucleotide polymorphisms (SNPs, a variation in a single base pair in a DNA sequence) are believed to contribute to less than 3% of obesity cases worldwide.[144] However, genome-wide association approaches have revealed more than a hundred SNPs (e.g., fat mass and obesity-associated gene, FTO)[145,146] that are associated with BMI and common obesity.[144] FTO SNPs are associated with increased appetite, and greater energy, and protein intake. Carrying a SNP makes individuals genetically more prone to developing obesity, but may also require exposure to unhealthy behaviors and environmental factors (e.g., increased consumption of dietary saturated fat content) to develop the disease.[147] Obesity cases solely explained by gene mutations are scare, at 8% of the obese population, where the remaining 92% of obesity cases are a consequence of a complex interactions between environment, behavior, and genetic factors.[147–149] Systematic reviews showed that the BMI seems to have a high heritability (reported a range from 47

to 90%) among twins and family members (from a 24% to 80%) regardless of sex, with higher heritability in the first years of life compared to adulthood.[150,151] Overall, different literature in genetics and obesity have concluded that, besides the genetic heritability of obesity, genetics are dramatically altered by behavior and environmental factors, but how much these factors alter the genetic risk of obesity requires further investigation.[147–149]

Behavioral Factors

Exercise

Exercise has a positive impact on weight management and it has been shown to revert some of the metabolic abnormalities of HFD-induced obesity.[124] Specifically, exercise increases the mitochondrial content in muscle tissue by stimulating the transcription factor peroxisome proliferator-activated receptor gamma coactivator-1α (PGC1-alpha), which coactivates DNA binding transcription factors NRF-1 and NRF-2 to enhance mitochondrial biogenesis.[152] Also, exercise augments the oxidative capacity of muscle by increasing TCA cycle, and fatty acid ß-oxidation enzyme activity (e.g., CS and ß-HAD, respectively).

Aerobic exercise has shown positive effects on mitochondrial oxidative enzyme activity (increased CS activity and ß-HAD activity) in obese individuals. Hey-Mogensen et al.,[153] have shown that obese non-diabetic men (n=14) following a 10-week aerobic training regimen at 65% VO_{2peak} experienced an improvement in CS activity when compared to baseline (pre 78.5 ± 4.3, post 102.4±9.1 U/mg dry muscle weight; $p< 0.01$). Others[36] have found that 8-week aerobic training enhanced beta-oxidation capacity by more than 120%, and reduced the production of toxic lipid intermediates DAG and

ceramides by 15% and 42%, respectively, in the vastus lateralis of obese adults. In a 16-week moderate aerobic exercise program or caloric restriction treatment, elderly overweight and obese individuals experienced an increase in muscle ß-HAD activity by 30.7% ± 6.8% compared to baseline values in both exercise and caloric restriction groups.[154] CS activity did not improve but this could have been related to the higher baseline CS activity observed in the exercise group compared to the ones who received a caloric restriction program for 16-weeks.[154] However, in a short aerobic (70% VO2max) program with obese young adults, both CS and ß-HAD activity was significantly increased but only CS activity reached significance.[155] Nonetheless, others[156] have not found significant improvements in CS activity in overweight and obese adults participating in a moderate or high-intensity interval training programs. Overall, the literature indicates that mitochondrial oxidative capacity can be enhanced following an aerobic exercise program in rodents and human trials. The literature in humans is not as clear as it is in animal studies to establish which exercise intensity, duration, and modality are effective to enhance musculoskeletal mitochondrial oxidative capacity in obese individuals.

Aerobic exercise has been shown to revert the mitochondrial oxidative capacity impairments (reduced CS activity and increased ß-HAD activity) observed in HFD consumers. An 8-week aerobic exercise training intervention was enough to increase CS activity after a 12-week HFD (60% of the energy from fat) experiment in C57BL/6J female mice.[157] In human trials, aerobic training programs showed contradictory results compared to the rodent models. Battaglia et al.,[158] compared the effect of an aerobic

training program (10 consecutive days, 1 hour a day at 70% VO_{2peak} on a cycle ergometer) in lean and obese male adults to recuperate the deleterious effects of a short-term isocaloric HFD (~70% Fat, 15% carbohydrate, and 15% protein) on skeletal muscle energy metabolism. Participants were fed an HFD for 3-days before the 10-day exercise intervention and consumed the same HFD during the last three days (days 8 through 10) of the exercise intervention. They evaluated *vastus lateralis* samples for CS and ß-HAD activity and found that the exercise program reverted the short-term HFD-induced alterations in CS activity (increased CS activity) in the obese group. ß-HAD activity also increased but did not reach significance ($p=0.07$). Although, more human studies are needed, it appears that exercise training elicits an improvement in mitochondrial activity in obese individuals when compared to baseline measurements of CS and ß-HAD activity.

Overall, the literature suggests that aerobic exercise is a viable treatment to correct impaired mitochondrial oxidative capacity in obese individuals. Exercise has the capacity to increase CS activity, while maintaining a physiological level of ß-HAD activity. Therefore, exercise may be a successful treatment to revert HFD-induced mitochondrial dysfunction (reduced CS activity, and exacerbation of ß-HAD activity). However, more research is necessary to understand the impact of training (type, intensity, and duration) needed to recuperate CS and ß-HAD activity in the presence of HFD-induced obesity.

Diet

Diet, in particular a HFD, has been implicated as a significant behavioral aspect to induce obesity since the 1950s.[104,159,160] Dietary fat is the macronutrient with more energy per gram (9 kcal per gram), and fewer energetic requirements to digest, metabolize, and store.[161] When there is an increase in caloric intake through dietary fat, it is positively correlated with gain weight.[162] But the source of those calories is as crucial as the amount of energy that individuals consume for increasing body weight and adiposity. As summarized by Miller, obesity is a problem of overeating certain types of fat; specifically, saturated fat is thought to be one of the most harmful types of fat that increase body weight and adipose tissue.[163] A study comparing in obese women a 16-week balanced HFD (50% of the energy from fat with a proportion of 1/3 each for saturated fat, SFA; monosaturated fat, MUFA; and polyunsaturated fat, PUFA) to a HFD supplemented with SFA (palmitate), HFD + MUFA, and HFD + PUFA groups, HFD + SFA group resulted in different effects on fat oxidation. The obese women fed with the balanced HFD experienced a 6% increase in fat oxidation compared to those consuming the HFD with SFA.[164] The groups with additional either MUFA or PUFA did show greater fat oxidation, not higher than the balanced HFD diet. [164] Others have indicated that supplementing the diet with PUFA (e.g., fish oil) may protect against weight gain and adiposity in obese individuals when compared to SFA (e.g., palmitate).[165–167] Supplementing the diet with high-MUFA fat components (e.g., oleic acid) had also been shown to improve central adiposity and body weight over a period of 4 to 12 weeks.[168–170] After 9-weeks of breakfast supplementation with 25 ml of extra-virgin olive oil (high in

MUFA) in obese women, Candido et al., found a significant decrease in body weight and total fat mass compared to the control group (25 ml of soybean oil, rich in linoleic acid a PUFA).[169] Others,[170] have found high-oleic acid (high MUFA) from peanuts to be inversely correlated with body fat mass ($r = - 0.264$, $p = 0.042$). The results of PUFA and MUFA consumption suggest that fat quality, over quantity, may be an important determinant of the body weight gain and adiposity outcomes in HFD-induced obesity and comorbidities.

Consequences of the exposure of a harmful HFD go beyond the increased body weight and adiposity. An excess of dietary fat intake has been associated with increased body weight and adipose tissue content, as well as metabolic abnormalities (e.g., insulin resistance) in various tissues.[171,172] In rodent models, prolonged exposure to a HFD has been shown to suppress the activity of the beta subunit of ATP synthase in the muscle mitochondria[173] and reduced lipolysis.[174] HFD-induced accumulation of the lipids in the cytosol of non-adipose tissue cells (e.g., myocyte), or lipotoxicity, lowers the oxidative capacity of mitochondria and enhances the accumulation of lipids (e.g., ceramides),[175] which ultimately leads to impairments in the insulin sensitivity within the myocytes.[176] In *in-vitro* studies with C2C12 myotubes (often use cell lines to test different responses in the skeletal muscle cells), PUFA at nutritional doses is protective from lipotoxicity rather than inducer compared to SFA.[177–180]

As summarized in a meta-analysis, the dietary composition is a determinant of diet-induced obesity, low-fat diets are a better dietary intervention than low-carbohydrate diets to induce weight and fat loss.[181] Overall, the literature supports that dietary

composition is one of the significant non-modifiable drivers of obesity in addition to the dietary caloric intake. Besides being frequently described as an energy-balance disorder, dietary fat appears to be a crucial macronutrient in the onset of obesity and metabolic abnormalities (e.g., lipotoxicity). It may trigger multiple metabolic mechanisms involved in the development and maintenance of obesity. Further, the fat types will condition the consequences on the energy metabolism in different tissues. SFAs will have a greater capacity to develop lipotoxicity and, therefore, more hurtful than PUFA in the skeletal muscle.

Environmental Factors

Gut Microbiota

The gut is a complex and dynamic environment colonized by a microbial community, known as gut microbiota. The gut microbial community is defined as the 10^{13} microbes dominated by anaerobic bacteria and contains ~1000 species with an estimated 100 times more genes than the human genome.[58] This niche of microorganisms has been described as a forgotten organ, with metabolic, immunological, and protective functions.[182,183] These functions at local and systemic levels include the maintenance of gut permeability, synthesis of vitamins, nutrient metabolism, regulation of energy storage, and modulation of the immune system.[58] The composition of the gut microbiota varies across individuals and can be unique enough that is serves as a sort of fingerprint.[184] Nonetheless, metagenomic studies which also study the functional capacity of the gut microbes have shown that most individuals share about 40% of their microbes.[15] This supports the idea of a core microbiome.

Bacteria dominate the gut microbiota and primarily come from the phyla *Bacteroidetes, Firmicutes, Actinobacteria,* and *Proteobacteria.* Still, viruses and *Achaea* are present too.[185] Different methods of classifying microbes have been proposed in an effort to define a healthy gut microbiota. Most generally, greater diversity in microbial species is thought to be as a marker of healthy gut microbiota.[186,187] Enterotype (a cluster of bacteria dominated by one genus) classification proposed by Arumugam et al.,[188] classifies human gut microbiota into three groups: *Bacteroides* (enterotype I), *Prevotella* (enterotype II), or *Ruminococcus* (enterotype III). Another classification method proposed is the *Bacteroidetes* to *Firmicutes* ratio, with a lower ratio being associated with obesity.[65] Both methods have received scrutiny in recent years. Enterotype classification is currently under debate due to gut microbiota intra-individuality and composition fluctuations,[189,190] while the *Bacteroidetes/Firmicutes* ratio is questioned due to the lack of replicability across studies.[191,192]

There are some species of microbes that have been found in high abundance in healthy individuals compared to unhealthy individuals.[193,194] A high abundance of Gram-negative, butyrate producers like *Roseburia intestinales, Eubacterium halii,* and *Faecalibacterium prausnitzii*, and Gram-negative, propionate and acetate producers like *Akkermansia muciniphila, Bifidobacterium spp,* and *Bacteroides spp* have been associated with health.[195] *Eubacteriaceae, Prevotella, Bacteroides,* and *Ruminococcus,* in high abundance, have also been suggested as markers of a healthy gut microbiota. [196]

Overall, an increase in bacteria that produce short-chain fatty acids (SFCAs; butyrate, propionate, and acetate) is considered a sign of a healthy gut microbiota

composition. SCFAs elicit endogenous signals for important regulatory roles (e.g., stimulation of anorexigenic hormones such as GYY) including the promotion of intestinal cell differentiation and intestinal barrier function while also serving as an energy source for colonocytes.[197–199] In addition, the obese gut microbiota has also been characterized by a reduced relative abundance of the commensal butyrate-producing bacteria gram-negative bacteria.[200] Oral butyrate administration in HFD-induced obese mice has been shown to stimulate lipolysis and increase ß-oxidation of fatty acids, thereby supporting the anti-obesogenic effects SCFAs.[201–205] In a recent review,[206] provides supporting role of the dietary and bacteria-derived SCFAs as positive signaling molecules as anti-obesity effects, particularly butyrate, at local by binding G-coupled-protein receptors causing secretion of anorexic hormones (e.g., peptide YY). At a systemic level, epigenetically regulate expression of relevant adipokines in obesity such as leptin. Adding to the existing literature, the increased presence of fecal SCFAs concentration in obese individuals,[207,208] but a low plasma SFCA levels are characteristic of obese individuals and might be of interest to calculate the ratio of SCFA fecal and plasma as gut microbiota healthy marker in obese individuals.

In the last decade, the gut microbiota has been highlighted as a determining factor in the development of obesity and obesity-related disorders with an overall reduction in microbial diversity when compared to lean individuals.[64,186,187,209,210] The gut microbiota was first linked to obesity by Bäckhed and colleagues; they found that germ-free (GF) mice colonized with gut microbiota from chow-fed mice experienced an 57% increase in total fat mass despite a significant reduction in their food intake.[210] This change in fat

mass occurred in the absence of significant changes in biomarkers of adipogenesis or lipogenesis. Later, another study supported this data by showing an increased capacity of gut microbiota in obese individuals to harvest more energy from food.[209]

Gut microbial dysbiosis is defined as a decrease in community diversity and an imbalance between pathogenic and beneficial microbes.[211] The state of gut dysbiosis has been highlighted as a key factor in the development and severity of metabolic diseases such as obesity.[65] The shift towards a harmful microbiota is influenced by a combination of different factors including diet, drugs, the health of the intestinal mucosa, interactions with the immune system, and lack of exercise. Despite these known links, the question remains as to whether gut microbial dysbiosis is a cause or a consequence of obesity. Further, the mechanisms regulating microbiota quality and diversity are multifactorial with behavioral factors such as poor diet or lack of exercise dominating over genetics in the shaping of gut microbiota structure and function.[212]

Gut Microbiota, Diet, and Obesity

Diet is a major contributor to the maintenance of the gut microbiota.[213–215] Short-term variation in the quantity and quality of food intake causes acute modifications to the gut microbiota.[214] Conversely, long-term variation in dietary intake and quality may cause significant modifications in gut microbial composition, with either positive or deleterious health consequences.[216]

Macronutrients

Proteins

High-protein diets are widely used in weight management treatments among obese and overweight individuals.[217] However, the impact of these diets on gut microbiota in obesity remain understudied. Kiilerich et al.,[218] found in C57BL/6J female mice that a high-fat high-protein diet (44.9% of the energy was from protein) exerted a protective effect against increased body weight beyond that of a high-fat, high-sucrose diet-fed (46.8% of the energy from fat) animals. Gut microbial composition analysis in this study showed that each diet had a different effect on the gut microbial community by two clearly defined community bacterial structures. *Lactobacillus* genus was more common in the high-fat, high-sugar diet vs. high-fat, high-protein diet, suggesting that protein had a positive impact on gut microbial composition by increasing a health-related genus. Another study[219] that compared C57BL/6J mice fed different proportions of protein (20%, 30%, or 40% of the energy from a whey-protein supplement) while on an HFD (45% of the energy from fat) for 21-weeks found that any increase in the protein significantly increased lean mass and *Lactobacillacae* in line with the Kiilerich study. Increasing protein content to account for 20-30% of the dietary energy also increased the composition in *Bifiodobacteriaceae* compared to HFD alone. As briefly summarized by Lopez-Legarrea et al., there are a few studies on the impact of high-protein diets on gut microbiota, besides the use of high-protein diets as anti-obesogenic treatment elsewhere.[220] The rest of the literature focuses on protein supplementation in healthy or athletic populations and its impact on gut microbiota composition. Moreno-Perez et al.,[221]

found that endurance athletes fed a diet supplemented with a blend of beef hydrolysate and whey protein experienced different changes to the gut microbiome when compared to a maltodextrin-supplemented control group. At the genus level a higher proportion of *Bacteroides* genus was found in the protein group compared to the maltodextrin control group. The protein supplemented groups (beef and whey) showed a 3.8-fold decrease in *Bifidobacterium longum* compared to the baseline sample (baseline abundance = 0.057 ± 0.16%; post treatment abundance = 0.015 ± 0.05%; $p = 0.021$). This species has been used as a probiotic for its anti-obesity effects in high-fat diet-induced animal models of obesity.[222,223] In contrast, others[224] have shown that a 3-week supplementation with either casein or soy protein powder in 42 healthy individuals did not impact gut microbial composition, with diet explaining only 3% of gut microbiota community variance. Overall, the impact of protein seems to shift gut microbial composition towards a healthier structure. However, further research is needed to explain the potential detrimental or positive effects of high-protein diets on the gut microbiota. Studies should focus on the type (animal vs. plant-based) and quantity (high vs. low) of protein being delivered. This is especially important to study among obese individuals where dietary changes in protein could aid with treatment via gut microbial metabolism.

Fats

The effects of HFDs on gut microbial composition have been more extensively studied.[70,71,225–229] HFDs exerts profound changes in gut microbiota, a reduction of abundance *Bacteroidetes,* and an increase of certain *Firmicutes* species.[70,71,227–229] Cani et al,[70] found a reduction of *Lactobacillus spp* (*Firmicutes)* whereas others[227–229] found

different *Firmicutes* class (*Erysipelotrichia, Mollicutes*), family (*Lachnospiracea*), and genus (*Mucispirillum)* members. Beyond total fat intake, the type of dietary fat could have a different impact on gut microbiota composition and adiposity.[71,230–233] SFA were found to be more detrimental to the gut microbial community structure, with greater community differences and elevated fat mass compared to chow-fed controls[231] and PUFA-supplemented HFDs.[71] The HFDs supplemented with either olive oil or safflower oil (rich in monounsaturated fat, MUFA), or palm oil (rich in SFA) have distinctive impacts on gut microbiota bacterial diversity. SFA-enriched HFDs have resulted in a reduction in gut microbial alpha diversity, increased the abundance of *Firmicutes*, and reduced the abundance of *Bacteroidetes* compared to PUFA-enriched diet.[232,233] Altogether, HFD-induced bacterial changes are dietary fat type-dependent, being more detrimental towards the bacterial diversity the SFA compared to the PUFA.

HFD (45% of the energy from fat) consumption for four weeks to induce obesity increased Gram-negative bacteria (*Firmicutes, Proteobacteria, and Actinobacteria)* and reduced *Bacteroidetes* in C57BL/6J mice.[208] Similarly, others[70,226,234] have indicated a decrease in *Bacteroidetes* among mice fed a HFD (40-72% fat). Moreover, they found that HFD-induced significant increase of gut permeability,[71,74,225] raising blood concentration of lipopolysaccharides (LPS; endotoxin of the outer membrane of Gram-negative bacteria). LPS induces metabolic endotoxemia, which ultimately is associated with an increased adipose tissue dysfunction and low-grade inflammatory state characteristics of obese individuals.[235,236] Interestingly, research has shown that following a short or long-term HFD, but not obesity itself, may be the driver of gut microbial shifts

in obesity-prone mouse models.[228,237,238] The review by Murphy et al.,[238] conclude that HFD-induced gut microbial changes are linear dose response to dietary changes and not obesity or genetics. In obese and lean rodents strains fed with a HFD Carmody[234] and Hildebrandt[228] showed that HFD-induced similar changes in both lean and obese rodents. Further, studies in monozygotic twins had shown that with identical energy intakes of SFA changed their gut microbiota by modulation of the *Bacteroidetes spp.*[239] Hence, HFD contributes to obese phenotype by altering gut microbial composition in a larger extend that the obese phenotype to the gut microbiota.

Overall, consumption of an HFD, in particular those with a higher composition in SFA, contributes to changes in the gut microbiota composition (increased *Firmicutes* and reduced *Bacteroidetes*). The literature showed that the microbial shift after an HFD disturbs the gut barrier by increasing its permeability. The increased permeability leads to a leak of LPS from the gut lumen into the bloodstream, thereby contributing to the low-grade inflammatory state and increased adipose tissue dysfunction in obese individuals. Therefore, HFD promotes adiposity and metabolic abnormalities which may be mediated by the gut microbiota.

Carbohydrates

The preferred nutrients for gut microbes are carbohydrates, and their main fermentation products are short-chain fatty acids (SCFAs), butyrate, acetate, and propionate, which exert metabolic signals in the host that are often associated with health.[240] Available literature indicates that an increased intake of complex carbohydrates and fiber had opposing effects on the gut microbiota when compared to HFD. These

changes included the amplifications of SCFA-producing bacteria in obese individuals.[241–245]

The impacts of carbohydrates on the gut microbiota for health are complex and depend on the type, amount, and frequency of intake. The Western diet,[246] is characterized by an increased intake of simple carbohydrates (e.g., sugar), which has been associated with an increased risk for metabolic diseases, obesity, and type 2 diabetes. Whereas an increase in complex carbohydrates (e.g., fibers), more abundant in plant-based diets,[247] has been associated with a reduced risk of developing metabolic diseases which are thought to be mediated by modification of gut microbiota composition.[248,249] Sugar consumption has been shown to inhibit the colonization of commensal *Bacteroides thetaiotaomicron*, an anti-obesogenic species,[250] increased abundance of *Proteobacteria*,[251,252] reduce alpha and beta diversity,[251,253] and depletion of SCFAs producing bacteria (e.g., *Lachnospiraceae* family).[253] The high-sugar-induced gut microbiota distribution favors a gut and systemic pro-inflammatory state by alteration of gut epithelial integrity and mucosa immunity.[254] In contrast, the long-term intake of complex carbohydrate intake and rich dietary fiber diets has been associated with a higher abundance of *Prevotella spp. and Bifidobacterium spp.,*[242–244,255] *Bacteroides spp.*[256] bacteria associated with improvements in metabolic abnormalities (e.g., glucose homeostasis), obesity, and comorbidities.

High-fiber and fiber supplemented diets have been shown to protect obese rodents against the negative impacts of a HFD through gut microbial modification, enhanced gut barrier function, and SCFAs production. Fiber as described by the CODEX Alimentarius includes any carbohydrate polymers derived from or contained edible plants which are

neither absorbed within the small intestine, nor hydrolysable by mammalian digestive enzymes in the small intestine,[257] can be classified by different factors, and the widely used one is their solubility.[258] Recently has been suggested that in disease the impact of soluble vs insoluble fibers.[259] Soluble fiber, have the capacity to dissolved in water and formation of a gel-type, typically includes pectins, gums, ß-glucans, and hemicellulose. Zhai et al.,[260] showed a dramatic increase in *Bacteroidetes* (from 7.83 to 25%) and *Akkermansia* (from 0.69 to 2.80%), and significant ($p<0.05$) decreases in weight, body fat, and liver adipose tissue lost after a 16-week HFD supplemented with soluble fiber. Similarly, others found the same bacterial species to be increased after a HFD supplemented with soluble fiber and consequent obesity-associated physiologic alterations (e.g., increased fat mass) improved.[255,261–263] Insoluble fiber, does not dissolve in water, includes cellulose, lignin and resistance starches. *Akkermansia spp*., *Oscillibacter spp., Bifidobacterium spp,, Bacteroidetes spp.,* and other SFCAs strain producers were significantly more abundant in the high-insoluble fiber diet than low- insoluble fiber diet.[264–266] As found by soluble fiber, insoluble fiber-induced changes in gut microbiota are associated with reduced adiposity and other cardiometabolic markers in diet-induced obese mice models.

Another carbohydrate-derived ingredient that has been shown to improve obesity outcomes are prebiotics. The 6th Meeting of the International Scientific Association of Probiotics and Prebiotics (ISAPP) defines dietary prebiotics as a selectively fermented ingredients that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health.[267] Prebiotics can

be found in the diet in fruits, vegetables, and grains. Currently, inulin, fructooligosaccharides (FOS), and galactooligosaccharides (GOS) are the most well-studied prebiotics in relation to obesity and cardiometabolic health outcomes.[255,268–270] Aligier et al.,[271] found that 4-weeks of HFD supplemented with inulin-type fructans protected male CB57L/J6 mice from increased adipose tissue (1.2g vs. 2.5g, $p<0.05$) and total body weight gain (4.8g vs. 5.8g, $p<0.05$) when compared to HFD without supplementation. Others found that the anti-obesity effect of inulin was driven by gut microbial composition changes, increased abundance of *Bifidobacterium spp.*[272] *Lactobacillus spp.*[272] and consequent improvements in adiposity in HFD-fed mice.[273,274] In human trials, the same protective effects have been shown in obese individuals.[275,276] Supplementing with prebiotic inulin increased the abundance of *Bifidobacterium* spp. and other SCFAs producers, improving gut permeability, inflammation, and other cardiometabolic parameters (e.g., c-reactive protein) affected in obesity.[277–283] It is not that clear the effects of prebiotic fiber reducing the adiposity by modulating the gut microbiota.[279] Largely, animal and human trials suggest potential promising use of prebiotic and dietary fiber types as nutritional treatments to improve adiposity and cardiometabolic alterations in obesity mediated by the modification of gut microbiota composition.

Probiotics

Another dietary intervention used to shift gut microbial composition for obesity management has been probiotics. According to the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), probiotics are live

microorganisms which when administered in adequate amounts confer a health benefit on the host.[284] In a systematic review of animal and human experimental studies, Ejtahed et al.,[285] concluded that *Lactobacillus spp.* and *Bifidobacterium spp.* are useful for weight management and reducing of fat mass. However, there is literature available that shows an increased in body weight (21.5±1.1 g vs. 17.1±1.1 g, $P<0.01$) and body fat ($p>0.05$) in female C57BL/6J mice after 20 weeks of HFD-induced obesity and supplementation with *Lactobacillus plantarum DSM 15313* compared to the control group fed with an HFD.[286] In line with these results, Yin et al.,[287] found that different *Bifidobacterium* strains have different impacts on weight management in HFD-induced obese Sprague-Dawley rats. They showed that those rats supplemented with *Bifidobacterium M12-4* experienced significant ($p<0.05$) increases in body weight compared to control or *Bifidobacterium L66-5* supplemented groups. As emphasized by Ejtahed et al.,[285] the diversity in the animal species (C57BL/6J mice vs. Sprague-Dawley rats), sex (female vs. male), sample size, and the duration of probiotic treatment (20 weeks vs. 6 weeks) in the literature makes the effectiveness of probiotics as anti-obesity treatment unclear. Further research is needed with homogenous methodology and replication of results to establish which probiotics could be used as a therapy to manage obesity.

Functional Food Ingredients

The biological effects of dietary bioactive ingredients have been investigated as treatment for improving of health.[288] Polyphenols are plant components with a variety of structural forms that provide polyphenols with different functional capacities. Polyphenols have exhibited anti-inflammatory, anti-estrogenic, cardioprotective,

chemoprotective, and neuroprotective health benefits across in-vitro, animal, and human studies.[49,288] The gut microbiota is often required to breakdown polyphenols into secondary bioactive metabolites before intestinal absorption and release into the bloodstream. [53] Among one of the most prominent families of the polyphenols is isoflavones found in soy, and to a lesser extent in lentils, beans, and chickpeas. These polyphenols have been highlighted for their anti-androgenic and hypolipidemic effects.[53] Genistein and daidzein, the main soy-derived polyphenols, exist primarily as polyphenol glucosides that require hydrolyzation to their secondary bioactive polyphenols in the gastrointestinal tract by ß-glucosidase enzymes expressed by gut microbes.[53,288] The bacterial species responsible for the biotransformation of soy isoflavones have not been fully identified, but *Slackia isoflavoniconvertens* is one of these microbes with ß-glucosidase activity.[54] The isoflavones and derived metabolites have been well studied for their resemblance of 17 ß-estradiol hormones and consequent estrogenic activity as anti-tumorigenic[289] and bone turnover-enhancing.[290]

Recently, anti-obesogenic effects have been associated with genistein.[48,80,291,292] Gen is an agonist of the ER alpha (ERα) and ER beta (ERß), which are cell-specific, meaning that the activation of the ERα/ß triggers different genomic and no genomic metabolic pathways in different tissues.[293,294] In the adipocyte isoflavones exhibits a inhibitory capacity of lipid accumulation by regulating the expression of lipogenic genes, signaling multiple pathways enhancing lipolytic enzymes, inhibition of adipogenesis, and downregulation of PPAR gamma.[48] Whereas understanding of the underlying causes and

mechanisms triggered by the stimulation of the ERα/ß in the skeletal muscle remain unclear.

Genistein supplementation has exhibited a reduction in body weight and energy intake in female C57BL/6J mice after 8 weeks on an HFD compared to either HFD alone or chow-fed animals.[50] Results were in line with ovariectomized female rats,[295,296] male rats,[51] and mice[297] with soy improving the bioavailability of isoflavones, and reduced adiposity. Some of the literature in this area has associated the decrease in body fat content with shifts in the gut microbial community.[296] Lu et al.,[52] found a reduction in body weight and adipocyte size in C57BL/6J male mice, alongside dramatic modifications of the gut microbiota including a decrease in *Firmicutes* and increase in *Bacteroidetes* after 12-week of HFD supplemented with genistein. Others,[296] found that 27 weeks of soy isoflavones supplementation (daidzein and genistein) reduced the *Firmicutes/Bacteroidetes* ratio and increased the relative abundance of healthy microbes such as *Prevotella* at the genus level, and *Ruminococcacea* at the family level in ovariectomized rats. In contrast, Lopez et al.,[81] found that C57BL/6J male mice fed a genistein-supplemented HFD for 6 months experienced a reduction in intestinal *Bacteroidetes* and increase in *Firmicutes* relative to an HFD-alone group. Besides the different microbial shifts observed among these studies, all the experiments results in an amelioration of adiposity while minimizing weight gain body.[80,81,296] A further and more in-depth analysis of the gut microbiota targeting lower taxonomic ranks (e.g. genera or species) could be of interest for new research to address the discrepancies among the literature on the impact of genistein on gut microbiota and associated obesity outcomes.

Gut microbiota, Exercise, and Obesity

Exe has been identified in the last decade as a new factor involved in the changes in the gut microbial community, and therefore function.[67,298–302] Lamoureux et al.,[303] found gut microbial community structure shifts in 30 taxa (e.g., *Bacteroides*, S24-7, and *Lactobacillus*) following 8-week three different exercise protocols (forced and voluntary exercise), but not overall diversity differences when compared to sedentary controls adult C57BL/6J mice (n=42; 11 male and 31 female). A 6-week exercise intervention, among adult rats, increased gut microbial richness which remained stable up to 25 days after the Exe intervention was terminated.[304] These results suggest that exercise may be an important treatment for acute modification of gut microbiota composition and structure. Further longitudinal research is necessary to confirm Mika et al.,[304] exercise-induced gut microbial changes long-term stability after an exercise intervention. The results by Lambert et al.,[305] found that 6 weeks of low-intensity exercise studying the impact of exercise in diabetic and non-diabetic mice increased the relative abundance of *Bifidobacterium spp., Lactobacillus spp.*[306] and *Clostridium cluster I* in diabetic mice independently from exercise. But exercise decreased relative abundance of *Bacteroides/Prevotella spp.* and *Metahnobrevibacter spp.* in healthy mice and diabetic mice. Overall, the animal literature on the impact of exercise on gut microbiota reports an increased in *Firmicutes*[305,307] phylum.

Human trials studying the impacts of exercise on gut microbial composition have predominantly described the effects of exercise in professional athletes.[96,308–311] Prolonged ultra-endurance exercise for 33 days enhanced alpha diversity and relative

abundance of butyrate-producing microbial species [311] compared to baseline gut microbiota characteristics. In line with these results, Clark et al.,[308] found that extreme exercise and diets, common among athletes, were correlated with a higher gut microbial diversity, which is considered a marker of health.[186] Interestingly, the taxa that increase after exercise treatment have not been consistent across all studies. Petersen et al.,[309] found *Prevotella* to increase among competitive cyclists, whereas, Grosicki et al.,[96] and Scheiman et al.,[94] found significantly great abundance of *Veillonella* among endurance runners. These differences suggest that the type of exercise may be important for shaping the gut microbiota.

In trials with obese humans, exercise has had a protective effect against obesity with the gut microbiota differing in relevant bacterial species related to health. These microbial changes have included increases in *Akkermansia muciniphila, Roseburia hominis,* and *Faecalibacterium prausnitzzi* among active women.[312] Allen et al.,[69] revealed that baseline differences in the gut microbiota of obese male and female individuals (n=14) disappeared after following a 6-week moderate-to-vigorous (60-75% of HR reserve) exercise protocol when compared with lean individuals (n=18). In a 3-week HIIT protocol in lean and overweight male adults did not find any significant differences in gut microbial diversity.[313] The scarce literature on exercise as a potential treatment to improve obese individuals' gut microbiota is unclear and requires further investigation with different exercise modalities and protocol lengths.

The mechanism behind the positive impact of exercise on gut microbiota remains unknown, specifically with regard to which intensity, mode, and duration of exercise is

the most effective at improving obesity-related outcomes and associated gut microbiota changes. There are variety of bacterial species that have been observed to increase after exercise in the different trials: animals (e.g., *Lactobacillus spp.*), athletes (e.g., *Veillonella spp*.), and obese (e.g., *Akkermansia mucinihila*) humans. Potential interaction with other factors may be of interest to study to analyze the possible synergetic effects of exercise in combination with other successful treatments that shift gut microbial composition.

Diet and Exercise Interaction and Gut Microbiota in Obesity

The combined effect of the two main factors involved in shaping the gut microbiota could elucidate new avenues for the development of treatments to improve obesity and associated metabolic abnormalities. In an attempt to use exercise as a treatment to increase the gut microbial diversity and shift it towards a more healthy microbial structure in high-fat diet-induced obesity, various rodent[314–316] studies have been published. Ribeiro et al., indicated that moderate-intensity exercise training was not enough to recuperate the gut microbial diversity lost during 8 weeks of HFD feeding in C57BL/6 male mice.[314] Whereas Denou et al.,[315] found that adult male C57BL/6 mice fed HFD for 6 weeks alongside high-intensity interval training (HIIT) experienced an increase in alpha diversity, *Bacteroidetes* phylum abundance, and *Bacteroidetes/Firmicutes* ratio compared to HFD non-exercise treatment group. Similar effects were found where exercise prevented HFD-induced weight gain by modification of gut microbiota.[316,317] On a 12-week HFD (40% of the energy from fat) + exercise or HFD alone intervention, exercise group prevented weight gain and increased the

abundance of *Faecalibacterium prausnitzi (Firmicutes* phylum)*, Clostridium spp.,* and *Allobaculum spp*.[265] Evans et al.,[268] found a body weight protective effect of exercise on HFD-fed C57BL/6J mice, as well as a reduction in *Firmicutes* abundance and increased in *Bacteroidetes*.

A few studies have investigated the potential synergetic effect of exercise and a healthy diet or functional dietary ingredients to revert HFD-induced gut microbial modifications and metabolic abnormalities. Rodent studies[79,318] have shown that the combination of functional ingredients and exercise can improve metabolic abnormalities, prevent weight and fat mass gain, and shift gut microbiota composition after a 4 to 7-week HFD (60% of the energy from fat). Nagano et al.,[318] studied the impact of voluntary wheel exercise, soluble fiber supplementation, and the synergic effect of exercise and fiber on HFD-fed C57BL/6N mice and found the synergic impact of exercise and fiber to be beneficial for increasing *Eubacteriaceae*. However, only the exercise group without the soluble fiber supplementation was able to reduce the abundance of *Erysipelotrichacea* and *Rikenellaceae*, two bacteria that have been associated with obesity.[226,319,320] So far, in the only human study, whey protein supplementation with combined aerobic and resistance exercise training had a significant impact on the gut virome (viruses) composition. The combination of whey supplementation and exercise training showed a trend towards increasing bacterial diversity.[321] Thirty-two male Sprague-Dawley rats were randomized to sedentary-soy, sedentary-casein, exercise-casein, or exercise-soy intervention groups.[322] Soy protein + exercise was found, compared to casein protein + exercise, in combination to exert a significant increase in ß-had activity among male

rats.[322] The sedentary-soy group showed higher ß-HAD activity compared to sedentary-casein and exercise-casein groups, without reaching statistical significance. Therefore, we could speculate that the additive effect of exercise and soy may be driven by the gut microbiota capacity to activate bioactive components of soy. Further research is needed to test previously shown positive dietary strategies and functional ingredients to prevent HFD-induced gut microbial dysbiosis in combination with exercise.[323] More studies including both the independent and the synergic effect of diet and exercise in shaping the gut microbiota are required to identify interventions with better treatment efficacy for obesity via the modification of gut microbiota.

Diet/Exercise – Gut Microbiota – Muscle

An emerging field of study aims to connect the gut microbiome and skeletal muscle mitochondria. [324–326] There is some literature available which has theorized about the gut-muscle axis and its importance for the host energy metabolism.[94,327,328] Bäckhend et al.,[329] were the first to suggest a gut microbiota-muscle axis based on correlations found between the gut microbiota composition and muscle metabolic pathways. In their animal study, germ-free (GF) wild-type C57BL/6J male mice (n=26) were fed a low-fat chow diet. After 6-10 weeks, half of the GF mice (n=13) were colonized with a conventional adult mouse gut microbiota, and either continued on their low-fat chow diet (GM + LFD; n=5) or switched to an HFD (GM + HFD; n=8 41% of energy from fat) for 2-3 weeks. The remaining thirteen GF mice were kept under GF conditions and feed with HFD (GF + HFD; n=8) or low-fat chow (GF + LFD; n=5). GF + -fed mice were significantly protected from HFD-induced obesity and adiposity (2.1± 0.5 g body weight)

compared to the GM + HFD mice (5.3±0.8 g body weight). When markers of muscle metabolism were evaluated, GF + HFD mice showed a 40% higher phosphorylation of AMPK, a 17% increase in carnitine palmitoyltransferase I (Cpt1, enzyme that catalyzes the entry of long-chain fatty acids to the mitochondria) activity, and a 4.5% increase in medium-chain acyl CoA dehydrogenase (Mcad, enzyme catalyzing the introduction of long-chain C8-C12 fatty acids into the mitochondria) in gastrocnemius muscle and liver mitochondria compared to the GM + HFD mice group. These results suggest that the presence of gut microbiota negatively impacts fatty acid oxidation in muscle and liver mitochondria when animals are fed an HFD. Other evidence for a gut microbiota-mitochondria axis comes from a study where rats were fed with human milk, cow milk, donkey milk, or no-milk for 4-weeks.[330] The liver mitochondria CS activity (umol/min/g) was increased in the human-milk and donkey milk groups compared to the control and cow milk groups. The cow milk group experienced a significantly increased expression of mitochondrial biogenesis transcription factors (PGC1-alpha and PGC1-beta) compared to the rest of the groups. Supplementation with milks affected 10 gut bacterial genera; *Streptococcus* and *Lactococcus* increased with donkey milk while *Coprobacillus* and *Parabacteroides* increased with human milk. These changes positively correlated ($r=0.6$, $p<0.05$) with mitochondrial oxidative capacity. *Blautia* abundance was enhanced by all milks and positively correlated ($r=0.6$, $p<0.05$) with fatty acid oxidation rate measured as the presence of palmitoyl-L carnitine (marker of fatty acid ß–oxidation, transported into mitochondria via carnitine palmitoyl transferase II to deliver palmitate for fatty acid oxidation and energy production), and fecal butyrate. Others did not analyze gut

microbiota composition but did indicate a benefit of combining dietary bioactive ingredients and exercise to increase metabolic activity in skeletal muscle as evidence by an increase in CS activity[331] and ß-HAD activity.[322] Together, these data provide evidence for a gut – mitochondria axis, since the modification of healthy gut microbial species (e.g., increased of *Blautia)* positively correlated with improvements in mitochondrial fatty acid oxidative capacity. Others,[328] have shown that metabolites derived from the gut microbiota enhance expression and activity of proteins involved in mitochondrial biogenesis in C57BL/6J mouse models. Gao et al.,[328] found that injection of sodium butyrate, one of the major SCFAs synthesized by the gut microbiota, increased fatty acid oxidation in skeletal muscle and protected from insulin resistance.

There is only one study in humans studying the gut microbiota-skeletal muscle axis. Scheiman et al.,[94] showed recently that a *Veillonella spp.,* obtained from endurance runners' gut microbiota has the capacity to recycle lactate derived from the muscle into propionate. *Veillonella atypica* and the lactate metabolic pathway enzymes were shown to be enriched before and after a marathon race in human athletes. To test *Veillonella spp* capacity to increase muscle performance, the researchers inoculated the *Veillonella atypica* from endurance athletes into mice. Animals were then run for 5 hours until exhaustion, and those treated with *Veillonella atypica* experienced a 13% increase in performance compared to the control group.

The relationship between the gut microbiota composition and the musculoskeletal oxidative capacity (TCA and fatty acid oxidation) is unknown. The recent literature on the gut - muscle axis suggests the axis as a potential new therapeutic target to improve

metabolic health in the obese population. The anti-obesogenic effects of exercise through the modification of gut microbiota remain uncovered, but the few studies available have shown promising results. Dietary treatments have been used elsewhere to revert obesity and metabolic abnormalities. Genistein has been recently shown promising results as an anti-obesogenic effect agent. This may be through the modification of gut microbiota but further work is needed to make causal links. This study aims to use exercise, genistein, or combined treatment to modify the gut microbiota and skeletal muscle oxidative capacity. To my knowledge, there are no studies that have assessed the synergetic role of diet and exercise on altering the gut microbiota and musculoskeletal metabolism in a state of diet-induced obesity.

CHAPTER 3

METHODOLOGY

Research Animals and Study Design

In this randomized controlled trial, one hundred and thirty-two adult male (n=63) and female (n= 69) C57BL/6 mice underwent one of five diet and/or exercise intervention for twelve weeks. All animals were purchased from Charles River (Wilmington, MA) and aged 4-5 weeks. Mice were provided with food and water *ad libitum* and maintained in a room with an alternating 12 h light/dark cycle which was kept at 22°C. All procedures were approved by the Institutional Animal Care and Use Committee at Midwestern University in Glendale, Arizona.

Treatment groups

The mice were randomly assigned into one of the following five groups: control (n= 27), high fat, high sugar diet (HFD; n=26), HFD + exercise (HFD + Exe; n=28), HFD + Genistein (HFD + Gen; n=27), or HFD + Exe + Gen (n=24). Whole *gastrocnemius* muscle were removed and maintained at – 80ºC until use on a sub-set of mice (n=49) with the following intervention distribution: control (n=10), HFD (n=10), HFD + Exe (n=10), HFD + Gen (n=10), and HFD + Exe+ Gen (n=9). The control group was fed with a standard chow diet (58% carbohydrate, 28.5% protein, and 13.5% fat) with untreated water. All the intervention groups were fed an HFD (20% carbohydrate, 20% protein, and 60% fat) purchased from Dyets (Dyets Inc, Bethlehem PA) and with 42 g/L of sugar (55% fructose / 45% sucrose) added to the drinking water. HFD + Gen and HFD + Exe + Gen groups were supplemented with 600 mg of Gen per kg diet. HFD + Exe and HFD +

Exe + Gen groups performed moderate intensity Exe 5 days per week on an electrically driven treadmill (Columbus Instr., Ohio) for a period of twelve weeks. The training regimen for Exe treatments consisted of a 3-week graded increase in Exe duration and intensity as follows: acclimation week 1, 10 min at 10 m/min; week 2, 20 min at 10 m/min; week 3, 30 min at 12 m/min; followed by treatment weeks 4–12, 30 min at 15 m/min.

Sample Collection, Processing and Laboratory Analyses

Collection of biological samples was conducted wearing the applicable personal protective equipment at Midwestern University (Glendale, AZ). The fecal pellets were collected at the time of euthanasia and immediately placed in a cryotube, frozen in liquid nitrogen, and stored at -80°C until processing. Collection of the gastrocnemius muscle samples was performed immediately at euthanasia. The muscle was placed in a tube, and immediately flat-frozen by placing in liquid nitrogen, followed by storage at -80 °C until use.

Gut microbiota

All fecal samples were transferred to Arizona State University facilities for processing and analyses. Frozen feces were thawed on ice before processing. Microbial DNA was extracted and isolated from a single fecal pellet from each mouse using the DNeasy PowerSoil DNA Isolation Kit (12888-100, QIAGEN, Hilden, Germany) according to directions provided by the manufacturer. Gene amplification and sequencing

were performed by the Center for Fundamental and Applied Microbiomics (CFAM) staff using equipment in the Genomics Core Lab at Arizona State University.

Bacterial community analysis was performed via next-generation sequencing on the MiSeq Illumina® platform (San Diego, CA, USA). Amplicon sequencing of the V4 region of the 16S rRNA gene was performed using the barcoded primer set 515f/806r[332] and following the protocol of the Earth Microbiome Project (EMP)[333,334] for library preparation. PCR amplifications for each sample were done in triplicate, and the DNA amplicons were pooled and quantified using Invitrogen Quant-iT™ PicoGreen® dsDNA Assay Kit (ThermoFisher, Waltham, MA, USA). A no template control sample was included during the library preparation as a control for extraneous nucleic acid contamination. A total of 240 ng of DNA per sample were pooled and then cleaned using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). The pooled sample was then quantified using the Illumina library Quantification Kit ABI Prism® (Kapa Biosystems, Wilmington, MA, USA) and diluted to a final concentration of 4 nM. The pooled sample was further denatured and diluted to a final concentration of 4 pM with 15% PhiX. Finally, the DNA library was loaded in the Illumina MiSeq and run using the version 2 module, 2x250 paired-end reads, and following the directions of the manufacturer.

Using the Quantitative Insights into Microbial Ecology 2 (QIIME 2) version 2019.1 the paired-end (forward and reverse files per sample) sequences were de-multiplexed and prepared for further diversity, community structure, and statistical analyses (see appendix E)[335]. First, using the Divisive Amplicon Denoising Algorithm 2

(DADA2)[336] pipeline, the de-multiplexed sequence files were filtered and trimmed by trimming the forward reads at 251 bp and the reverse reads at 0 bp after the visual inspection of the quality plots (quality score > 20-25). Next, forward and reverse reads were merged into one unique sequence file per sample and chimeric sequences and other errors formed during PCR amplification were removed. Lastly, the inferred samples were combined into one unified sequence table. Based on the output from the DADA2 analysis, we chose a rarefaction depth of 15,247 counts per sample we kept all the sequenced samples. Rarefaction provides a statistical adjustment for differences in sequence counts across samples to aid comparisons for alpha diversity. Taxonomy of the DADA2 output sequences were assigned using classifier the Greengenes 13_8 classifier 99% OTUs. Sequences were then aligned with MAFFT[337] and a phylogeny tree generated with FastTree .[338]

Mitochondrial oxidative capacity assays

In order to determine the oxidative capacity of the muscle mitochondria, the activity of two mitochondrial enzymes were assessed, citrate synthase (CS).[339] All muscle samples were transferred from Midwestern University to the Arizona Biomedical Collaborative building for further analyses. Frozen gastrocnemius muscle samples were transferred to a cold room at 4°C to perform the homogenization process and guarantee the integrity of the samples for downstream analyses. The muscle (5-20 mg) samples were homogenized and diluted in an average of 375.71±112 ul of homogenization buffer (see appendix B) at 1:40, for the preservation of the muscle cells, inhibition of protease activity, and prevention of oxidation of the targeted enzymes.

The tricarboxylic acid (TCA) cycle is the metabolic pathway prior to the electron transport chain and primary source of electrons (via the reduced forms of adenine dinucleotide, NADH, and flavin adenine dinucleotide, $FADH_2$), and therefore tightly coupled to the oxidative phosphorylation (OXHOP) capacity of the mitochondria. CS is the enzyme which catalyzes the first reaction of the TCA cycle, the condensation of acetyl-coenzyme A (acetyl-CoA) and oxaloacetate to form citrate. CS is used as a quantitative enzyme marker for TCA cycle function.

The CS activity of each sample was measured independently by adding to the homogenized sample an enzymatic cocktail (5,5'-dithiobis-2-nitrobenzoate (DNTB), acetyl CoA, oxaloacetate, Tris buffer, and homogenizing buffer; see appendix C) and measuring the absorbance in a spectrophotometer (Beckman Coulter DU 7830, Ca, USA) at 412 nm and at a stable room temperature (23-25°C). We calculated the average change in absorbance per minute for four minutes (Equation 1). The calculations were made based on the molar extinction coefficient for DTNB at 412 nm and the dilutions of the tissue, first in the homogenate and then in the assay volume. CS contributes 2 carbons to the 4 carbons of oxaloacetate resulting in citrate with 6 carbons. The hydrolysis of the thioester of acetyl CoA results in formation of CoA with a thiol group (CoA-SH). The thiol reacts with DNTB or also known as Ellman's reagent^{340} in the reaction mixture to form 2-nitro-5-thiobenzoate (TNB), which exhibits maximum absorbance 412 mm (Equation 2).[341]

Equation 1: ΔAbs/min ÷ 13.6 x Muscle dilution factor = CS μmol/min/ g of wet tissue

Equation 2: Oxaloacetate + acetyl-CoA $\xrightarrow{\textit{Citrate Synthase}}$ Citrate + H_2O

DTNB → TNB – CoA + NTB^- (λ_{max} = 412 mm)

Statistical Analyses

Sample size was determined from earlier projects on exercise and diet, with a minimum sample size of 7 mice per group to achieve level of significance ($p<0.05$). This sample size also considers the slight variability in the response of mice to the stimulus of exercise, since some animals respond better than others to exercise intervention. All variables were checked regarding their normality distribution using the Shapiro-Wilk test and logarithmically transformed if appropriate for model assumptions. For all alpha and beta diversity analyses, the Benjamini-Hochberg correction (adjusted *p*-values) was used to adjust for multiple comparisons. Gut microbiota analyses were assessed by QIIME2 and remaining analyses were performed in the R statistical software package, version 3.6.1. The relationships between mitochondrial enzymes, intervention groups were assessed by multiple regression analysis meeting assumptions. The mediation analysis was asses with Baron and Kenny[342] with four step various regression analyses and significance of the coefficients is examined at each step

Step 1: We conducted a multiple regression analysis adjusting for sex to assess the relationship between the treatment group (*X*) and each of the mitochondrial enzymes (*Y*). If proven the relationship significant we continued to step 2**. Step 2:** We conducted a multiple regression analysis with treatment group association with gut microbiota alpha diversity (*M*) with sex as a covariate. After proven the relationship significant, we continued to step 3. **Step 3:** We run a multiple regression analysis with gut microbiota alpha diversity predicting mitochondrial oxidative capacity (CS activity and ß-HAD). After proven the relationship significant, we continued to step 4. **Step 4:** We conducted a multiple regression analysis with group treatments and alpha diversity predicting mitochondrial enzymatic activity. After proven significant all the four steps, we assessed the gut microbiota mediator effect on mitochondrial enzymatic activity by assessing the indirect effect; this means that we calculated how much of change of every unit of the mitochondrial enzymatic changed by the treatment is mediated by the gut microbiota alpha diversity and full mediation was supported. If the fourth step was not proven significant, the results will support partial mediation.

Aim 1 statistical analyses

Hypothesis 1: Analysis of α-diversity (within-samples) was assessed using Observed OTUs, Faith's PD, Pielou's evenness Index, Chao1 Index, and Shannon Index metrics. Group differences in alpha diversity metrics between the treatment groups were performed using Kruskal-Wallis[343] non-parametric tests and median alpha-diversity values were visualized using distribution comparison plots (box-plots).

Hypothesis 2: To determine β-diversity (between-samples) Jaccard, Bray-Curtis, and weighted and unweighted UniFrac distance metrics were generated from QIIME2 and visualized using the ggplot package in R statistical software. Group differences in β-diversity metrics were assessed by permutational multivariate analysis of variance (PERMANOVA).[344]

Hypothesis 3: Taxonomy tables created with QIIME2 were uploaded into the Linear discriminate analysis of effect size (LEfSe)[345] analysis tool (Galaxy version 1.0).[346] The differential abundances of microbial species were compared across all intervention groups, and after removing the control group to determine differences in abundance between the HFD treatments. LEfSe is a validated algorithm to evaluate the differential abundance of microbial taxa most likely to explain the differences among study groups. LEfSe utilizes non-parametric Kruskal-Wallis rank-sum tests and consequent pairwise Wilcoxon rank-sum testing to detect which taxa are differentially abundant. Then, linear discriminate analysis is performed to calculate the effect size of identified taxa with an alpha value of 0.05 (adjusted for multiple comparisons) and LDA score > 2. We choose for the pairwise Wilcoxon test the stricter option, all-against-all, to test all the comparison possible between groups of the identified taxa. Visual outputs of LDA scores as bar plots and cladograms were provided by LEfSe to visualize group differences.

Aim 2 statistical analyses

Hypothesis 1: Intervention effects on CS kinetic activity were assessed by a multiple regression analysis adjusting for sex as a covariate (equation 4). We hypothesize

there is a significant association between the intervention group and CS activity after adjusting for sex.

Equation 4: $y = ß_0 + ß_1X_1 + ß_2X_2 + e$

(y = CS activity, $ß_0$ = intercept, X_1=group, X_2 = sex, e = error)

Aim 3 statistical analyses

Hypothesis 1: We assessed the association between CS activity and α-diversity using a multiple regression analysis adjusting for sex and treatment group (equation 6). We hypothesized that gut microbiota alpha diversity will significantly associate to CS activity after adjusting for sex and intervention group.

Equation 5: $y = ß_0 + ß_1M + ß_2X_1 + ß_3X_2 + e$

(y= CS activity, $ß_0$ = intercept, m=alpha-diversity index, x_1=group, x_2 = sex, e = error)

Hypothesis 3: We hypothesize that the gut microbiota mediates the relationship between diet and exercise and the mitochondrial oxidative capacity adjusting for sex. We assessed with a multiple regression analysis if gut microbiota alpha diversity was correlated with mitochondrial oxidative capacity (CS activity) (Equation 6). Furthermore, we assessed using a multiple regression analysis if gut microbiota alpha diversity predicts the mitochondrial oxidative capacity (CS activity) after adjusting for sex and intervention group (Equation 7). We calculated the indirect effect of gut microbiota as a mediator by subtracting the coefficients of the impact of intervention group on mitochondrial oxidative capacity to the coefficient of the gut microbiota alpha-diversity effect on the mitochondrial oxidative capacity.

Equation 6: $Y = ß_0 + ß_1M_1 + ß_2X_2 + e$

(Y= CS activity, $ß_0$ = intercept, M_1= alpha-diversity index, X_2= sex, e = error)

Equation 7: $Y = ß_0 + ß_1X_1 + ß_2M + ß_3X_3 + e$

(Y=CS activity, $ß_0$ = intercept, X_1= group, M= alpha-diversity, X_3= sex, e = error)

CHAPTER 4

RESULTS

A total of 143 C57BL/6 mice were used in the study (Figure 3). Eighty-three mice fecal pellets were available: control (n=17), HFD (n=16), HFD + Exe (n=18), HFD + Gen (n=17), and HFD + Exe + Gen (n=15) groups. Phenotypical characteristics of the mice are outlined in Table 1. We performed a Kruskal-Wallis test to evaluate the differences of adipose tissue and liver weight among the intervention groups, and we used pairwise-Wilcoxon tests for post-hoc analyses. We found that control and HFD + Exe + Gen groups had significantly ($p<0.05$) lower adipose tissue and liver weights than HFD, HFD + Exe, and HFD + Gen groups. Muscle biopsies were performed on a sub-set of mice (n=49) with the following intervention distribution: control (n=10), HFD (n=10), HFD + Exe (n=10), HFD + Gen (n=10), and HFD + Exe+ Gen (n=9).

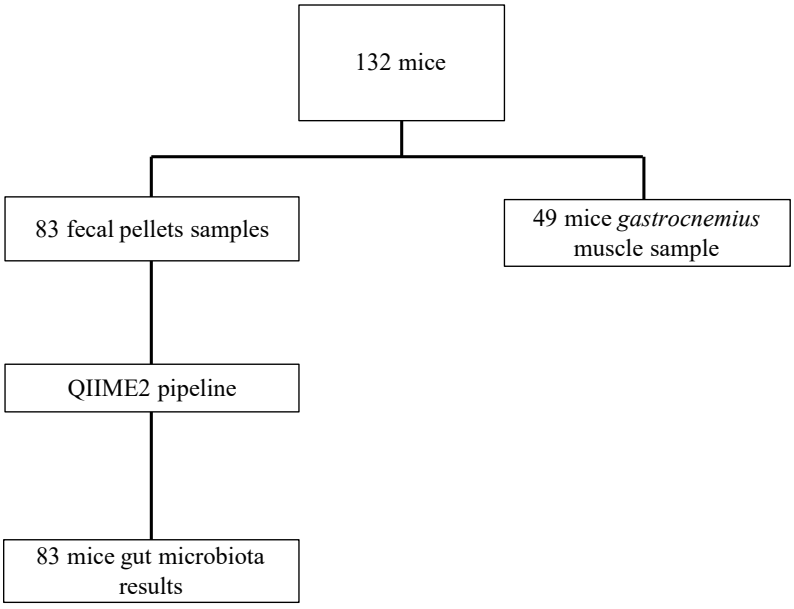

Figure 2. Study flow chart.

Table 1. C57BL/6 mice phenotypical characteristics and inflammation markers.

	Group				
	Control	HFD	HFD + Exe	HFD + Gen	HFD + Exe + Gen
Male, n	8	8	9	8	6
Female, n	9	8	9	9	9
Baseline body weight (WT), g[§]	20.55 (18.21, 22.53)	20.1, (17.84, 22.96)	19.98, (17.73, 21.44)	19.78, (18.39, 23.23)	19.41, (17.10, 20.8)
Post intervention body WT, g[§]	28.21, (24.05, 37.67)	42.76 (32.51, 49.15)	38.52 (32.02, 44.41)	37.32 (27.97, 43.62)	31.07 (25.91, 36.98)
[a]Heart WT, g	0.146 ±0.26	0.162 ±0.29	0.160 ± 0.03	0.140 ±0.02	0.136 ±0.02
[a]Liver WT, g	1.157 ±0.30	1.909 ±0.90	1.430 ±0.45	1.094 ± 0.26	0.959 ±0.22
[a]Kidney WT, g	0.342 ±0.07	0.400 ±0.11	0.394 ±0.10	0.324 ± 0.5	0.319 ±0.06

[a]Adipose tissue WT, g	1.50 ±1.23	3.657 ±1.68	3.571 ±1.53	3.936 ±3.98	2.027±2.09
[b,c][Il-6], pg/mL	27.51 ± 15.62	96.04 ± 14.02	58.16 ± 41.76	62.44 ± 45.89	52.09± 28.14

All values are mean ± standard deviations, or as median (IQR)[§]. [a]The weight values for heart, liver, kidney and adipose tissue were post-mortem. [b]The serum concentrations for IL-6 were measured after the 12-week intervention.[c] The values of IL-6 were based on 7-8 mice per group. WT, weight; IL-6, interlukin-6

GUT MICROBIOTA

Alpha Diversity

The control group had significantly greater microbial richness (the number of species in the samples; Observed OTUs and Chao1 index) compared to HFD (Kruskal-Wallis H = 12.21, $p<0.01$) and HFD + Gen (Kruskal-Wallis H = 10.89, $p<0.01$) (Figure 3). Richness did not differ between control and HFD supplemented with exercise groups, HFD + Exe (Kruskal-Wallis H=1.41, p=0.23) and HFD + Exe + Gen (Kruskal-Wallis H=2.03, p=0.15). Control group (Kruskal-Wallis H=10.90, $p<0.01$), HFD + Exe (Kruskal-Wallis H =8.96, $p<0.05$) and HFD + Exe + Gen (Kruskal-Wallis H=10.31, $p<0.05$) groups had significantly greater richness compared to HFD + Gen. To assess if phylogeny played a role in the richness differences, we measured phylogeny impact on alpha-diversity by Faith PD alpha diversity metric (Figure 5). We found that phylogeny was significantly ($p <0.01$) driving differences in the control, HFD + Exe and HFD + Exe + Gen compared to HFD and HFD + Gen. The control group, as expected, differed from the HFD group with a greater diversity as measured by Faith's PD metric; however, control group has a significantly higher alpha diversity to HFD + Gen. The analysis of the gut microbial evenness within the samples, Pielous' evenness, did not reveal any significantly ($p>0.05$) differences among the intervention groups. This remained true when evaluating community evenness in conjunction with abundance (Shannon Index). Therefore, Exe and its combination with Gen may be interventions with a greater capacity to prevent gut bacteria richness HFD-induced reduction compared to genistein alone, but not over the gut microbiota species distribution in the environment (evenness).

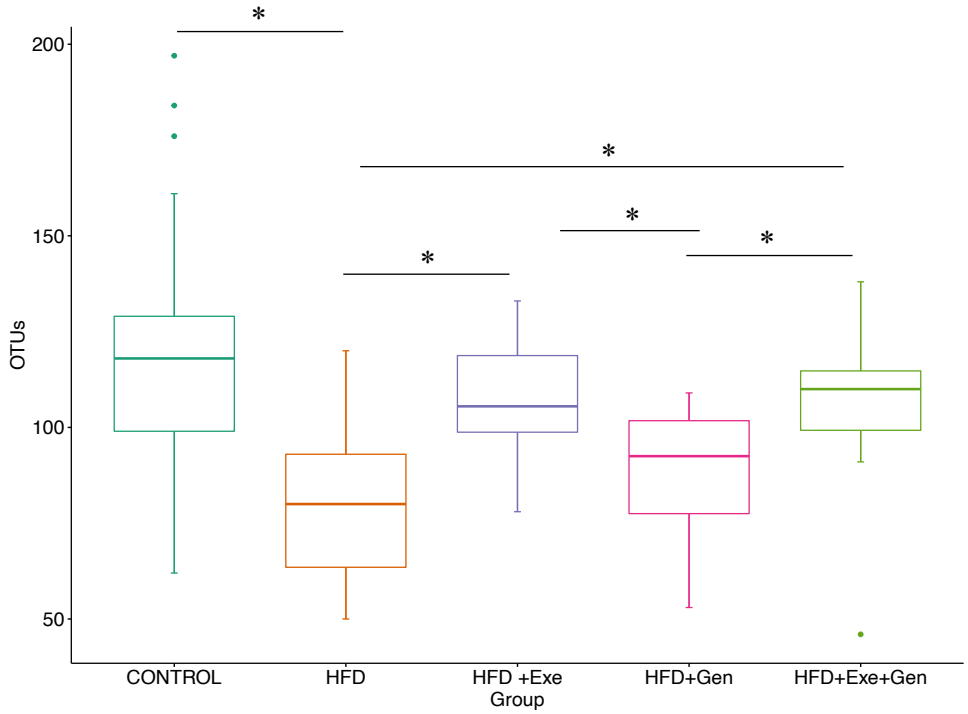

Figure 3. Alpha diversity boxplot for Observed Operational Taxonomic Units (OTUs). (*) denotate significant group differences ($p<0.05$) after Benjamini-Hochberg correction for multiple comparisons. Treatment groups are as follows: Control; High-fat, High-Sugar diet (HFD); HFD + Exercise (HFD + Exe); HFD + Genistein (HFD + Gen); HFD + Exe + Gen.

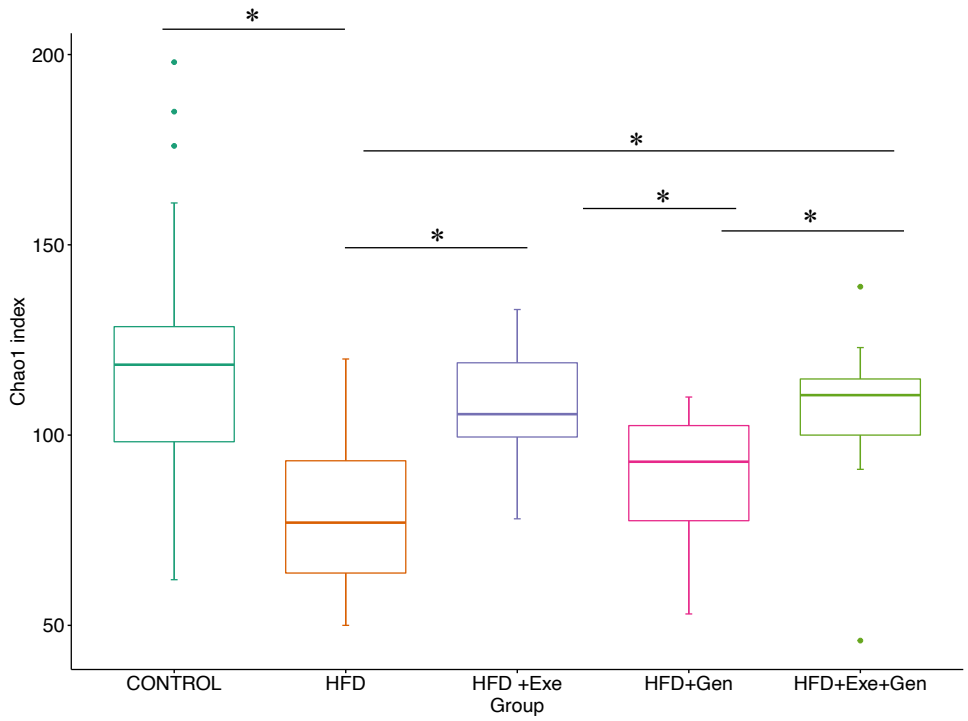

Figure 4. Alpha diversity boxplot for Chao1 Richness Index. (*) denotes significant group differences ($p<0.05$) after Benjamini-Hochberg correction for multiple comparisons. Treatment groups are as follows: Control; High-Fat, High-Sugar diet (HFD); HFD + Exercise (HFD + Exe); HFD + Genistein (HFD + Gen); HFD + Exe + Gen.

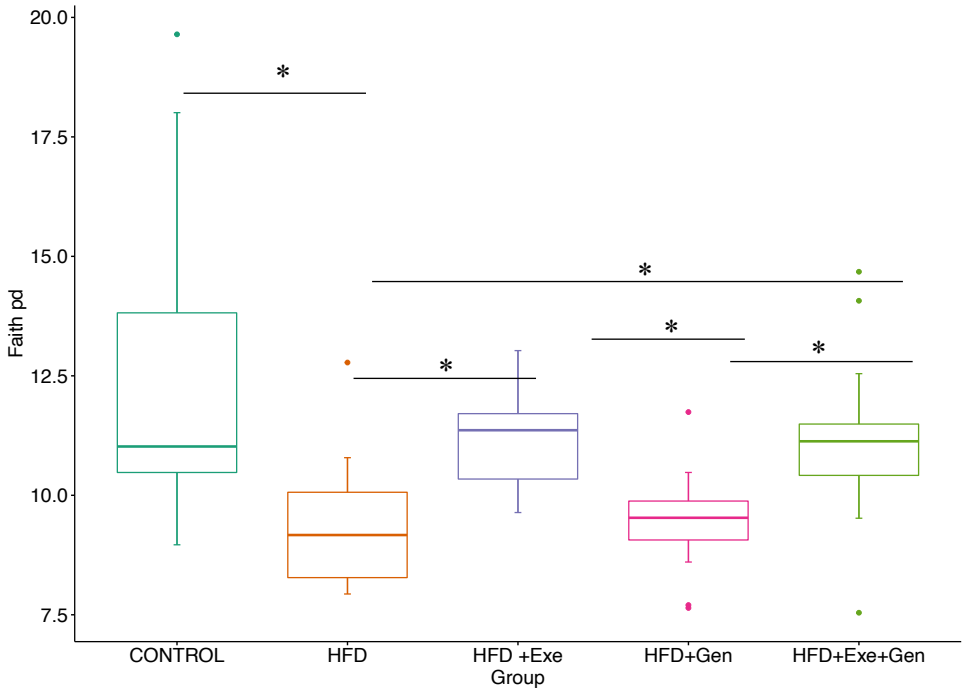

Figure 5. Alpha diversity boxplot for Faith Phylogenetic Diversity. (*) denotate significant group differences ($p<0.05$) after Benjamini-Hochberg correction for multiple comparisons. Treatment groups are as follows: Control; High-Fat High-Sugar diet (HFD); HFD + Exercise (HFD + Exe); HFD + Genistein (HFD + Gen); HFD + Exe + Gen.

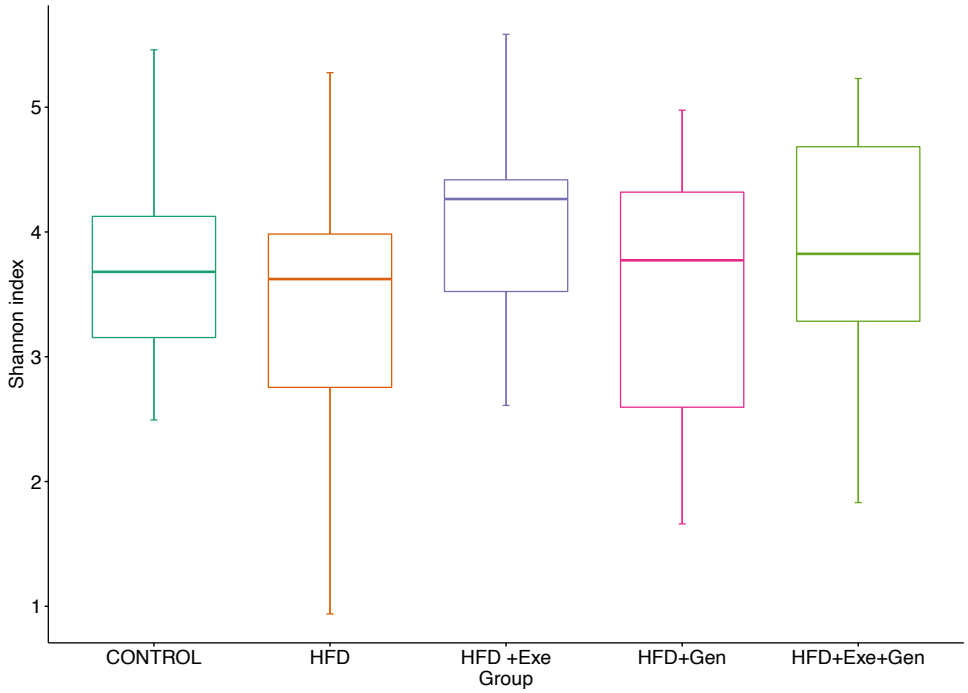

Figure 6. Alpha diversity boxplot for Shannon Index. (*) denotate significant group differences ($p<0.05$) after Benjamini-Hochberg correction for multiple comparisons. Treatment groups are as follows: Control; High-Fat High-Sugar diet (HFD); HFD + Exercise (HFD + Exe); HFD + Genistein (HFD + Gen); HFD + Exe + Gen.

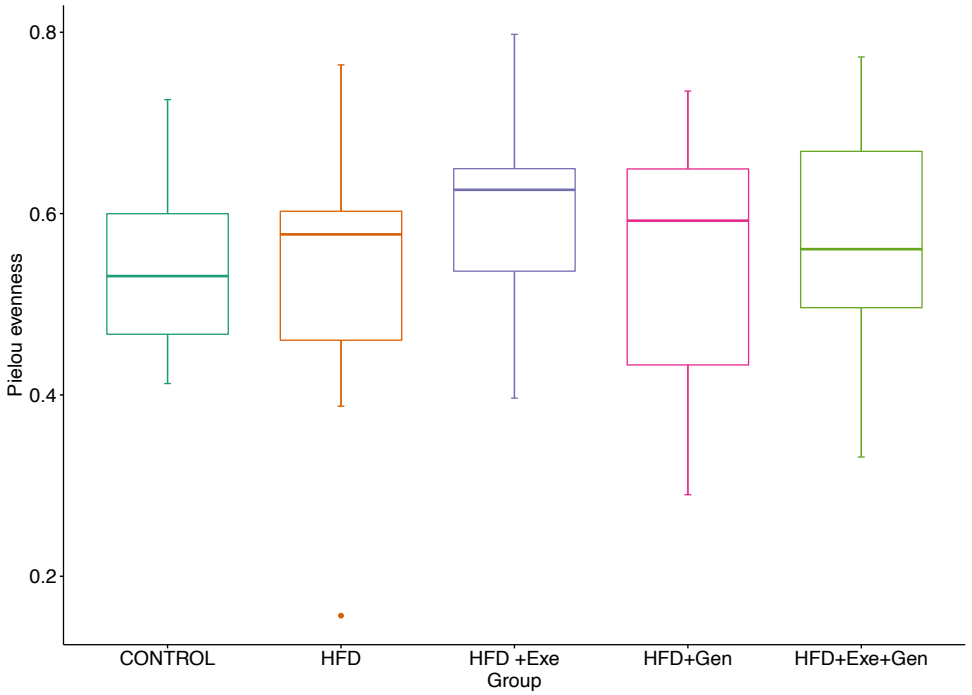

Figure 7. Alpha diversity boxplot for Pielous evenness. (*) denotate significant group differences ($p<0.05$) after Benjamini-Hochberg correction for multiple comparisons. Treatment groups are as follows: Control; High-Fat High-Sugar diet (HFD); HFD + Exercise (HFD + Exe); HFD + Genistein (HFD + Gen); HFD + Exe + Gen.

Beta Diversity

Jaccard distance (Figure 8), measurement of the presence and absence of microbes showed that all groups significantly differed from each other. When phylogeny was considered, via unweighted UniFrac (Figure 9), we found the same results. All groups differed in their microbial community structure. Bray-Curtis dissimilarity matrix (Figure 10), community structure differences which based the analysis on abundance, suggested that HFD + Exe and HFD + Gen groups did not differ from HFD structure. Whereas, the combined intervention was significantly different from HFD, as well as HFD + Exe and HFD + Gen. The control group had a significantly different structure compared to all other intervention groups. These results suggest that the combined treatment may have had a positive effect over HFD, but this effect was not enough to reach that of the control group. When phylogeny was added to the analysis by weighted UniFrac (Figure 11), we found same results. HFD + Exe + Gen and Control groups differed from all intervention groups, whereas HFD + Exe and HFD + Gen groups did not differ from HFD group.

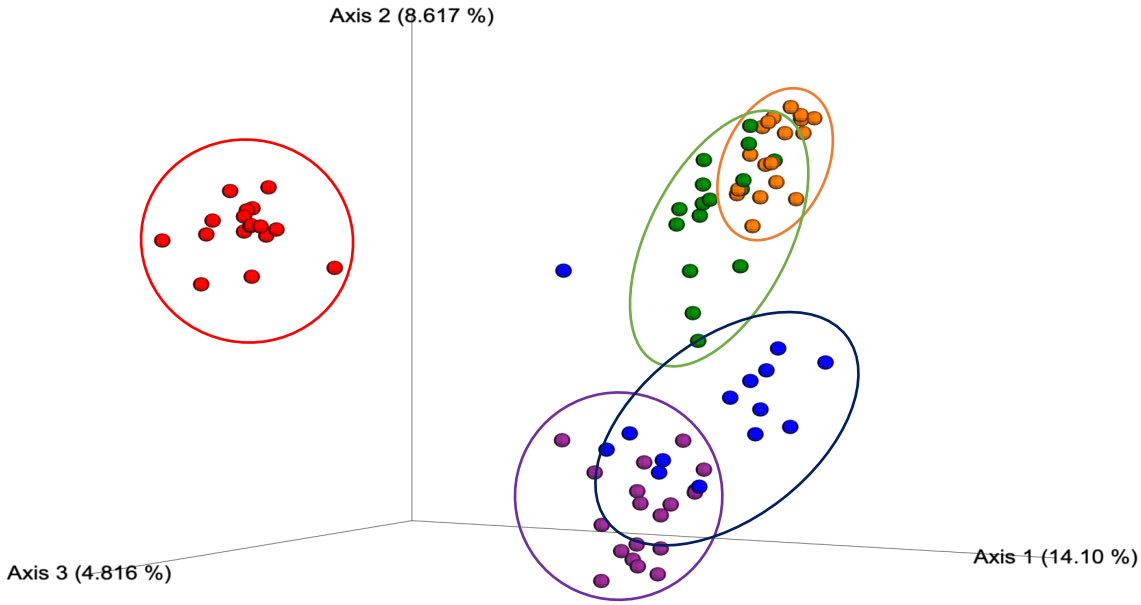

Figure 8. Beta-diversity PCoA plot of between group distances using Jaccard distance matrix values. Treatment groups are as follows: control, red; High-Fat High-Sugar diet (HFD), blue; HFD + Genistein (HFD + Gen), purple; HFD + Exercise (HFD + Exe), orange; HFD + Exe + Gen, green. Significant group differences ($p<0.05$) after Benjamini-Hochberg correction for multiple comparison were observed for all pairwise treatment comparisons.

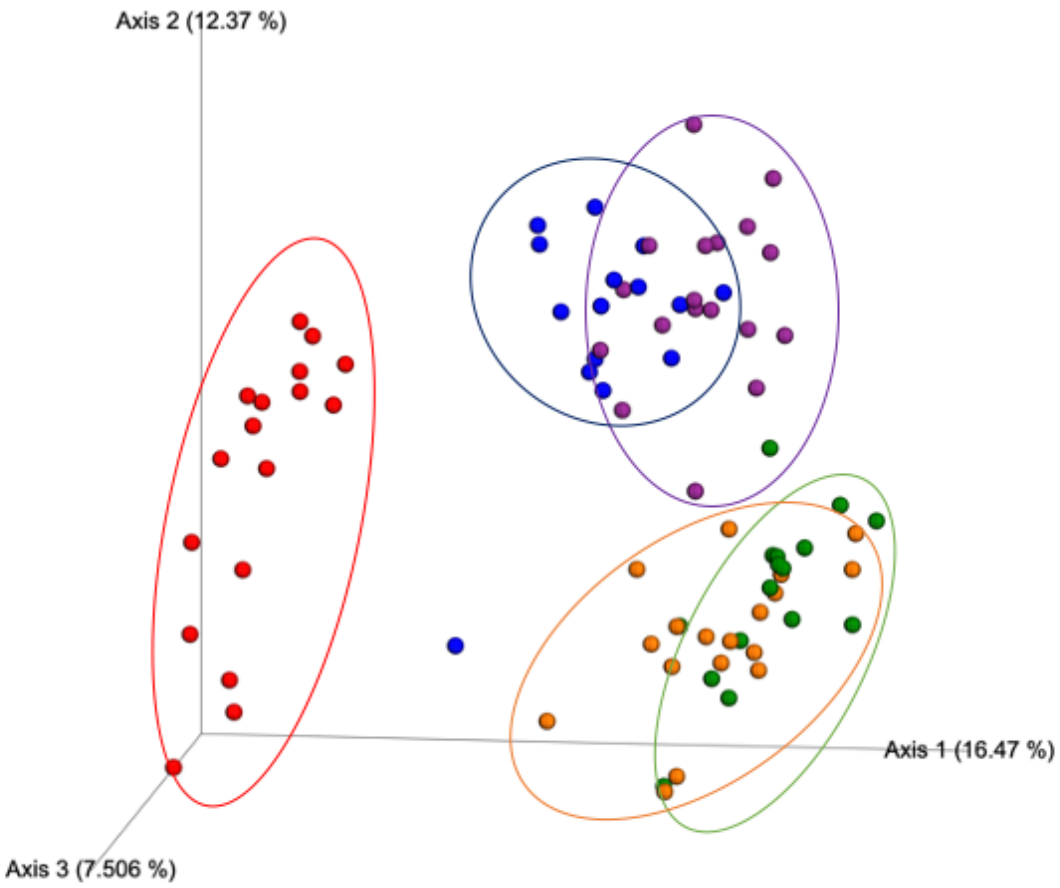

Figure 9. Beta diversity PCoA plot of between group distances using unweighted UniFrac distance matrix values. Treatment groups are as follows: control, red; High-Fat High-Sugar diet (HFD), blue; HFD + Genistein (HFD + Gen), purple; HFD + Exercise (HFD + Exe), orange; HFD + Exe + Gen, green. Significant group differences ($p<0.05$) after Benjamini-Hochberg correction for multiple comparison were observed for all pairwise treatment comparisons.

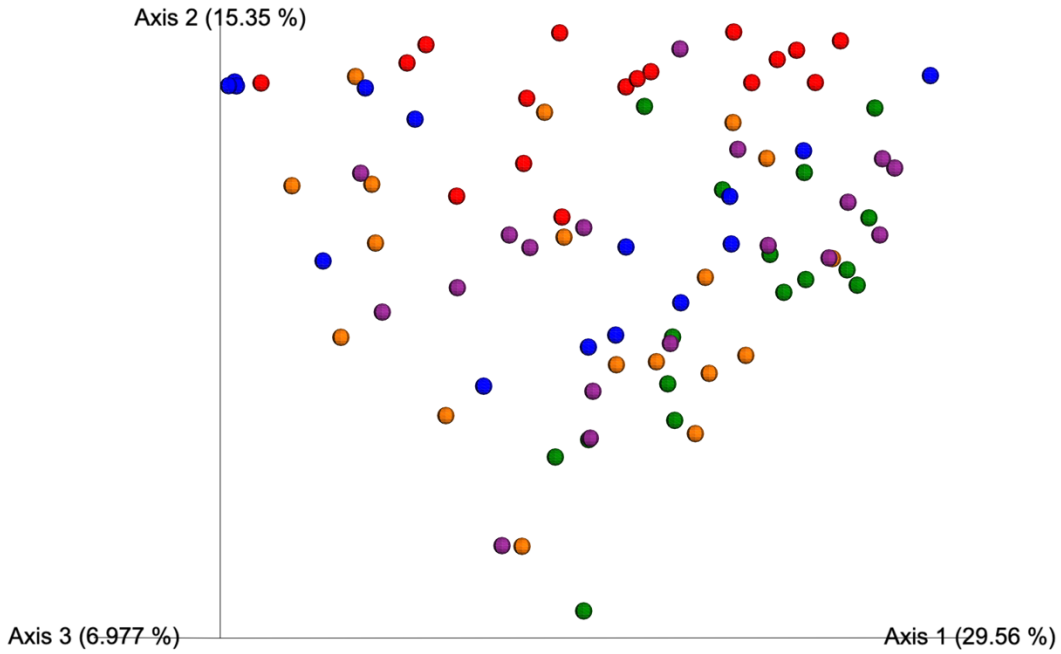

Figure 10. Beta diversity PCoA plot of between group distances using Bray-Curtis distance matrix values. Treatment groups are as follows: control, red; High-Fat High-Sugar diet (HFD), blue; HFD + Genistein (HFD + Gen), purple; HFD + Exercise (HFD + Exe), orange; HFD + Exe + Gen, green.

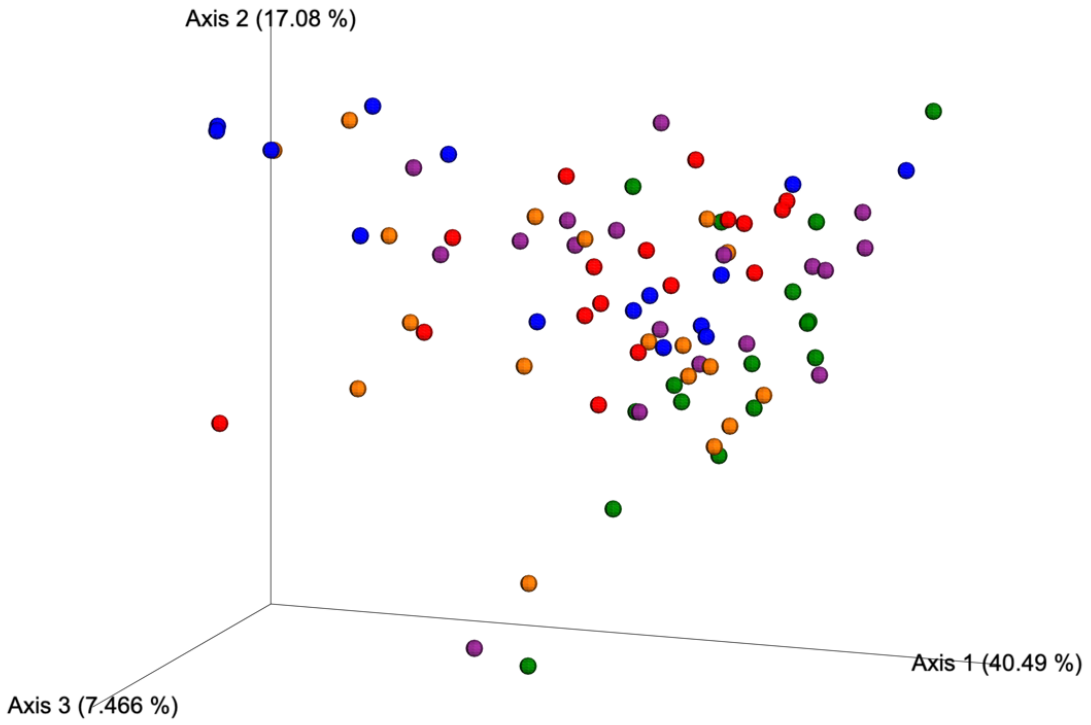

Figure 11. Beta diversity PCoA plot of between groups distances using weighted UniFrac distance matrix values. Treatment groups are as follows: control, red; High-Fat High-Sugar diet (HFD), blue; HFD + Genistein (HFD +Gen), purple; HFD + Exercise (HFD + Exe), orange; HFD + Exe + Gen, green.

Differential Abundance

All-against-all LEfSe analysis was utilized to identify the genera that most likely explained the differences between all intervention groups. This analysis identified that the gram-positive genus, *Ruminococcus*, was differentially more abundant in the control group when compared to all other intervention groups (Linear Discriminant Analysis (LDA) score >2, $p<0.05$, Wilcoxon rank-sum test, Figure 12). In order to assess the impacts of genistein, exercise, and combined intervention (Exe + Gen) on gut microbiota, we compared HFD vs HFD + Exe, HFD + Gen, and HFD + Exe+ Gen. The HFD + Exe + Gen group had a significantly greater abundance of *Ruminococcus* when comparing to all HFD groups (Linear Discriminant Analysis (LDA) score >2, $p<0.05$, Wilcoxon rank-sum test) (Figure 13).

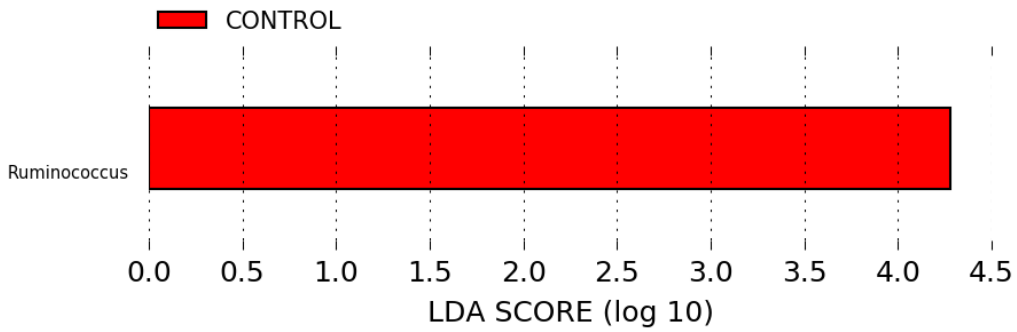

Figure 12. Effect size of differentially abundant genera of *Ruminococcus* after using all-against-all Linear discriminate analysis of effect size (LEfSe) analysis between Control, High-Fat High-Sugar diet (HFD), HFD + Exercise (HFD + Exe), HFD +Genistein (HFD + Gen), and HFD + Exe + Gen groups. The phylogenetic features were tested by LEfSe to reveal the genera which significantly differed between the different groups.

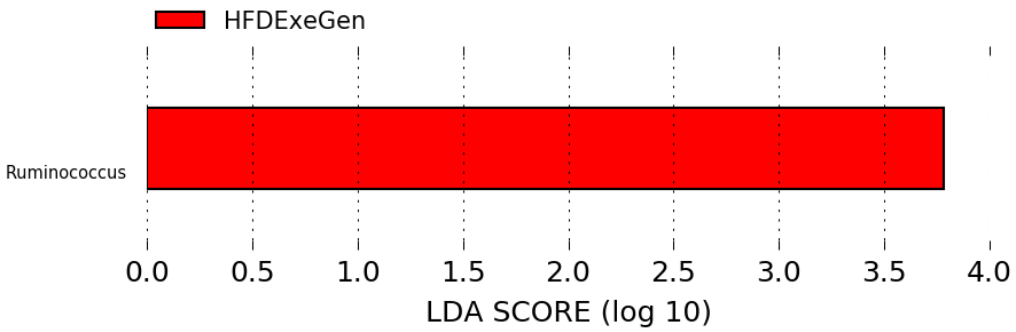

Figure 13. Effect size of differentially abundant genera of *Ruminococcus* all-against-all Linear discriminate analysis of effect size (LEfSe) analysis between High-Fat High-Sugar diet (HFD), HFD + Exercise (HFD + Exe), HFD +Genistein (HFD + Gen), and HFD + Exe + Gen groups. The phylogenetic features were tested by LEfSe to reveal the genera which significantly differed between the different groups.

MITOCHONDRIAL OXIDATIVE CAPACITY

Citrate Synthase

The regression model for CS activity ($p=0.1612$, $F_{9,39}= 1.56$, $R^2=9.5\%$) did not reach significance (Table 3). We did not find any statistically significant differences among the intervention groups ($p>0.05$, Figure 18); CS activity in HFD + Exe + Gen (23.20 ± 16.52 uml/min/g), control (18.88 ±10.13 uml/min/g), HFD (14.88 ±7.11 uml/min/g), HFD + Exe (20.44±12.96 uml/min/g), and HFD + Gen (17.68±11.18 uml/min/g). There was no significant synergistic effect between Gen and Exe ($p>0.05$), suggesting that the combined treatment did not prevent the HFD-induced CS activity decline. However, sex might play a role in the CS activity since the interaction regression model showed significant results for sex ($p=0.005$) withmale CS activity (24.1 ± 9.37 uml/min/g) being significantly higher compared to that of females (14.1 ± 12.7 uml/min/g). However, there was no significant association ($p=0.52$) between microbial richness and CS activity. As expected, based on the non-significant results found when studying Exe, Gen, and their interaction on CS activity, we did not find any significant mediation of microbial richness on CS activity ($p=0.34$).

Table 2. Citrate Synthase (CS) kinetic activity (uml/min/g wet tissue) differences among Control, HFD, HFD + Exe, HFD + Gen, and HFD + Exe + Gen.

Independent variable	Correlation coefficient	Standard Error	T statistic	p-value
Intercept	15.3102	4.974	3.078	**0.00381 ***

HFD	-1.6339	7.0355	-0.232	0.81727
HFD + Exe	-0.7847	7.0355	-0.112	0.91177
HFD + Gen	-4.9084	7.0355	-0.698	0.48953
HFD + Exe + Gen	2.3003	7.0355	0.327	0.74545
R^2 =0.09532			SEE =11.12	
F-ratio =1.562			p=0.1612	

Table 3. Exercise, Genistein, and their combined interaction effects on Citrate Synthase (CS) kinetic activity (uml/min/g wet tissue) after adjustment for sex.

Independent variable	Correlation coefficient	Standard Error	T statistic	p-value
Sex; Male	10.7178	3.6028	2.975	**0.00536***
Exe	-4.7065	9.9497	-0.473	0.63883
Gen	4.7104	9.497	0.473	0.63855
Exe:Gen	-0.3754	7.1675	-0.052	0.95853
R^2 =0.1588			**p=0.041***	
F-ratio =2.793				

CHAPTER 5

DISCUSSION

This study examined the impacts of Exe, dietary supplementation with Gen (600 mg Gen/kg diet), and their combined effects on gut microbial community diversity and mitochondrial oxidative capacity in male and female adult C57BL/6 mice consuming a HFD for 12 weeks. Additionally, we explored the mediation of gut microbiota through Gen and Exe and the mitochondrial oxidative capacity relationship. The number of gut bacteria (richness; Observed OTUs, Chao1, and Faith PD) was significantly higher in the Exe and the combined group (Exe + Gen) than other intervention groups (HFD and HFD + Gen). The bacterial community's diversity differed among all five groups (Jaccard distance, Bray-Curtis dissimilarities, weighted and unweighted UniFrac), indicating each treatment's unique effects on the gut microbiota community structure. LEfSe analysis revealed a greater abundance of the genus *Ruminococcus* in the control group when compared to all other groups. When comparing all HFD groups against each other, we found that in the HFD + Exe + Gen group, the genus *Ruminococcus* was significantly more abundant. This suggests that in our study Exe + Gen is more successful changing the gut microbiota towards control group gut microbiota composition than Gen or Exe alone. Although the mitochondrial oxidative capacity (CS activity) did not differ significantly among groups, the combined treatment (Gen + Exe) had the highest CS activity while the lowest CS activity was observed in the HFD group.

Beneficial effects of exercise on gut microbiota have been shown in various animal studies fed with a HFD.[70,85,314,316] In contrast to the treatment with Gen alone, we found that Exe (HFD + Exe) and its combination with Gen (HFD + Exe + Gen) prevented

the decline of gut microbial richness (Observed OTUs and Faith's PD) as compared to the HFD and HFD + Gen groups; however, when accounting for the evenness and abundance of the species present (Shannon Diversity Index), the difference disappeared. These data suggest that exercise may impact the number of bacterial species, but not how evenly distributed or abundant the species are in the gut bacterial community. Others have reported no differences in alpha diversity in mice following voluntary exercise.[79,304,307,347] Conversely, forced exercise protocols have yielded diverse results.[303,314,315,348,349] Ribeiro et al. showed that C57BL/6J male mice fed a HFD with an 8-week, low-to-moderate (50% VO_{2max}) aerobic exercise regimen (30 min/day, 5 days/week) did not experience a modification of gut microbial richness.[314] Whereas other studies have shown that 4-weeks of endurance training without dietary modifications[348] and 6-weeks of high-intensity interval training (HIIT) after a HFD[315] improved the alpha-diversity (Shannon index, accounts for number of species and evenness) and the Bacteroides/Firmicutes ratio in eight-week-old male C57BL/6J mice. The intensity of exercise has been previously discussed as a determining factor for changing gut microbiota in C57BL/6J mice.[350] Other studies have found forced exercise to impact the bacterial richness, but not microbial abundance and evenness (Shannon Index) when compared to sedentary or voluntary exercise groups in C57BL/6J mice.[350] In rats forced to exercise while being fed a HFD with similar study length and protocols to ours, contrary results were observed, including decreased alpha diversity (Shannon Index),[66] no differences in alpha-diversity at the species level (Shannon Index),[304,351] and an increase in bacterial richness at the family level.[352]

Similar to our results, human trials have shown exercise to have a significant trend in increasing richness and gut microbial alpha-diversity evenness and relative abundance.[309,311,353–355] Allen et al., studied the differences between lean and obese individuals, but they did not find any alpha diversity differences in a 6-month exercise intervention in a previously sedentary obese group, which showed that exercise significantly shifted alpha-diversity in obese individuals towards a healthy gut microbiota.[69] However, Kern et al., did find an increase in gut bacterial richness in overweight and obese individuals, which were randomized into moderate or vigorous-intensity exercise programs for 6-months.[353] The vigorous-intensity exercise group experienced greater shifts in alpha-diversity factors (richness, evenness, and relative abundance) than the moderate exercise group; suggesting a higher exercise intensity might significantly impact to a greater extend bacterial alpha-diversity than moderate exercise intensity interventions. In athletic populations exercising at high intensities for long periods, studies have elucidated a significantly greater alpha diversity and different community structure than control individuals.[96,308,309,311,356] Overall, human and rodent literature suggest that aerobic forced exercise might be a viable treatment to consider to prevent, improve, and reestablish gut microbial alpha-diversity in the presence of a poor diet.

Forced exercise indicated a distinctive beta-diversity compared to all groups as found by others in rodents[79,317,320,349] and human models.[69,353,357] Rodents fed with a HFD and following different exercise interventions, forced[79,317] and voluntary,[320] had significantly different community structure compared to HFD groups. In line with the

rodent literature, we found that Exe groups clustered closer in the PCoA plots. The Gen + Exe and Exe groups had more similar gut microbial communities, but this shift induced by exercise was still significantly different. This suggests an independent effect of HFD, Gen, Exe, and Exe + Gen on the gut microbiota beta-diversity. A study which used a 50% VO_{2max} aerobic exercise regimen over 8-weeks did not experience a modification of community structure in male C57BL/6J mice fed an HFD.[314] Fewer studies in humans have analyzed the gut microbiota beta-diversity in the context of different exercise regimens.[69,353] Structured 6-month exercise programs in previously sedentary obese individuals changed microbial beta-diversity significantly compared to control group.[69,353] Further, Kern et al. explained that the metabolic activity of exercise-induced functional gut microbial changes upregulated amino acid and carbohydrate metabolism degradation.[353]

The heterogeneity of exercise impacts on gut microbiota alpha- and beta-diversity might be explained by different factors. In both animal and human models, the intensity (low-to-moderate vs. high-intensity) and mode of exercise (voluntary vs. forced)[350] training likely help explain the different results among the literature.[302] Our results add to the existing science that forced exercise may be a more effective treatment to significantly prevent decline in the gut bacterial richness (alpha-diversity) and beta-diversity compared to voluntary exercise.[79,304,350] This suggests that exercise had a distinct impact on the gut bacterial alpha- and beta-diversity compared to diet as discussed elsewhere.[349,352] It remains to be discovered if these changes have a positive[303,305,316] or null effect on obese individuals' health.[358]

Genistein has emerged as a potential therapy for obesity[291] with beneficial effects on adipose tissue, glucose metabolism, and gut microbiota across a wide range of doses (2-600 mg/kg of body weight) in animal studies[81,83,297,359] and in human (50-500 mg/kg of body weight) clinical trials.[360,361] Contrary to expected findings, supplementing a HFD with 600 mg of Gen per kg of diet did not prevent the reduction of gut microbiota alpha-diversity (Shannon Index, Observed OTUs, Chao1, Faith's PD, Peilous' evenness) or significantly modify the relative abundance of bacterial taxa. There is evidence of 24-weeks on a HFD supplemented with genistein (e.g., 3 mg kg of body/weight/day) or a combined isoflavone supplement (supplement based on genistein and daidzein) to be significantly effective at increasing gut microbial alpha-diversity (Observed OTUs and Shannon Index) compared to a HFD alone in C57BL/6J mice.[52,81,295,296] As found in the rodent literature, forty five male and female obese individuals were randomized to genistein group or placebo/control group for 2 months. The obese individuals supplemented with 50 mg/day of genistein showed increased alpha-diversity (Shannon Index) and different bacterial abundance compared to the control group.[361] The main difference between our results and the literature are the length of the exposure (28 weeks[82] vs. 12 weeks) to obtain changes in the gut bacterial alpha diversity. Given all these findings, supplementation with genistein may require a longer (e.g., 24-weeks) chronic exposure to successfully modify alpha diversity in the gut microbiota.

In terms of community structure (beta-diversity), we found gut microbiota beta-diversity differences among the Gen group which was similar to other findings.[80,82,362] Our results suggested that there was a distinctive effect of Gen on the microbial

community structure. Others have found that genistein significantly impacted community structure (weighted UniFrac) and increased the relative abundance of health-associated bacteria, *Prevotella* and *Akkermansia* in rodents.[81,83] Similarly, in human obese subjects, 50 mg genistein/day significantly changed gut bacterial beta-diversity; data further suggested that this change might have been driven by an increase in the relative abundance of *Ruminococcus Bromii*, *Helicobacter apodemus, Lactobacillus reuteri, Bacteroides acidifaciens,* and *Akkermansia muciniphila* compared to baseline and control groups.[361] We did not find significant changes in the relative abundance of gut microbes in the HFD + Gen group when compared to the other groups. Further research should identify the optimal length and dose of genistein for beneficial gut microbial changes in HFD-induced obesity to disclose genistein's independent effects on microbial diversity (alpha and beta) and bacterial taxa compared to exercise for the improvement of obesity and associated diseases.

The combined treatment of isoflavones and exercise is a widely used treatment to reduce menopausal symptoms, and its cardiometabolic consequences (e.g., increased LDL cholesterol).[363,364] The potential combination of both treatments to modify gut microbiota diversity in obesity has not been studied before. We found that Exe and Gen impacted the gut microbial community differently than either treatment alone. Our results showed significant differences between the HFD + Exe and HFD + Exe + Gen, which clustered separately in the PCoA plots. The Gen group clustered further apart from the Exe groups (HFD + Exe, and HFD + Exe + Gen) and closer to the HFD group. As previously suggested by literature investigating exercise, diet, and gut

microbiota,[316,352,365] it could be that exercise provides additional and independent beneficial changes to isoflavones effects on gut microbiota composition. Our results add to the existing literature that combining exercise and genistein changes gut microbiota significantly, as evidenced by the highest gut bacterial richness and different beta-diversity found in the HFD + Exe + Gen group compared to all groups. However, the control animals alpha and beta diversity differed significantly from all other groups; this suggests that even the combined treatment does not completely prevent HFD-induced gut microbial structure changes. Therefore, more studies are needed where exercise and genistein's synergistic effects are combined to promote improvements in gut microbiota diversity and community structure during states of metabolic disease.

We found interesting results when we analyzed the taxonomy driving differences between groups with LEfSe. The combined treatment group (HFD +Exe +Gen) was dominated by the genus *Ruminococcus*, when compared to all other HFD groups. Interestingly, when we compare all the groups against each other, *Ruminoccocus* dominated the control group. These results suggest that the combined treatment may drive taxa dominance toward a healthy control microbiota as found by others.[81,91,366] The increased abundance of *Ruminococcus spp*. could be related to its involvement in the conversion of soy isoflavones from glycosides to the aglycone form, the bioactive metabolite of isoflavones.[367] Our results indicated that the combination of Exe and Gen is significantly driven by *Ruminococcus spp.* when compared to all other HFD groups. *Ruminococcus spp.* have been found to be increased in other populations when supplementing with genistein[91,361] and reduced on a HFD.[366]

The phylum *Firmicutes* seems to change with the combination of exercise and bioactive ingredients; since in humans another species belonging to the phylum *Firmicutes, Veillonella atypica,* significantly increases after exercise.[94,96,354] If exercise promotes the increase of *Ruminoccocus*, a higher abundance of this genera should benefit HFD with genistein supplementation by increasing its conversion to its bioactive metabolite and consequent beneficial health effects (e.g., reduction of inflammation). Therefore, the combined lifestyle approach of genistein and exercise, may be an important approach to prevent HFD-induced gut microbial disturbances by modifying gut microbial community structure and richness. Researchers who have studied other bioactive ingredients in combination with exercise, found that the combination of cellulose nanofiber (insoluble fiber) and voluntary exercise significantly increased *Ruminococcaceae* (*Ruminococcus* family; *Firmicutes* phylum) in male C57BL/6N mice.[318] However, more research is required to confirm or reject the relevance of *Ruminococcus* in relation to the effect of exercise and genistein and how the results of mice are transferable to humans. Further analysis should include the concentration of secondary genistein metabolites, such as aglycones, 5-hydroxy-equol in the gut and blood samples to measure the synergistic effect of genistein and exercise on the conversion of bioactive isoflavone metabolites before and after a lifestyle intervention.

The CS activity results did not confirm our hypothesis that Exe + Gen would be able to significantly prevent the decline in CS activity in the musculoskeletal mitochondria of mice fed with a HFD. Despite the non-significant group differences, the HFD had the lowest CS activity (18.88 ±10.13 uml/min/g wet tissue) compared to all

intervention groups. HFD + Exe + Gen (23.20 ± 16.52 uml/min/g wet tissue) was the group with the highest CS activity followed by the HFD + Exe (20.44±12.96 uml/min/g wet tissue). These results are in line with the literature available on exercise[368–370] and functional ingredients[371,372] and their combination[331,373] to significantly change CS activity in the skeletal muscle mitochondria in rodents. To our knowledge, studies on the synergistic effect of genistein with exercise to prevent the decline in CS activity in the presence of a HFD has never been investigated before.

The impact of exercise for improving the skeletal muscle CS activity during a HFD has been widely supported elsewhere.[368–370,374–376] Cheng et al., showed that CS activity increased by 2-fold in the last two weeks of 6-weeks aerobic forced exercise in adult rats fed with a HFD.[368] The % of fat from the total calories in a six-week study by Cheng et al. was 76 %, which was slightly higher than in our 12-week study (60% of calories from fat).[368] Others had found that CS activity increases with the length of exposure to a HFD in mice.[377,378] A study with a 12-week voluntary exercise protocol in adult male rats fed with a HFD (40% of the energy content from fat) found a significant increased CS activity compared to control group.[379] In the same line, Barbose de Queiroz and colleagues showed in male Wistar rats an improved CS activity after 4 weeks on a HFD while exercising compared to a sedentary group on the same diet, but not when comparing HFD + Exe to a standard diet + Exe group.[370] The differences found in the mentioned research may relate to the hypothesis that the type of fats (saturated vs. polysaturated vs. unsaturated vs. trans-saturated)[380–382] have a different impact on mitochondrial oxidative capacity, with saturated fats being more hurtful to mitochondrial

oxidative capacity than monosaturated fats (the one used in this study). Also, the length of exposure to a HFD might play a role in the CS activity results. In our study, the HFD + Exe + Gen group had the lowest CS activity decline compared to the HFD group, but this difference did not reach significance after the 12-week intervention. It might be of interest in future projects to measure CS activity at baseline and every two weeks rather than only at the end of the intervention to understand the how activity changes in real-time.

To our knowledge, our study is the first to measure the impact of genistein on CS activity in the skeletal muscle mitochondria. Genistein has been shown to modify different mitochondrial metabolic pathways in various cell types (e.g., hepatocytes or cancer cells).[383–385] In the skeletal muscle, supplementation with genistein increased the 5'-adenosine monophosphate-activated protein kinase (AMPK) phosphorylation (a regulator of the oxidation capacity of the mitochondria in the skeletal muscle cells)[386] and the expression of genes involved in fatty acid oxidation in obese individuals supplemented with 50 mg genistein/day for 2 months.[361] Other functional ingredients have been shown to have the capacity to prevent the decline of or improve skeletal muscle mitochondrial CS activity.[372,387] Myricanol, a Chinese berry extract, has been shown to revert reduced CS activity in C57BL/6J mice myotubes after palmitic acid injection to mimic HFD-induced fat metabolism consequences in the skeletal muscle cells.[387] Similarly, 8-weeks of an HFD supplemented with sesame prevented the decline of CS activity compared to the HFD alone and no significant differences were found when compared to chow diet + sesame in adult C57BL/6J mice.[372] The mechanism

responsible for the CS activity regulation by genistein and other functional ingredients remains undetermined. Future research should include analysis of the mRNA of key co-transcription factors activators (e.g., PGC-1 alpha), transcription factors and protein expression in addition to the CS activity to determine the metabolic pathway stimulated by genistein to change the CS activity.

The full mechanism of genistein effects on skeletal muscle remains poorly understood, but a potential estrogen receptor (ER) dependence has been postulated. Genistein is an agonist of the ERα and ERß, which are cell-specific, meaning that the activation of the ERα/ß triggers different metabolic pathways in different tissues.[293,294] The literature in rodents treated with estradiol (a more potent agonist for the ERα/ß compare to Gen) had shown an increased CS activity and mitochondrial oxidative capacity.[388,389] But no studies are looking at genistein's capacity to stimulate ERα/ß and enhance the CS activity. Future studies studying the impact of genistein effects on skeletal muscle CS activity would benefit from determining the expression of ERα/ß to define the ERα/ß dependency for genistein metabolic effects in the myocyte's mitochondrial oxidative capacity.

We did not find significantly higher CS activity when supplementing the HFD with Exe and Gen. There is a lack of literature on the potential synergistic effects of genistein and exercise on the mitochondrial oxidative capacity in myocytes. Nonetheless, exercise increases the ER expression[390] in myocytes, and genistein is a potent ER agonist. We can hypothesize that the higher CS activity seen in our study in the HFD + Exe + Gen group could be due to the synergistic effect of both treatments on the ER and the consequent

stimulation of CS by PGC-1alpha. Other functional ingredients (e.g., polyphenols) have shown a synergistic effect with exercise on the CS activity and higher expression of key regulators of mitochondrial biogenesis, AMPK and PGC-1 alpha, in obese rats fed with a HFD.[331] The combination of resveratrol and exercise increased mitochondrial biogenesis and resulted in a higher CS activity compare to either treatment alone in male mice.[391,392] Overall, more research assessing genistein alone, and in combination with exercise, is required to confirm our results and further elucidate the mechanism behind their actions on skeletal muscle mitochondrial oxidative capacity.

The muscle used in our study, the *gastrocnemius* muscle, might have a role in our study's CS activity results. The muscles' mitochondrial oxidative characteristics are determined in part by the predominant isoform type of myosin heavy chain (MHC) protein expressed throughout the skeletal muscle. Briefly, in rodents, the *gastrocnemius* muscle is composed primarily of type IIb MHC isoform, a fast-twitch fiber, characterized by a low number of mitochondria and capillary content with glycolytic energetics (i.e., less oxidative capacity).[393] Whereas, in other muscles such as the *soleus*, the dominant fiber type isoform is MHC I/IIa, slow-twitch which is characterized by high mitochondrial and capillary content with a high oxidative energetics.[393] Smith et al., found a significant increase in the CS activity of rat *soleus* muscle (MHC type IIa and I; slow-twitch vs. gastrocnemius which is a predominantly MHC IIb fiber type muscle; fast-twitch) after 3-4 weeks of HFD plus forced exercise (54.2 ± 3.2 umol/min/g wet tissue) compared to control (43.8 ± 1.8 umol/min/g wet tissue).[376] Similarly, others found a significant increase in CS activity when analyzing the *soleus*[368,370,374,376,379] in rodents fed

a HFD following a forced exercise program. A few others have found that rats on a HFD exercising for 6 or 11 weeks[369] significantly increased their CS activity compared to controls when measured in red *vastus* or *gastrocnemius* myocytes (fast-twitch). Fiber typing of the muscle tissue samples to determine MHC isoform type in each of the samples should be considered a complementary analysis in future research projects to interpret further the effects of exercise, exercise and genistein, and genistein on the skeletal muscle's mitochondrial CS activity.

We did not find gut microbiota richness to be a mediator of Exe and Gen effects on CS activity. Other rodent data have shown a potential gut – muscle axis with microbially-synthesized metabolites (e.g., SCFA, bile acids) signaling on/in the skeletal muscle. Lahiri et al., found a reduced muscle mass in germ free mice (i.e., mice without gut microbiota) compared to mice with gut microbiota, suggesting a potential gut microbiota relevance in the skeletal muscle mass homeostasis.[394] Others have found that skeletal muscle metabolic intermediates such as lactate[94] are utilized by the gut bacteria, providing preliminary data on the potential crosstalk between both organs. Other key axes have been described in the literature such as gut – liver,[395] gut – adipose tissue,[396] and even gut – metabolic inter-organ crosstalk[397] as novel relevant targets in the prevention and treatment of obesity and metabolic syndrome. Our study found Exe + Gen to be the only intervention that significantly prevented a HFD-induced gut bacterial richness decline, and HFD-induced weight increases in adipose tissue and liver. These preliminary results suggest that diet and exercise have synergistic beneficial effects on a variety of organs (i.e., liver, and adipose tissue) which may be more potent than in

skeletal muscle. Future research should explore how the adipokines, myokines, and other metabolites in these different organs interact with each other and how the gut microbiota play a role in mediating crosstalk between tissues and how these interactions influence the prevention and treatment of obesity.

Strengths and limitations

Strengths of this research include that this was a well-controlled study of diet and exercise, with a large sample size (n=132), and both sexes being represented. The amount of genistein used (600 mg/kg/diet; ~4–7 μM serum genistein)[398] was comparable to a human dose when consuming soy milk (~2–4 μM serum genistein)[399] facilitating the translation of our results to future human trials. The utilization of a robust and widely accepted mitochondrial oxidative biomarker (CS), measuring the carbohydrate metabolism help to understand the impact of the HFD on the mitochondria oxidative capacity.

Limitations of the present study were the absence of baseline fecal pellets which limited the ability to assess differences within groups before and after the intervention. The muscle fiber type could play a role in the CS activity results we found. The majority of the literature studying the CS activity studied a less glycolytic fiber types (MHC type IIa and type I) compared to glycolytic fibers which are predominantly found in the *gastrocnemius* muscle utilized in our study. Further research should consider combining the use of enzymatic kinetic assay with muscle MHC fiber typing techniques, protein expression (e.g., estrogen receptors, CS) to complement the results found in the enzymatic analysis.

CHAPTER 6

CONCLUSION

Our results suggest that both Exe and Gen had a positive impact on shifting the gut microbiota; however, the Exe treatment may have had a greater capacity to change alpha diversity and preventing the HFD-induced gut bacterial richness decline. Exe, Gen, and their combined effects differentially influenced the gut microbial community as indicated by the significantly different beta-diversity across all groups. Exe + Gen changed gut microbial community structure, specifically by increasing the abundance of *Ruminococcus,* which was also the dominant microbial taxa in the healthy control animals. These results suggest a potential synergistic effect of Exe and Gen on gut microbiota. Our study's preliminary results add to the poorly explored therapeutic field of combining genistein and exercise to prevent HFD-induced obesity by the modification of gut microbiota. Further, our data could be useful to inform further study of the impact of lifestyle factors on gut microbiota in human trials.

CS activity was significantly affected by 12-weeks of an HFD; however, none of the treatments had a significant impact on enzyme activity. These results may be the result of the muscle type analyzed which had a lower mitochondria content. The CS activity analysis could benefit from determining the MHC isoforms of the muscle samples to understand the skeletal muscle's oxidative metabolism. Similarly, the type of fat utilized in the study was high in monounsaturated fats. Future studies should incorporate more saturated fat to see if this elicits different responses. Further research must be done on the Gen and Exe combination to confirm the trend shown in CS activity in our study and elucidate mechanisms. Lastly, measuring the secondary metabolites of

genistein will help to understand the availability of more bioavailable metabolites and their mechanistic interactions with tissues.

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APPENDIX A

MIDWESTERN UNIVERSITY SCHOOL OF MEDICINE IACUC APPROVAL FORM

MIDWESTERN UNIVERSITY (MWU)-GLENDALE

OFFICE OF RESEARCH AND SPONSORED PROGRAMS

IACUC – APPLICATION FOR USE OF ANIMALS

FORM B

The Institutional Animal Care and Use Committee (IACUC) meets monthly. This completed form must be received no later than the 1st day of the month of which the application will be reviewed (e.g., an application is due on August 1st in order to be reviewed at the August meeting).

PLEASE SUBMIT APPLICATIONS ELECTRONICALLY TO: AZORSP@MIDWESTERN.EDU.

1. TYPE OF PROTOCOL SUBMISSION

IACUC PROTOCOL NUMBER: 2880

CRC PROTOCOL NUMBER: ____

Make a selection for section A & B

A. ☒ **New Protocol** **B.** ☒ **Research Protocol**

☐ **Triennial Resubmission** ☐ **Teaching Protocol**

☐ **Amendment to an approved protocol** ☐ **Clinical Protocol**

Animals owned by MWU? ☒ **YES** ☐ **NO**

if you are purchasing research animals through MWU's Business Services select YES

Animals housed at MWU? ☒ YES ☐ NO

Please attach informed consent forms and copies of any documents being used for recruitment.

2. PRIMARY INVESTIGATOR & FUNDING INFORMATION

The principal investigator (PI) or Instructor must be the primary contact person for the protocol. Course coordinators should be listed as secondary PI's.

C. Principal Investigator(s): Layla Al-Nakkash, Tom Broderick

Phone Numbers:

LA: 623-572-3719 Office TB: 623-572-3664 Office

LA: 623-572 -3473-Lab TB: 623-5–537-6022-Lab

LA: 623-760-8249-Emergency TB: 623-523-3069-Emergency

Department/College: Physiology, AZCOM

Protocol Title: Diabetic obesity results in cognitive impairment.

Funding Agency: Midwestern Arizona Alzheimer's Consortium

Grant Number: AZ0049

Grant Title: Diabetic obesity results in cognitive impairment: Evaluation of the gut-brain and bones effects in response to exercise and genistein treatment

Species & Strain: Mouse, C57BL/6J

Total number of animals requested for duration of project (3 years): 100

3. USDA PAIN & DISTRESS CATEORY

*Indicate the appropriate letter based on category descriptions found in **Appendix I**. Check the category that is most invasive if there is a question of possible overlap.*

D. USDA CATEGORY ASSIGNMENT: ☐A ☐B ☐C ☒D ☐E

Does your application include any of the following?

E. ☐ Survival Surgery **☐ Multiple**

Survival Surgery ☒ Food or Fluid Regulation

☐ **Prolonged Restraint** ☐ **Hazardous Agent Use**

☐ **Non-Centralized Housing** *(study or housing of animals outside of Foothills Animal Facility spaces for more than 12 hours)*

☐ **Radioactive material *in vivo*** *(may require Radiation Safety Committee Approval)*

☐ **Use of dangerous drugs and/or chemicals** *(may require Biosafety Committee approval)*

☐ **Use of select agents/toxins** (requires Institutional Review Entity approval)

☐ **Use of pathogenic organisms** *(may require Biosafety Committee approval)*

☐ **Potentially dangerous recombinant DNA** *(may require Biosafety Committee approval)*

☐ **Non-surgical procedures that ***do not*** require the use of analgesic, pre-anesthetic and/or anesthetic**

☐ **Non-surgical procedures that ***require*** the use of analgesic, pre-anesthetic and/or anesthetic**

☐ **Trauma** *(i.e., involves conscious discomfort)*

☒ **Euthanasia**

Date of Radiation Safety Committee Approval: ____

Date of Biosafety Committee Approval: ____

4. PROTOCOL INFORMATION

Additional rows can be added to each table

F. List all procedures performed on the animals that take place outside of the animal facility:

Procedure/Description	Location/Room	Duration
Exercise training (treadmill running)	Foothills 137	30 minutes
Euthanasia by CO_2 gassing and cervical dislocation, followed by tissue harvest.	Foothills 137	Under 1 minute for euthanasia. Tissue harvest ~ 15 minutes per mouse.

Please note: any location of procedures outside of an approved animal facility (Foothills Animal Facility or Large Animal Facility) will be designated as a Study Area and will be subject to semiannual inspection by the IACUC. Teaching/Clinical applications must include the length, time, and number of visits.

G. Fill in the table detailing each species, strain, gender (if specified in your application) and the number of that classification of animal that will be used to complete this protocol:

Species	Strain	SEX ~~Gender~~	# To Be Used
Mouse	C57BL/6J	Male	50
Mouse	C57BL/6J	Female	50

**When ordering animals you must use the exact species/strain in your order form to business services so it can be verified with the IACUC Administrator*

H. List <u>ALL</u> personnel who will be handling and using animals for this protocol:

Personnel Name	Animal Use They Will Participate In	Training/Experience
Layla Al-Nakkash	Feeding mice genistein-based diet Euthanasia and tissue harvest	Completed relevant CITI training. 13 years of experience in rodent-based research
Tom L Broderick	Feeding mice genistein-based diet Exercise training of mice Euthanasia and tissue harvest	Completed relevant CITI training. 32 years of experience in rodent-based research
Jeff Plochocki	Euthanasia and tissue harvest	Completed relevant CITI training. 14 years of experience in rodent-based research
Charles Schaefer	Exercise training and feeding of mice Tissue harvest	Completed relevant CITI training. 5 years of experience in rodent-based research

Following protocol approval, animal ordering and/or use will be permitted following the submission of signed Training Certification sheets (Form B5) for all personnel involved.

<u>5. PROTOCOL DETAILS</u>

BRIEFLY ADDRESS EACH OF THE FOLLOWING:

I. Abstract of Research/Teaching Plan. Please include in this description:

a. A concise description of the overall goal, specific aims, and scientific merit and justification of this project. (No more than 1 page in length)

● The association between type 2 diabetes (T2DM) and the development of Alzheimer's disease (AD) is well-established. A metabolic pathologic feature of T2DM is impaired insulin use in tissues (insulin resistance- or IR). Interestingly, systemic IR and defective brain insulin signaling (IR in brain) are common features of AD, and patients with T2DM have a 50-75% increased risk of developing AD. The link between increased dietary fat/sucrose intake and risk for AD/cognitive dysfunction has also been made. Cognitive impairment in AD risk appears to be greater in females compared to males. Further, both diabetes and increased consumption of dietary fat are associated with abnormal bone development and gastrointestinal dysfunction.

● These disorders result in complex and challenging diseases to understand and therefore provision of effective treatments is similarly difficult. We plan to use a mouse model of diet-induced obesity (DIO) mouse to induce and mimic the diabetic state. We will examine the effects of either consuming soy or participating in exercise (or both) on key markers of brain function, gut and bone health. Our study will provide valuable information as to the connection between the gut, brain and bone health and will also allow us to determine differences between males and females.

●Based on this rationale, we hypothesize that adminstration of genistein, a naturally occurring phytoestrogen with anti-diabetic effects, or exercise will improve outcomes in the DIO mice. We predict both genistein supplement and exercise combined will have additive beneficial effects. We hypothesise that male and female mice will respond benefically to genistein and exercise yet, we predict the mechanisms will be varied.

Our aims are to:

(1) examine the role of genistein and exercise in the expression of brain proteins associated with synaptic function and memory,

(2) determine the effects of genistein and exercise on gut leakiness and the microbiome, and

(3) assess the effects of genistein and exercise on structural bone characteristics in the DIO mouse.

Four-week-old C57BL/J6 mice (male and female) will be used and assigned to the following groups.

1. Control mice (fed standard diet)
2. DIO (fed high fat diet with fructose/sucrose added to the drinking water)
3. DIO + genistein treatment
4. DIO + exercise
5. DIO + genistein + exercise

MICE: A total of -100 mice will be used (10 mice per group x 5 groups x sex (2) for a total of 50 males and 50 females (Jackson Laboratories).
SEX: NIH guidelines require studies to utilize sex-dependent comparisons of animal models, thus proposing sex-dependent mechanisms in future grant applications will be of importance.
HIGH SUGAR: This will consist of adding 42g/L to the drinking water (55% fructose/45% sucrose).
DIO: Diet will consist, from calories, 60% fat, 20% protein and 20% carbohydrate (Dyets Inc, Bethlehem PA). Genistein supplement (600 mg genistein/kg diet) will added into the DIO diet (Dyets Inc. Bethlehem, PA
EXERCISE: Training will consist of low intensity performed on a treadmill. Duration will 30 min/day 5 days/week (Mon-Fri) for the duration of the study. Intensity of running will be moderate (10 m/min) which is equivalent to the guidelines provided by the American Heart Association for moderate exercise level and duration.

At the end of the 4-month treatment period, mice will be euthanized by CO_2 gassing followed by cervical dislocation. The following will be performed:

1. Serum measures.
2. Protein expression (western blot) in the brain
3. Bone measures for bone health
4. Gut proteins/fecal measures.

b. The individual protocols utilizing animals. All experimental protocols performed in live animals should be described in detail, including

where the procedure or protocol will be performed, the duration of each protocol and the animal handling after the protocol, etc. A table(s) detailing the animal groups, based on experimental procedures, along with numbers required is strongly suggested. Additionally, provide a succinct description of protocols utilizing tissues harvested from euthanized animals. This succinct description should clearly justify the use of the animals for these protocols.

Species	Strain	SEX ~~Gender~~	Group	# To Be Used
Mouse	C57BL/6J	Male/Female	Control	20
Mouse	C57BL/6J	Male/Female	DIO	20
Mouse	C57BL/6J	Male/Female	DIO + genistein	20
Mouse	C57BL/6J	Male/Female	DIO + exercise	20
Mouse	C57BL/6J	Male/Female	DIO + genistein + exercise	20

c. Teaching/Clinical Applications: Please provide information on: 1. if animals will be transported by personnel other than their owner, 2. if the animals will be hospitalized during the course, 3. if animals will expose humans to potential zoonosis or risks associated with animal exposure, 4. if special housing requirements or restrictions will be necessary, 5. if special diets or dietary restrictions are necessary, and 6. if animals will be returned to their owners following their participation in the course/activity. Please attach informed consent forms and copies of any documents being used for recruitment.

Pertaining to point 5:
See groups in the above table. No dietary restrictions. However, mice (see groups above) will be fed a diet high in fat to induce diabetes and obesity (consisting of 60% fat, 20% protein, 20% carbohydrate) leading to the typical phenotype associated with eating fat

(obesity, visceral obesity, decreased exercise capacity, hyperglycemia, hyperinsulinemia, IR, etc.)

In some of the groups mentioned in the above table, mice will also be given genistein. Genistein will be incorporated into the high fat diet.

II. Provide the rationale for the appropriateness of the species selected and a justification of the number of animals to be used. Justification for numbers of animals must include power analyses unless sufficient justification is provided for not performing a power analysis.

Animal model of study. Male and female mice of the C57BL/6J are selected because they represent a relevant model for the study of DIO and the development of cognitive impairment. Mice fed a diet high in fat and sugar (fructose/sucrose) develop insulin resitance in the hypothalamus, decreased synaptic plasticity, increased amyloid beta deposition and altered cognitive function. Therefore, this model is representative model of the human condition.

Justification for the number of animals.
Since this project has not begun, there are no data to run a power analysis. However, extrapolating from our earlier exercise and diet studies, a minimum ample size of 7 mice/group would be needed for this MAAC-funced project to achieve a level of significance. This sample size also considers the slight variability of mice within the groups to the stimulus of exercise (i.e. some mice respond better than others to exercise).

III. Describe the procedures for ensuring that discomfort, distress, pain and injury will be limited to that which is unavoidable in the conduct of scientifically sound research. Describe the use of analgesic, anesthetic and tranquilizing drugs and/or comfortable restraining devices (where appropriate) to minimize discomfort, distress, pain and injury.

Euthanasia: Mice will be gassed with CO_2, and then subjected to cervical dislocation after treatment. Tissues and plasma will be harvested. In the event mice become ill or injured during the exercise session or with treatment, they will be euthanized in the same fashion.

IV. If chemicals, toxins, radiologic/radiation-emitting material or pathogenic organisms are to be administered to live animals, describe the route of administration, the viability of any pathogenic organism, the care

and handling of the treated animals, the administration precautions/safety requirements, and the procedures to be followed by personnel caring for the treated animals. In addition, provide a statement describing the toxicity/health hazard/exposure limit and radiation/biological half-life of the chemicals or toxins and or the pathogenicity of the organism. State any possible risks to other facility animals and personnel. Provide information regarding the proper disposal of the carcasses.

Genistein, being a naturally-occurring phytoestrogen, is well-tolerated by mice. Other than accelerating the risk of developing obesity and associated cardio-metabolic, -vascular dysfunction, a diet high in fat is also well-tolerated by mice.

V. Describe the activities that involve survival surgery including provisions for pre- and post-operative care. Provide the following information:

- **aseptic techniques that will be employed**
- **a brief description of each of the surgical procedures employed**
- **the housing and care of the animal after surgery**

Note: Some survival surgical procedures involving rodents may be performed outside the Animal Facility with committee approval provided that aseptic technique can be assured, and all pre- and post-surgical care is acceptable. *However, the investigator's laboratory will then be defined as a Study Area which is subject to evaluation by the IACUC on a semiannual basis.* No animal may be involved in more than <u>one</u> operative procedure from which it is allowed to recover unless justified for scientific reasons.

N/A

VI. Describe non-surgical protocols or procedures that require the use of analgesic, preanesthetic and/or anesthetic agents, including the care and handling of the animal after the procedure.

N/A

VII. In the fields below, provide a description of the methods and sources consulted to determine whether or not alternatives exist for: 1) this application in general, AND 2) for procedures which may cause more than momentary or slight pain or distress. ***Please note: A painful procedure is defined by the USDA as "any procedure that would reasonably be expected to cause more than slight or momentary pain and/or distress in a human being..." Acceptable methods of euthanasia are not considered painful.***

The minimal information should include the databases searched AND/OR other sources consulted (e.g. a professional organization, such as the AMA, or a veterinarian), the date of the database search, the years covered by the search, the major key words and/or search strategy used, and the findings of the search. The information should be such that the IACUC can readily assess whether the search topics or other sources consulted were appropriate and whether the database search was sufficiently thorough. If an alternative exists but is not used, provide specific scientific justification for the decision.

Databases searched: PubMed

Date of search (years covered in the database: October 2 – 2017

Key words: exercise, genistein supplementation, Alzheimer's

Findings of search: As expected, when either of the key words were individually enter, hundreds of citations were generated. When all three key words, only one entry from the "Medical Hypothesis" Journal (Elsevier) authored by McCarthy appeared. The conclusion was that "lifestyle" approach combining diet/exercise should be evaluated. Therefore, no alternative methods exist.

VIII. Provide information on the veterinary care of the animals involved, noting any special requirements for housing, cage cleaning, watering, etc. ***Please note that all research animals must be housed in the Animal Facility. If they are to be housed in the investigator's laboratory for more than 12 hours, prior approval is required by the IACUC. Furthermore, the laboratory will be designated as a Study Area subject to semiannual inspection by the IACUC.***

No special care is required and mice will be cared for according to recommendations of the NIH. Mice will be housed at the Foothills vivarium and evaluated for daily signs of illness Mice will be closely monitored for signs of distress as well and Drs. Broderick and Al-Nakkash will monitor the mice after the implanatation of the genistein-release pellets. Water and food ad libitum will be provided under standard conditions: 12 hour light-dark cycle, room temperature at ~22-23°C. Exercise training will be conducted under Dr. Tom Broderick's supervision in his lab (137). Following the treatment protocol, all mice will be euthanized in laboratory 137 of the Foothills Science Center. Any concerns relating to animal care will be immediately discussed with Tim Martin, DVM

IX. Describe any euthanasia method that will be used and the reasons for their selection. If the method of euthanasia is not consistent with the most current recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (see ORSP website or http://www.avma.org for current guidelines), provide scientific justification for it. and/or anesthetic agents, including the care and handling of the animal after the procedure.

Mice will be gassed with CO_2, followed by cervical dislocation. This method is quick and efficient and is approved by the Panel on Euthanasia of the American Veterinary Medical Association. Tissues and plasma will be harvested. In the event mice become ill or injured during the exercise session (which has never been the case), they will be euthanized in the same fashion.

Principal Investigator

☒ I certify that the activities described above do not unnecessarily duplicate previous experiments and are consistent with animal welfare regulations and standards for animal use described by the USDA (9CFR Parts I, II, III) and by the PHS (Guide for the Care and Use of Laboratory Animals).

☒ I agree that any significant changes in my ongoing research protocol will be submitted to the IACUC for appropriate review and action.

☒ In addition, I will ensure that all personnel involved in animal use related to this protocol have been educated as to the health risks entailed and have been offered participation in the Animal User Occupational Health Program of MWU. (A copy of the Animal User Occupational Health Program is available for review in the Office of Research and Sponsored Program Office).

☒ I certify that appropriate pain-relieving drugs have been or will be used throughout the activity to relieve pain or distress whenever it occurs, including postoperative or post procedural care and immediate treatment of unexpected injuries.

☒ I certify that all personnel performing any procedures on animals are qualified and trained to perform such procedures, UNLESS OTHERWISE STATED & JUSTIFIED IN THE PROTOCOL (i.e. students are being trained to perform the procedures).

Layla Al-Nakkash

10/30/17

Principal Investigator Date

Tom Broderick

10/30/17

Principal Investigator Date

Course Coordinator Date

Upon notification of action by the Institutional Animal Care and Use Committee, you are authorized to place orders for animals though the Animal Ordering Process. Orders will be placed provided that all personnel have been trained and that caging and animal care can be provided.

10-30-17

Date

John VandenBrooks

Approved

IACUC CHAIR

APPENDIX B

MUSCLE HOMOGENIZING BUFFER FOR CITRATE SYNTHASE

Reagents and instruments

Reagent	Company (product number)	Instrument	Company (product number)
Bovine Serum Albumin 10% (BSA)	Fisher Bioreagents 100g (BP1605-100)	**Pipettes 5 ul – 5 ml**	Thermo Scientific F1-ClipTip™ Variable Volume Single Channel Pipettes
Ethylenediamine Tetraacetic acid (EDTA) 14.3 mol	Fisher 100g (S311-100)	**stir and heating plate**	VWR® Professional Stirrers, 230V
Glycerol	Fisher Bioreagents 1L (BP229-1)	**Scale**	Sartorius Secura® Analytical Balance 220 g x 0.1 mg
K_2HPO_4	MP Biomedics, LLC 500 mg (151945)	**Stainless steel lab spatula**	
KOH	Fisher chemical 500 g (P250-500)	**Weighing paper**	VWR® Weighing Paper (12578-121)
2-Mercaptoethanol	Fisher 100 ml (O3446I-100)	**pH meter**	Fisherbrand™ accumet™ AE150 Benchtop pH Meter
Ultra-pure H_2O		**Bickers 50 – 100 ml**	
		Parafilm	Parafilm M All Purpose Laboratory Film (PM992)
		Graduated cylinder 50 ml	
		50 ml tubes	
		100 ml glass bottle	

Protocol

1. Calibrate the pH meter: We will need to calibrate the pH meter following manufacturer instructions. Once we calibrate the pH meter, we will start weighting the reagents for the buffer.
2. Prepare KOH 1 M and 0.5 M solutions by dilution in 50 ml tubes.

3. Pipette into a bicker 1 ml of the K_2HPO_4, a potassium salt and an inorganic phosphate used to maintain a constant pH.
4. Prepare 2-mercaptoethanol stock: Pipette under a security hood .7 ml of 14.3 mol 2-mercaptoethanol into a bicker. Add 9.3 ml of ultra-pure H_2O for a final volume of 10 ml.
5. Weight 1 g from 10% BSA and dissolved in 8 ml of ultra-pure water on the stir-heated at plate. Bring to final volume of 10 ml.
6. Prepare EDTA stock: weight 0.931 g of EDTA on the scale and dissolved in 25 ml of ultra-pure H_2O using the stir-plate (no heat needed). Add 3 drops of 1 M K_2HPO_4 for buffering and help dissolving. The EDTA is used as a protease inhibitor.
7. Using a graduated cylinder measured 25 ml of glycerol.
8. Once all the reagents are all measured and pre-weighted, we will mix them in a 100 ml bicker. Leave the glycerol for the end. Once we add the glycerol we will measure the pH. We will use KOH to increase the pH to 7.34 or to reduce the pH with HCL to pH of 7.34.
9. Save the buffer in a glass 100 ml glass container in the freezer -20 ^{o}C.

APPENDIX C

CITRATE SYNTHASE ASSAY

Reagents and instruments

Reagent	Company (product number)	Instrument	Company (product number)
Tris base 1 kg	Fisher BP152-1	**Pipettes 5 ul – 5 ml**	Thermo Scientific F1-ClipTip™ Variable Volume Single Channel Pipettes
DTNB	Alfa aesar (A14331)	**Scale**	Sartorius Secura® Analytical Balance 220 g x 0.1 mg
Oxalacetic acid 98%	Sigma (O-4126)	**Stainless steel lab spatula**	
Acetyl-CoA	Sigma (A2056 or A2181)	**Weighing paper**	VWR® Weighing Paper (12578-121)
Ultra-pure H_2O		**pH meter**	Fisherbrand™ accumet™ AE150 Benchtop pH Meter
HCl	Fisher Scientific (A466-250)	**Bickers 50 – 100 ml**	
		50 ml tubes	
		100 ml glass bottle	
		Spectrophotometer	Beckman Coulter DU 7830
		2.5 ml cuvettes	PMMA (759080D)
		Ice bath	
		Parafilm	Parafilm M All Purpose Laboratory Film (PM992)
		wipes	Kimberly-Clark Professional™ Kimtech Science™ Kimwipes™ Delicate Task Wipers (06-666)

Protocol

1. Prepare the Tris buffer 100 mM, pH 8.3. Keep it in the refrigerator or freezer – 20ºC.

a. Calculate the number of moles of Tris that are required for the volume and mM that you decided. In this case 1 L and 100 mM.
b. Determine grams of Tris. Molecular weight of Tris = 121.14 g/mol. 121.14 x 0.1 = 12.114 g of Tris.
c. Dissolve the Tris (12.114 g) into the distilled water, 1/3 to ½ of your desire volume (1 L). 0.333 L to 0.500 L
d. Mix in HCl (e.g. 1 M HCl) until pH meter give you the desired final pH solution, pH 8.3
e. Dilute the buffer with water to reach the desired final volume (1L).

2. Weight the reagents
    a. 1mM DTNB: Weight 4 mg of DTNB in the scale and dissolve in 10 ml of 100 mM tris buffer, pH 8.3. Kept this reagent in a dark bottle or wrap the falcon tube with foil paper and stored in the refrigerator for a couple of days.
    b. 10 mM Oxaloacetate: Dissolve 13.2 mg of Oxaloacetate in 10 ml of 100 mM tris buffer, pH 8.3. Requires to be prepare fresh daily.
    c. 3 mM Acetyl-CoA: Dissolve 3.1 mg of Acetyl-CoA in 1 ml of ultra-pure water. Prepare fresh daily.
3. Procedure
    a. The homogenized samples thawed them on the ice bath or bucket with ice.
    b. Centrifugate the homogenate for 5 minutes at 0.5 g^{-1} at 4 Celsius degrees.

c. Set the spectrophotometer to 412 nm, zeroing with a reagent cocktail (all the reagents without a sample + homogenizing buffer) without the sample. The enzyme should appear plateau in the spectrophotometer.

d. Prepare triplets of the reagent cocktail per sample. Average the three samples absorbance to calculate the enzyme activity. Pipette 5-50 microL of tissue homogenate into the 1 ml cuvette of reagent cocktail. Make sure that you only pipette the sample just before doing the reading. Mix gently inversion (e.g., on a piece of parafilm). Place the cuvette in the spectrophotometer and start a timer.

e. Follow the change in absorbance for 2-4 minutes, recording the change every 15-30 seconds. After a short lag time, the enzyme will reach its maximal velocity and the change in absorbance will remain constant for a couple of minutes. It is this period of maximal steady activity that is used in the calculations. Some troubleshooting and adjusting of the sample volume may be necessary to find a velocity which is neither too slow nor too fast. If too little enzyme is put into the system, very little change will be detected, and the change will probably be inconsistent. If too much enzyme is put into the system, the enzyme will overpower the system, producing a large change in absorbance at first and then dying off to ever smaller changes as the reaction proceeds. We usually look for absorbance changes of 0.02-0.04 units every 15 seconds.

f. Determine the average change in absorbance per minute after the reaction has reached its maximal steady state. Calculate the results on the basis of the molar extinction coefficient for DTNB at 412 nm (13,600) and the dilutions of the tissue, firs in the homogenate and then in the assay volume.

Δabs/min / 13.6 x Dilution factors muscle x volume in the cuvette/muscle weight = micromole/min/g

Example
Change in absorbance every 15 sec 0.03
Change in absorbance per minute 0.12
Dilution in muscle homogenate 1:20
Volume of muscle homogenate used 5 microl
Dilution of muscle in assay volume 201

12 / 13.6x20x201

Where 201 comes from 1005 (1 ml of cocktail + 5 ml of the muscle)/5 micro l that you are using as volume of muscle homogenate.

Expected values

Rat tissue

- Heart 125 micromol/min/g
- Plantaris 10-30 micromol/min/g
- Soleus 20-35 micromol/min/g
- White vastus 10-15 micromol/min/g

Human muscle, mixed:

- Untrained 10-20 micromol/min/g
- Trained 30-60 micromol/min/g

APPENDIX D

QIIME 2 CODE

#Before running QIIME2 make sure that you are using the lasted version at qiime2.org#

#Open a terminal window and make sure to know in which directory currently are you#

ls

#are you in the folder where your fastq files are? If you are you need to activate Qiime2#

source activate qiime2-2019.1

#if you are not in the fastq files directory you need to move to the fastq file folder#

cd directory name

#You need to know if your sequence files (fastq) are multiplexed (sequences ha not yet been assigned to samples) or demultiplexed (samples are been assigned to samples). There are different types of FASTQ files and each one requires a different import protocol. In this project the files are CASAVA 1.8 demultiplexed format.
File name format 1_44_L001_R1_001.fastq.gz
Therefore, we will skip the demultiplex step.#

#importing fastq files#

```
qiime tools import \
  --type 'SampleData[PairedEndSequencesWithQuality]' \
  --input-path fastq\  #name of the folder where your fastq files are#
  --input-format CasavaOneEightSingleLanePerSampleDirFmt \ #do not change this#
  --output-path fastqSoy.qza #you can give the name that you want to your output folder#

#visualize your imported data#
qiime demux summarize \
  --i-data fastqSoy.qza \
  --o-visualization fastqSoy.qzv
```

#visualize your file in qiime.view website. Use the tab interactive quality plot to visualize the quality of each sample before denoting your data.
Look to the median quality score (q20-q25 should be fine). Analyze the plot, look for any peak drop in the quality score. Thats should be case where you will need to trim.

Write down the sequence base where the drop of the peak score happens for the next step.#

#quality control with DADA2 DADA2 is a pipeline for detecting and correcting (where possible) Illumina amplicon sequence data. As implemented in the q2-dada2 plugin, this quality control process will additionally filter any phiX reads (commonly present in marker gene Illumina sequence data) that are identified in the sequencing data, and will filter chimeric sequences.
#

```
qiime dada2 denoise-single \
  --i-demultiplexed-seqs fastqSoy.qza \
  --p-trim-left 0 \
  --p-trunc-len 251 \
  --o-representative-sequences fastqSoy-dada2.qza \
  --o-table table-dada2.qza \
  --o-denoising-stats stats-dada2.qza


#create a visualization of the output created#
qiime metadata tabulate \
  --m-input-file stats-dada2.qza \
  --o-visualization stats-dada2.qzv

#create a feature table. The output of the first code will provide us with the sampling depth necessary for the alpha and beta diversity analysis#
qiime feature-table summarize \
  --i-table table-dada2.qza \
  --o-visualization table.qzv \
  --m-sample-metadata-file SoyExeMetadata.txt
qiime feature-table tabulate-seqs \
  --i-data fastqSoy-dada2.qza \
  --o-visualization fastqSoy-dada2.qzv

#create a phylogenetic tree#
qiime phylogeny align-to-tree-mafft-fasttree \
  --i-sequences fastqSoy-dada2.qza \
  --o-alignment alignedfastqSoy.qza \
  --o-masked-alignment maskedfastqSoy.qza \
  --o-tree unrooted-treeSoy.qza \
  --o-rooted-tree rooted-treeSoy.qza
```

#diversity analysis: alpha and beta diversity. We chose the sampling depth 15247. We kept all the samples with a sequence count >15,000#
qiime diversity core-metrics-phylogenetic \
  --i-phylogeny rooted-treeSoy.qza \
  --i-table table-dada2.qza \
  --p-sampling-depth 15247 \
  --m-metadata-file SoyExeMetadata.txt \
  --output-dir core-metrics-results

#alpha diversity significance#
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-results/faith_pd_vector.qza \
  --m-metadata-file SoyExeMetadata.txt \
  --o-visualization core-metrics-results/faith-pd-group-significance.qzv

qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-results/evenness_vector.qza \
  --m-metadata-file SoyExeMetadata.txt \
  --o-visualization core-metrics-results/evenness-group-significance.qzv

#beta diversity significance#
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file SoyExeMetadata.txt\
  --m-metadata-column sex \
  --o-visualization core-metrics-results/unweighted-unifrac-body-site-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file SoyExeMetadata.txt \
  --m-metadata-column sex \
  --o-visualization core-metrics-results/unweighted-unifrac-subject-group-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance --i-distance-matrix core-metrics-results/jaccard_distance_matrix.qza --m-metadata-file SoyExeMetadata.txt --m-metadata-column sex --o-visualization core-metrics-results/jaccard-distance-matrix.qzv

qiime diversity beta-group-significance --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza --m-metadata-file SoyExeMetadata.txt --m-metadata-column sex --o-visualization core-metrics-results/bray-curtis-matrix.qzv

```
qiime diversity beta-group-significance  --i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza --m-metadata-file SoyExeMetadata.txt --m-metadata-column sex --o-visualization core-metrics-results/weighted-unifrac-subject-group-significance.qzv


#plot the results#
qiime emperor plot \
  --i-pcoa core-metrics-results/unweighted_unifrac_pcoa_results.qza \
  --m-metadata-file SoyExeMetadata.txt \
  --p-custom-axes PreWeight  \
  --o-visualization core-metrics-results/unweighted-unifrac-emperor-PreWeight.qzv

qiime emperor plot \
  --i-pcoa core-metrics-results/bray_curtis_pcoa_results.qza \
  --m-metadata-file SoyExeMetadata.txt \
  --p-custom-axes PreWeight \
  --o-visualization core-metrics-results/bray-curtis-emperor-PreWeight.qzv


#taxonomy analysis#
qiime feature-classifier classify-sklearn \
  --i-classifier gg-13-8-99-515-806-nb-classifier.qza \
  --i-reads fastqSoy-dada2.qza \
  --o-classification taxonomy.qza

qiime metadata tabulate \
  --m-input-file taxonomy.qza \
  --o-visualization taxonomy.qzv

qiime taxa barplot \
  --i-table table-dada2.qza \
  --i-taxonomy taxonomy.qza \
  --m-metadata-file SoyExeMetadata.txt \
  --o-visualization taxa-bar-plots.qzv

#organize the metadata file per group instead of per samples#
qiime feature-table group --i-table table-dada2.qza --p-axis sample --m-metadata-file SoyExeMetadata.txt --m-metadata-column Group --p-mode median-ceiling --o-grouped-table table-group.qzv --output-dir table-results


#create a taxa plot per group#
```

qiime taxa barplot --i-table table-group.qzv.qza --i-taxonomy taxonomy.qza --m-metadata-file SoyExeMetadata.txt --o-visualization taxa-bar-plots-group.qzv

APPENDIX E

R CODE

```
###DISSERTATION STATISTICAL ANALYSIS
#Setworking directory
setwd("~/Dropbox (ASU)/Dissertation/Results")
#load the metadata document
library(readxl)
metadata = read_excel("metadata_GM.xlsx")
#if you want to visualize the names of the variables
#names(metadata)

###Descriptive stastistics#####
#body weight, heartweight, liver, kidney, and adipose. glucose, insluin and IL6 levels
#Check for normality distribution
normality_test = lapply(metadata,5, shapiro.test)

#not possible to transform. Run non-parametric statistical analysis for glucose, insulin, body weight
#following the next tutorial https://rcompanion.org/handbook/F_08.html
if(!require(psych)){install.packages("psych")}
if(!require(FSA)){install.packages("FSA")}
if(!require(lattice)){install.packages("lattice")}
if(!require(multcompView)){install.packages("multcompView")}
if(!require(rcompanion)){install.packages("rcompanion")}

#Kruskal-wallis PostWT differences between groups.
kruskal.test(PostWeight~Group, data = metadata) #kruskal wallis p<0.05. Signifcantly differences
boxplot(PostWeight~Group, data = metadata, main = "Weight gain", xlab = "Group", ylab = "g") #visualization

#Post-hoc test: Dunn test for multiple comparisons of groups
DT = dunnTest(PostWeight~Group, data = metadata, method = "bh")
write.table(DT, file = 'DT_WTgain.csv', sep = ",", row.names = F, col.names = T)
        #we found differences between HFD + Exe + Gen vs. HFD, vs. HFS + Gen vs. HFD + Exe
        #No differences HFD vs. HFD + Exe, HFD + Gen. HFD + Exe vs. HFD + Gen. HFD + Exe + Gen vs. CONTROL
describeBy(metadata$PostWeight, metadata$Group)


####AIM 1:GM output from QIIME2 format
setwd("~/Dropbox (ASU)/Dissertation/Results/QIIME2/core-metric-results/")
#######tutorial used from the following link https://forum.qiime2.org/t/tutorial-integrating-qiime2-and-r-for-data-visualization-and-analysis-using-qiime2r/4121
library(readxl)
```

```
library(qiime2R) #package to transfer qiime2 artifacts to R
library(tidyverse)
#upload the metadata
metadataq = read_q2metadata("metadata.tsv")
metadata = read_excel("metadata_variables.xlsx")
#merge the metadata with the sequence ID with the metadata with the group and other
variables
metadata = metadata %>% left_join(metadataq)
head(metadata)
view(metadata)
#upload the qiime artifacts that we will be plotting
rarefication = read_qza("rarefied_table.qza")
OTU = read_qza("observed_otus_vector.qza")
faithpd = read_qza("faith_pd_vector.qza")
pielous = read_qza("evenness_vector.qza")
chao1 = read_qza("chao1.qza")
shannon = read_qza("shannon_vector.qza")
#move the samples names into a new column in each of the outputs so we can merge all
of them into the metadata file
OTU = OTU$data %>% rownames_to_column("SampleID")
faithpd = faithpd$data %>% rownames_to_column("SampleID")
pielous = pielous$data %>% rownames_to_column("SampleID")
chao1 = chao1$data %>% rownames_to_column("SampleID")
shannon = shannon$data %>% rownames_to_column("SampleID")

##check if the # of samples in the metadata match with the ones in the different metrics
gplots::venn(list(metadata=metadata$SampleID, chao1=chao1$SampleID)) # ==
gplots::venn(list(metadata=metadata$SampleID, faithpd=faithpd$SampleID)) # +2
samples in the metadata
gplots::venn(list(metadata=metadata$SampleID, pielous=pielous$SampleID)) # +2
samples in the metadata
gplots::venn(list(metadata=metadata$SampleID, OTU=OTU$SampleID)) # +2 samples
in the metadata
gplots::venn(list(metadata=metadata$SampleID, shannon=shannon$SampleID)) # +2
samples in the metadata

#merge to the metadata all the values so we can run the analyses and plot the results
based on groups
metadata = metadata %>% left_join(OTU)
metadata = metadata %>% left_join(pielous)
metadata = metadata %>% left_join(faithpd)
metadata = metadata %>% left_join(chao1)
metadata = metadata %>% left_join(shannon)
View(metadata)
```

```
###Figures GM
#bar plots of the relative frequencies of the taxonomy at level 2 (phylum level)
https://forum.qiime2.org/t/creation-of-barplot-with-excel/17245/13
#Load the packages required and set up the working directory
library(tidyverse)
library(RColorBrewer)
library(cowplot)
library(qiime2R)
library(MicrobeR)
library(readxl)
#Working directory
setwd("~/Documents")
##load all the required objects from qiime2 feature table, metadata, taxonomy
#Feature table
table = read_qza("table-dada2.qza")
count_tab = table$data %>% as.data.frame()
#Metadata
metadata = read.delim("~/Documents/metadata.txt", header=TRUE)
metadata = metadata %>% rename(SampleID = "sample_id") #use SampleID as sample
identifier

#Taxonomy
taxonomy = read_qza("taxonomy.qza")
tax_tab = taxonomy$data %>% as.data.frame() %>%
separate(Taxon, sep = ";", c("Kingdom", "Phylum", "Class", "Order","Family", "Genus",
"Species")) %>% column_to_rownames("Feature.ID") %>% select(-Confidence)

#Colllapse feature table at phylum level
tab_phy = Summarize.Taxa(count_tab, tax_tab)$Phylum %>% #the following 4 lines of
code prune the taxonomy to cotain phylum names only
  rownames_to_column("tax") %>% filter(!grepl("NA", tax)) %>% #remove taxa without
kingdom or phylum level annotation
  mutate(tax =gsub("\\[|\\]", "", tax), #remove square brackets
         tax=gsub("k_.*p_", "", tax)) %>%
  column_to_rownames("tax")
#Plot taxa using group means
taxa_barplot(features = tab_phy, metadata = metadata, category = "group", normalize =
"percent", ntoplot = 7) + ylab("Relative Frequencies %") +ggsave("relative
frequencies.pdf", height = 4, width = 11, device = "pdf")

####beta-diversity PCoA plots
#Jaccard
jaccard = read_qza("jaccard_pcoa_results.qza")
```

```
jaccard$data$Vectors %>% select(SampleID, PC1, PC2) %>% left_join(metadata)
ggplot(aes(x=PC1C2, color=group)) + geom_point(alpha=7) + scale_fit_brewer(palette
='GnBu')

##BOXPLOTS for alpha diversity
setwd("~/Dropbox (ASU)/Dissertation/Results/QIIME2/alpha diversity tables")
chao1 = read_excel("chao.xlsx")
ggboxplot(chao1, x="Group", y="chao1", color="Group", palette ="Dark2", xlab =
"Group", ylab = "Chao1 index", bxp.errorbar = TRUE, bxp.errorbar.width = 0.04,
font.label = list(size=30)) + theme(text = element_text(size = 16))

otu = read_excel("otus.xlsx")
ggboxplot(otu, x="Group", y="observed_otus", color="Group", palette ="Dark2", xlab =
"Group", ylab = "OTUs", bxp.errorbar = TRUE, bxp.errorbar.width = 0.04, font.label =
list(size=30)) + theme(text = element_text(size = 16))

shannon = read_excel("shannon.xlsx")
ggboxplot(shannon, x="Group", y="shannon", color="Group", palette ="Dark2", xlab =
"Group", ylab = "Shannon index", bxp.errorbar = TRUE, bxp.errorbar.width = 0.04,
font.label = list(size=30)) + theme(text = element_text(size = 16))

pielou = read_excel("p.xlsx")
ggboxplot(pielou, x="Group", y="pielou_e", color="Group", palette ="Dark2", xlab =
"Group", ylab = "Pielou evenness", bxp.errorbar = TRUE, bxp.errorbar.width = 0.04,
font.label = list(size=30)) + theme(text = element_text(size = 16))

faith = read_excel("faith.xlsx")
ggboxplot(faith, x="Group", y="faith_pd", color="Group", palette ="Dark2", xlab =
"Group", ylab = "Faith pd", bxp.errorbar = TRUE, bxp.errorbar.width = 0.04, font.label =
list(size=30)) + theme(text = element_text(size = 16))


########AIM 2: CS and B-HAD############
##normality testing
library(ggpubr)
library(dplyr)
library(e1071)
library(readxl)
#upload the metadata. Make sure that you set up the path to the folder with the resuls of
aim 2.
setwd("~/Dropbox (ASU)/Dissertation/Results/CS and B-HAD")
metadata <- read_excel("metadata.xlsx")
shapiro.test(metadata$cs) #p=0.1423 data is normally distributed
skewness(metadata$cs, na.rm = T) #skewness = 0.64 slightly skewed to the right
```

```
kurtosis(metadata$cs, na.rm = T) #kurtosis=0.0344 <3 platykurtic. data are light-tailed or lack of outliers.

###Create the model first
model = lm(cs~group + sex + group:sex, data = metadata) #adjusting for sex and testing for interaction between sex and group.
summary(model)
#there is no significant differences among groups. And sex is driving the difference p<0.05.
#F statistics is quite low (what we want is to be as far as possible from 1) F=1.562 9,39
#R2, sex is exlaining 9.53% of the variance, which is low.

#Check the residuals plots (residuals should be center on zero) and normality.


###Regression asumptions. Here more information about the steps: shorturl.at/mnxI2
##Assumption 1: linearity of the data. Multiple linear regression requires the relationship between
#   the independent variable and depdent variables to be linear. The linearity assumption will be tested with scatterplots.

##Assumption 2: Normality of the data. erors between observed and predicted values (residuals) should be normally distributed
#    Check with a Q-Q plot.
##normality testing for the results
qqPlot(metadata$residuals, col.lines = "red", cex = 1, ylab = "Residuals", xlab = "Quantiles") #four graphics will appear in the plot tab. Q-Q plot are of interest.
#there is a more or less strainght line with the residuals.
#   Therefore, we conclude that the residuals are normally distributed.
shapiro.test(metadata$residuals) #not normally distributed. the p<0.05

##Assumption 3: No multicollinearity in the data. Independence of the IV. Check by Variance Inflation Factor (VIF) and correlation matrix.
# when VIF is > 10 we assume there is multicollinearity, therefore, we can continue with the model build.
library(car)
car::vif(model) #our model have a value of VIF = 1. We assume no multicollinearity.
#correlation
required_packages <- c('MASS', 'rcompanion', 'lsr', 'vcd', 'DescTools')
for (p in required_packages) {
  if(!require(p,character.only = TRUE)) {
    install.packages(p, dep = TRUE)
  }
}
```

```
ContCoef(metadata$group, metadata$sex) #check the correlation between the two IV in the model.
# the correlation coefficient is 0.04299336


##Assumption 4: Homoscedasticity. Variability of the residuals needs to be small across all the IV.
#   check by a sactterplot of residual vs. predicted values.
#residuals = the observed value and the mean value that the model predicts for that observation.
#observed values in the model are called fitted values. Residuals are named residuals in the model.
#Save the residuals and the fitted values in the metadata as new columns
metadata$residuals = model$residuals
metadata$fitted = model$fitted.values
#check the three values, actual values, fitted, and residuals
metadata %>% select(cs,fitted, residuals) %>% head() #the residuals do not look good. high numbers

plot(model, 1)
#the line should be a straight line close to zero.
# there is a line close to zero, but its not a strainght line, its slightly curved.
# But the values are randomly distributed throughout the plot.



###plot in bar a graph bar the mean and standard deviation.
library(psych)
library(tibble)
descriptives = describeBy(metadata$cs, metadata$group)
descriptives
control_des = as.data.frame(descriptives$CONTROL)
HFD_des = as.data.frame(descriptives$HFD)
HFDExe_des = as.data.frame(descriptives$`HFD +Exe`)
HFDGen_des = as.data.frame(descriptives$`HFD+Gen`)
HFDExeGen_des = as.data.frame(descriptives$`HFD+Exe+Gen`)
descriptives_group = rbind(control_des, HFD_des, HFDExe_des, HFDGen_des, HFDExeGen_des)
descriptives_group = descriptives_group %>%
add_column(group=c("control","HFD","HFD + Exe", "HFD + Gen", "HFD + Exe + Gen"), .before = "vars")

descriptives_sex = describeBy(metadata$cs, metadata$sex)
descriptives_sex
```

```
male_des = as.data.frame(descriptives_sex$Male)
female_des = as.data.frame(descriptives_sex$Female)
descriptives_plot = rbind(male_des, female_des)
descriptives_plot = descriptives_plot %>% add_column(sex= c("Male", "Female"),
.before = "vars")

##plot the results
library(ggplot2) #plot figure packages
sex_cs = ggplot(data=descriptives_plot, aes(x=sex, y=mean)) +
  geom_errorbar(aes(x = sex, ymin=(mean-sd), ymax=(mean + sd), width = 0.5)) +
geom_bar(stat = "identity", fill = c("Female"="navy blue", "Male"="light blue"))
#important write first the error bar code and then the bar plot, the errorbar will anked to
the bar.
sex_cs = print(sex_cs + labs(y="Citrate Synthase (umol/min/g)", x ='Sex') + labs(fill =
"Sex")) + theme(axis.title = element_text(size = 16, face = "bold"), axis.text.x =
element_text(size = 14))
sex_cs

group_cs = ggplot(data = descriptives_group, aes(x=group, y=mean)) +
geom_errorbar(aes(x = group, ymin=(mean-sd), ymax=(mean+sd), width =0.5)) +
geom_bar(stat = "identity")
group_cs

####Testing indepenent effect of each treatment and treatment interaction. Does exe, gen,
and their combined effect have an effect on CS?
##We already compare against control. They do not prevent the HFD-induced CS
decline.
#load the packages necessary to run the regression model
library(readxl)
setwd("~/Dropbox (ASU)/Dissertation/Results/CS and B-HAD")
#upload the metadata file
metadata <- read_excel("metadata.xlsx")
###Create the model first
model = lm(cs~sex + exe + gen + exe:gen, data = metadata) #adjusting for sex.
summary(model)
#no correlation between the CS and GM richness
mediation_2 = lm(cs~richness, data = metadata)
summary(mediation_2)

##test the differences in gain weight among the groups adjusting for  sex
setwd("~/Dropbox (ASU)/Dissertation/Results")
library (readxl)
metadata_w = read_excel("metadata_w.xlsx")
##Adipose_WT is not normally distributed
```

```
#running kruskal-wallis
kruskal = kruskal.test(Adipose_Wt~Group, data = metadata_w)
pairwise.wilcox.test(metadata_w$Adipose_Wt, metadata_w$Group, p.adjust.method =
"BH")
#There are not significant differences between Exe + Gen and control.
#The rest are sign different to each other.
##therefore, adipose tissue it might play a role.

#what about the liver weight
kruskal_liver = kruskal.test(Liver_Wt~Group, data = metadata_w)
kruskal_liver
pairwise.wilcox.test(metadata_w$Liver_Wt, metadata_w$Group, p.adjust.method =
"BH")

#what about the liver weight
kruskal_heart = kruskal.test(Heart_Wt~Group, data = metadata_w)
kruskal_heart
pairwise.wilcox.test(metadata_w$Heart_Wt, metadata_w$Group, p.adjust.method =
"BH")

#il6. Not significnatly different among groups.
kruskal_il6 = kruskal.test(SiL6~Group, data = metadata_w)
kruskal_il6
pairwise.wilcox.test(metadata_w$SiL6, metadata_w$Group, p.adjust.method = "BH")

#anova for the adipose tissue weight
anova_at = aov(Adipose_Wt~Group, data = metadata_w)
summary(anova_at)
TukeyHSD(anova_at)
##Results
#There is significant differences between HFD vs. Control, HFD + Exe vs. Control, HFD
+ Gen vs. Control
#descriptives for adipose tissue weight
metadata_w %>% group_by(Group) %>% summarize(mean = mean(Adipose_Wt, na.rm
= TRUE), sd = (sd(Adipose_Wt, na.rm = TRUE)))

#anova for the adipose tissue weight in healthy vs. no healthy
anova_h = aov(Adipose_Wt~Group_Comb, data = metadata_w)
TukeyHSD(anova_h)
###Results
#There is not significant differences between HFD vs. Healthy groups. Combining the
groups does not add any benefit to the analysis.
```

APPENDIX F

CITRATE SYNTHASE NORMALITY DISTRIBUTION STATISTICAL ANALYSIS

The CS results were normally distributed (Shapiro-Wilk test $p>0.05$), but slightly skewed to the right (skewness = 0.64). All the linear regression assumptions were met. 1. Linearity of the data dummy coding. 2. Residuals were normally distributed (Figure 14). 3. No multicollinearity of the data was confirmed ($r = 0.04$ and variance inflation factor =1). 4. Homogeneity of residuals variance was established (Homoscedasticity; Figure 15).

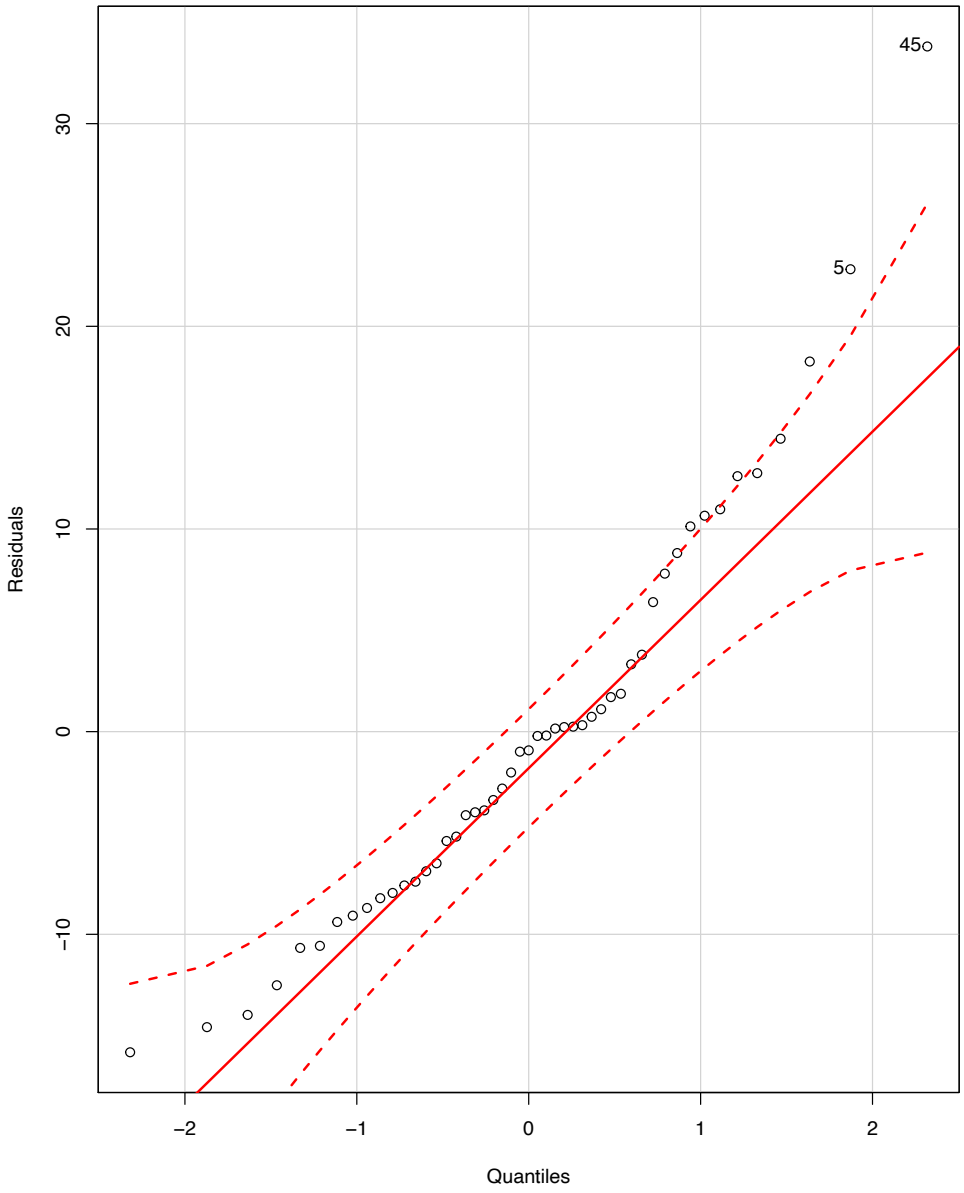

Figure 14. Q-Q plot normal distribution of regression residuals in the citrate synthase regression model.

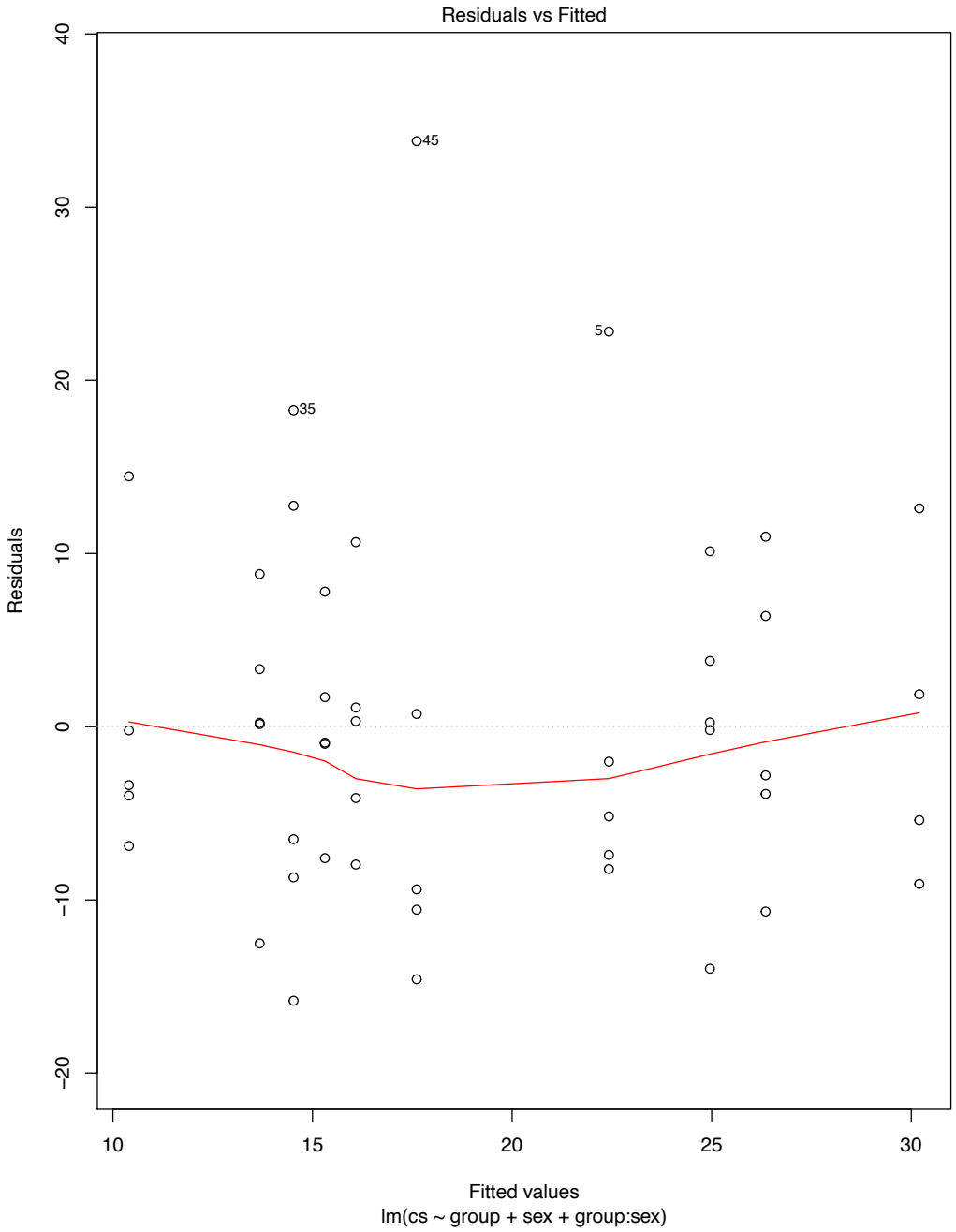

Figure 15. Homogeneity of residuals variance (Homoscedasticity) in the citrate synthase regression model.