Examining the Effects of a High Fat Diet on the Development of Metabolic Syndrome and Gut Leakiness in Male Sprague-Dawley Rats

by

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ABSTRACT

The prevalence of obesity and obesity-related disorders have increased world-wide. In the last decade, the intestinal microbiome has become a major indicator of metabolic and gastrointestinal health. Previous research has shown that high-fat diet (HFD) consumption can alter the microbial composition of the gut by increasing the abundance of gram-positive bacteria associated with the onset of obesity and type 2 diabetes. Although, the most common form of obesity and metabolic syndrome intervention is exercise and diet, these recommendations may not improve severe cases of obesity. Thus, an important relevance of my project was to investigate whether the intake of an organometallic complex (OMC) would prevent the onset of metabolic and gastrointestinal complications associated with high-fat diet intake. I hypothesized that the consumption of a HFD for 6 weeks would promote the development of metabolic and gastrointestinal disease risk factors. Next, it was hypothesized that OMC treatment would decrease metabolic risk factors by improving insulin sensitivity and decreasing weight gain. Finally, I hypothesized that HFD-intake would increase the abundance of gram-positive bacteria associated with gastrointestinal disease. My preliminary data investigated the effects of a 6-week HFD on the development of hepatic steatosis, intestinal permeability and inflammation in male Sprague Dawley rats. I found that a 6-week HFD increases hepatic triglyceride concentrations, plasma endotoxins and promotes the production of pro-inflammatory cytokines in the cecum wall. I then investigated whether OMC treatment could prevent metabolic risk factors in male Sprague-Dawley rats fed a HFD for 10 weeks and found that OMC can mitigate risk factors such

hyperglycemia, liver disease, impaired endothelial function, and inflammation. Lastly, I investigated the effects of a 10-week HFD on the gastrointestinal system and found an increase in liver triglycerides and free glycerol and alterations of the distal gut microbiome. My results support the hypothesis that a HFD can promote metabolic risk factors, alter the gut microbiome and increase systemic inflammation and that OMC treatment may help mitigate some of these effects. Together, these studies are among the first to demonstrate the effects of a soil-derived compound on metabolic complications. Additionally, these conclusions also provide an essential basis for future gastrointestinal and microbiome studies of OMC treatment.

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CHAPTER ONE

INTRODUCTION

Brief History of Metabolic Syndrome

Abdominal obesity has been associated with the development of cardiovascular disease and type 2 diabetes since the 1950's when Vague (1956) suggested its role in systemic inflammation [Vague, 1956]. In the 1980's, the development of obesity was then associated with markers of hypertriglyceridemia, glucose intolerance and insulin resistance in studies on obese men and women [Kissebah et al., 1982; Fujioka et al., 1987]. Due to the persistence and discoveries from past studies on obesity, in the late 1980's, "syndrome X" was introduced to properly describe the risk factors of cardiovascular disease [Reaven, 1988]. An accumulation of studies ranging from the 1950's to late 1990's proposed similar concepts of metabolic disorders. However, it was not until the early 2000's that metabolic syndrome (MetS), now regarded as a non-communicable disease and global epidemic, was characterized [Saklayen et al., 2018; Oda, 2012].

According to the World Health Organization (WHO), metabolic syndrome is defined by abdominal obesity, insulin resistance, hypertension and hyperlipidemia [Parikh et al., 2004]. In the United States, the prevalence of metabolic syndrome has increased from 25.3% to 34.2% in adults 18 years or older between 2007-2012 [Moore et al., 2017]. The development of metabolic syndrome is linked to an array of factors associated with lifestyle and diet. Previous research has shown that genetic predisposition, lack of physical activity, and an increase in the consumption of high-calorie-low fiber foods modulate metabolic syndrome in people [Oda, 2012].

The increasing prevalence of metabolic syndrome in humans indicates the need for studies to examine possible causes and risk factors. To investigate the progression of metabolic syndrome, researchers have used animal models to adequately mimic aspects of obesity, diabetes, dyslipidemia, hypertension, fatty liver disease [Panchal et al., 2011] and intestinal dysbiosis. The use of these models has demonstrated the need for novel therapeutic treatments and prevention methods for metabolic syndrome and its comorbidities.

Metabolic Syndrome and the Gut Microbiome

The gut microbiome and its broad genetic make-up consists of trillions of microbes living on the surface of our skin, in our mouths, noses, and digestive systems [Harley et al., 2012; Cho et al., 2012; Cani et al, 2016]. In a healthy adult, the intestine harbors a diverse community of bacteria where the predominant genus of microbiota present includes *Bacteriodetes*, *Firmicutes, Actinobacteria* and *Proteobacteria* [Cho et al., 2012]. Under normal healthy conditions, bacteria in the gut outnumber host cells and have a significant impact on the host physiology [Harley et al., 2012]. Diet-induced changes in the composition of the gut microbiome (unbalanced ratio of *Bacteriodetes* and *Firmicutes*) have been associated with the development of cardiovascular disease, hypertension, obesity and hyperglycemia; symptoms associated with MetS and type 2 diabetes [Wang et al., 2019]. The microbiota living symbiotically with their host are generally non-pathogenic and have evolved with humans to combat colonization by harmful bacteria while extracting energy from non-digestible dietary polysaccharides.

Many of the soluble dietary fibers fermented by colonic bacteria are used as a source of energy for the gut microbes [Ganapathy et al., 2013]. Moreover, the gut microbiota may be the fundamental link in a pathway of events that defines the development of obesity and insulin resistance after high fat/high calorie intake. Fluctuations in the gut microbiota have proved to be detrimental to metabolic health by degrading the intestinal barrier and increasing intestinal permeability through the transcription of pro-inflammatory cytokines. Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and interleukin-1beta (IL-1B) can cause cell shedding (tight junction degradation) and increase intestinal permeability [Bischoff et al., 2014]. This allows the lipopolysaccharides (LPS) released from gram-negative bacteria to cross the intestinal epithelium into the lamina propria. LPS and pro-inflammatory cytokines can flow via systemic circulation and thus promote metabolic endotoxemia and systemic inflammation [Raetz et al., 2002].

Previous studies have attempted to examine a link between the gut microbiota and insulin resistance [Backhed et al., 2007; Yan et al., 2016] and found that the gut microbiome can influence energy balance. However, to the best of my knowledge, my dissertation research is the first study to propose preventing diet-induced MetS by preventing gut dysbiosis induced by high fat intake. Dead gram-negative bacteria can release endotoxins that cross the intestinal wall and enter the bloodstream [Wheeler et al., 2015]. Consequently, endotoxins stimulate the production of TNF-α [Wang et al., 2002], which is associated with an increase in cell shedding of the intestinal barrier, thereby preventing tight junctions from sealing the gaps left behind. During microbial infection or

tissue damage, the innate immune system promotes inflammation as a physiological response to pathogens [Baker et al., 2011].

Local cells release pathogen-associated molecular patterns (PAMPs) [Baker et al., 2011]; releasing cytokines that activate the transcription factor, nuclear factor-kappa beta (NF-kB) and promoting localization of macrophages to the site of infection. Additionally, antimicrobial molecules and leukocytes terminate pathogens while preventing activation and localization of macrophages to the damaged site, thereby inhibiting inflammation. One of the many cytokines that NF-kB activates is interleukin-1 beta (IL-1B), which is produced by monocytes and released by an elevated concentration of lipopolysaccharides [Patel et al., 2015]. The relationship between the gut microbiome and symptoms associated with MetS induced by poor nutrition is not well understood. Elucidating these relationships may lead to the development of probiotics therapies to help prevent or reverse the pathophysiological consequences of poor nutrition.

Study Species

The Sprague-Dawley (SD) rat is a Wistar-hybrid generated in the 1920's by Robert S. Dawley [Brower et al., 2015]. New strains were developed in the 1950's and 1980's by Charles River Laboratories and Harlan Laboratories, respectively, with varying genetic compositions [Brower et al., 2015]. Since then, Sprague Dawley rats have been used to model human conditions such as obesity, diabetes, cancer and cardiovascular disease. To examine the pathophysiological mechanisms of metabolic disease states, researchers developed dietary approaches to induce metabolic syndrome in animal models. Previous research from our laboratory has shown that Sprague-Dawley rats

develop risk factors associated with metabolic syndrome during 6 weeks of HFD-intake [Sweazea et al., 2010]. More specifically, SD rats showed an increase in adiposity, developed hyperglycemia, insulin-resistance and endothelial dysfunction [Sweazea et al., 2010]. Similarly, other studies have shown that longer HFD treatments in SD rats increased similar markers of metabolic syndrome such as obesity and dyslipidemia [Dobrian et al., 2000; Ghibaudi et al., 2002].

Studies have reported that the gut microbiome has a pivotal role in the development of metabolic syndrome in humans [He et al., 2017]. It is evident that changes in the gut microbiome can influence disease progression in animal models [Franklin et al., 2017]. Rodent models have been proposed to be a better representation of the human gut microbiome in comparison to the germ-free mouse model [Wox-Oxley et al., 2012]. Humanized rat models have a gut microbiota more abundant in the major phyla found in humans; *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Proteobacteria* [Wox-Oxley et al., 2012]. Likewise, studies from our lab have also shown that SD rats on a control diet have similar microbial composition to healthy humans [Crawford et al., 2019] and show a similar decrease in commensal bacteria during the consumption of HFD subsequently leading to plasma endotoxemia.

The SD rat provides the ability to properly examine the physiological mechanisms and development of metabolic disease. Additionally, as a long-standing biomedical model, SD rats are utilized to investigate the importance of the gut microbiome in health and the progression of obesity, diabetes and gastrointestinal disorders. Together, murine studies offer evidence which supports the use of rodent models in biomedical research to better understand and predict disease causing factors of metabolic syndrome.

Dissertation Overview

The aim of my dissertation is to investigate the effects of a high fat diet on the development of metabolic syndrome and gut microbial dysbiosis. Additionally, my dissertation focused on the effects of a natural health product on alleviating metabolic and gastrointestinal complications associated with high fat diet intake. I tested the hypothesis that a high fat diet will increase adiposity, promote glucose intolerance, insulin resistance, decrease gut microbial diversity and increase the development of pro-inflammatory cytokines in the gut. I also hypothesized that supplementation with an organometallic complex (OMC) would decrease risk factors associated with metabolic syndrome and promote gut microbial diversity. In Chapter 2, I treated adolescent male Sprague-Dawley rats with HFD for 6 weeks. I then examined the effects of HFD-intake on gut microbial composition, the production of lipopolysaccharides, hepatic triglycerides and pro-inflammatory markers in the cecum and liver. In Chapter 3, I investigated OMC supplementation during a 10-week chow and HFD. In all study animals, I examined body weight, glucose tolerance, insulin resistance, hepatocyte injury, oxidative stress and endothelium-dependent vasodilation. I correlated these markers of metabolic syndrome to the progression of cardiovascular disease and obesity. In Chapter 4, I further investigated the effects of HFD on the gut microbiome. I examined differences in microbial composition in rats fed either a chow or HFD for 10 weeks. In Chapter 5, I used the same models of metabolic syndrome supplemented with OMC previously mentioned in Chapter 3 to investigate the effects of OMC supplementation on gastrointestinal physiology. I examined inflammation in the cecum and small intestine in response to HFD as well hepatic triglycerides and free glycerol concentrations.

CHAPTER 2

SIX-WEEK HIGH FAT DIET PROMOTES SIMPLE STEATOSIS, INTESTINAL INFLAMMATION AND ENDOTOXIN PRODUCTION WITHOUT OBESITY IN MALE RATS

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ABSTRACT

Energy-dense foods can alter gut microbial diversity. However, the physiological effects of diet-induced microbial changes on the development of nonalcoholic fatty liver disease (NAFLD) remain debatable. We hypothesized that high-fat intake for 6 weeks would promote intestinal dysbiosis by increasing gram-positive bacteria, inducing the intestinal production of pro-inflammatory cytokines and subsequent hepatic lipid infiltration in young male rats. Six-week old male Sprague-Dawley rats were divided into two groups and fed either a standard rodent chow or a 60% high-fat diet (HFD) for 6 weeks. Chromogenic endotoxin quantification assays indicate an increase in lipopolysaccharide concentration in the plasma of HFD rats ($p = 0.032$). Additionally, Western blot analyses of the cecum showed significantly greater protein expression of the transcription factor NF-kB ($p = 0.037$) and the pro-inflammatory cytokine IL-1β ($p = 0.042$) in rats fed HFD. Linear discriminate analysis of effect size (LEfSe) showed greater abundance of *Firmicutes* and *Actinobacteria* in the samples collected from the cecum of HFD rats compared to chow. Consistent with the development of steatosis, Oil-Red-O stained area was increased in liver sections from HFD rats. Hepatic triacylglycerol concentrations ($p < 0.001$) and plasma alanine aminotransferase ($p < 0.001$) were significantly increased in HFD-fed animals compared to chow. These findings show that a short duration of high

fat consumption can have profound deleterious effects on gastrointestinal health and the inflammatory state of these young male Sprague-Dawley rats.

INTRODUCTION

The prevalence of metabolic syndrome has risen 35% since 2012 and over two-thirds of Americans meet the criteria of obesity, dyslipidemia, insulin resistance and/or hypertension (Moore et al., 2017). Nonalcoholic fatty liver disease (NAFLD) is a major complication of obesity that affects approximately 24% of the global population and overweight or obesity in adolescence increases the risk of developing NAFLD in adulthood (Younossi et al., 2018). Recent studies have noted a link between obesity and the gut microbiome composition early in life, which is shaped by how babies are delivered (i.e., vaginal or c-section), breastfeeding, as well as administration of probiotic and antibiotic supplements (Angelakis and Raoult, 2018). While high fat-induced NAFLD and changes to the gut microbiome have been well-studied in adults, very little research has focused on adolescent populations.

The trillions of bacteria that comprise the gut microbiota have important roles in maintaining homeostasis within the intestinal lumen (Cani et al., 2008; Harley & Karp, 2012; Cox et al., 2015). Typically, the intestinal microbiota of a healthy individual includes the following phyla: gram-negative *Bacteroidetes* (*Bacteroides* spp.) and *Proteobacteria* (*Escherichia coli* spp.) as well as gram-positive *Firmicutes* (*Clostridium* spp.) and *Actinobacteria* (*Bifidobacterium* spp.). Research has shown that a high fat diet (HFD)-induced pathogenic state results in an imbalance (dysbiosis) of *Bacteroidetes* and *Firmicutes* and may be a cause of increased adiposity, body weight, glucose intolerance,

insulin insensitivity and hepatic lipid infiltration (Hansen et al., 2015; Zhu et al., 2015). Additionally, while *Bacteroidetes* are gram negative, relative abundance of this phylum typically increases with weight loss, indicating its role in a healthy gut flora.

In patients diagnosed with NAFLD, the intestinal microbiota is notably altered, and the increase of pathogenic bacteria can promote hepatic steatosis. A rise in the relative abundance of gram-negative bacteria can increase intrahepatic fat by altering appetite signaling, expression of genes involved in lipogenesis or by mechanisms which promote inflammation (Mokhtari et al., 2017). Interestingly, previous studies of hepatic steatosis in humans indicate a decrease in the relative abundance of *Bacteroides* and *Prevotella* (*Bacteroidetes* spp.), thereby increasing the metabolic dominance of other bacteria. In addition, this can enhance gut permeability and promote the release of endotoxins from gram-negative bacteria into the circulation (Mokhtari et al., 2017). Lipopolysaccharide (LPS) binding to toll-like receptor 4 (TLR4) on hepatocytes initiates the production of proinflammatory cytokines and thus promotes systemic inflammation (Mokhtari et al., 2017).

Previous studies have found that prolonged consumption of HFD is associated with changes in intestinal permeability that promote systemic inflammation. During HFD, the gut wall becomes permeable to pro-inflammatory cytokines and LPS, which foster metabolic endotoxemia and low-grade inflammation (Hansen et al., 2015; Netto Candido et al., 2018; Oliveira et al., 2018). Under normal conditions, the human intestinal epithelium sheds every 5 days due to differentiation and proliferation of stem cells located in the intestinal crypt (Bischoff et al., 2014). Tight junction proteins fill the gaps left after extrusion of cells, thereby maintaining the intestinal barrier. However, an

increase in intestinal permeability induced by high fat-high caloric intake can lead to inflammation, excessive cell shedding and a decrease in tight junction protein expression (Cani et al., 2008; Oliveira et al., 2018).

Kim et al. (2012) demonstrated that TLR-deficient male mice do not develop inflammation in response to an 8-week HFD (60% kcal fat), which indicates a causal role for microbial LPS in obesity-associated inflammation. Additionally, 8-week HFD increases the relative abundance of *Firmicutes* and *Proteobacteria*, induces macrophage production, increases inflammation of adipose tissue and levels of LPS in the intestinal lumen and plasma, thus increasing intestinal permeability. We have previously shown that adolescent 6-week old male Sprague-Dawley rats fed HFD for 6 weeks develop oxidative stress, impaired endothelium-dependent vasodilation, an increase in adiposity and glucose intolerance (Sweazea et al., 2010). The current study provides additional support for the investigation of a 6-week HFD on the development of metabolic diseases in adolescents. The results emphasize the importance of prevention methods for metabolic syndrome and its comorbidities.

Despite an increase in gut microbiome-related research in animal models, researchers remain confounded by the bacterial species associated with increased intestinal permeability and hepatic steatosis during HFD. Moreover, recent studies present inconsistencies in data with regards to relative abundance of bacterial species associated with the onset of metabolic disease, thus complicating treatment approaches. Therefore, the major goal of the present study was to examine the effects of a 6-week high-fat diet on microbial relative abundance and the expression of inflammatory factors in the cecum wall and the development of hepatic steatosis in adolescent rats.

Specifically, we hypothesized that high-fat, high-caloric intake for 6 weeks would promote intestinal dysbiosis and thus induce the intestinal production of pro-inflammatory cytokines, impair insulin signaling and promote hepatic steatosis in six-week old male Sprague-Dawley rats.

MATERIALS AND METHODS

Ethical Approval: All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee.

Animal Models: Samples for the current study were obtained from a subset of animals used in a larger study of the effects of high fat intake on vascular reactivity (Sweazea et al., 2010). As estrogen exerts vasoprotective effects prior to menopause, thus preventing the vascular effects of a high fat diet, only males were used. Six-week old adolescent Sprague-Dawley rats (*n*=37 total; 140-160 g initial body mass; Harlan Teklad) were randomly divided into two groups that were either maintained on their regular plant-based standard rodent chow diet ("Chow", *n*=18) or switched to a high fat diet (HFD, *n*=19) for 6 weeks. The chow diet was purchased from Harlan Teklad (2018S) and contained 24% protein, 58% carbohydrates and 18% fat (in % kcal). This diet served as a reference group as the animals were raised and maintained on this diet. The HFD contained 20% protein, 20% carbohydrate, and 60% fat (in % kcal) and was purchased from Research Diets Inc. (D12492; New Brunswick, NJ, USA). The fat content of the HFD consisted in 9% fat from soybean oil and 91% fat derived from lard. In contrast, 60% of the fat content of the chow diet was comprised of soy products (soybean oil and

meal) with the remainder derived from wheat and corn. The carbohydrate content of the chow diet was comprised of 54% wheat products, 40% corn products and the remainder soybean meal. This is in contrast to the carbohydrate composition of the HFD, which contained 63% corn and 37% sucrose. While the percent protein content of each diet was similar, the protein composition differed with 36% wheat, 34% corn and 26% soy proteins in the chow diet and 98.5% of protein comprised of a mixture of casein, lactic and 30 mesh in the HFD. With the high content of animal-based ingredients and soy, the HFD is a good model of a western diet high in saturated fats.

Rats were housed in pairs, maintained on a 12:12 hour light:dark cycle and were provided access to water and food *ad libitum*. Cages were supplied with tunnels and chew toys for enrichment. At the end of the six-week feeding protocol, non-fasted animals were euthanized by an overdose of sodium pentobarbital (200 mg/kg, i.p.), blood was collected by cardiac puncture, and pieces of cecum and liver were dissected and immediately placed either in liquid nitrogen for protein expression or in Optimal Cutting Temperature compound for histology. All samples were stored at -80ºC until analyses. Whole blood was centrifuged at 14,000 rpm for 10 minutes at 4ºC to separate formed elements from plasma. Plasma was stored at -80ºC until analyses. Sample size varied among experiments due to repeated use.

Morphometric Measurements: Morphometric data were collected from a subset of 6-8 animals per group. Body mass was measured (to the nearest hundredth) at the end of the 6-week feeding protocol prior to euthanasia. Epididymal fat pad mass was also measured (to the nearest hundredth) at the end of the dietary intervention to determine variation in

adiposity between chow and HFD-fed rats since the fat pad can easily be removed and examined objectively (Sweazea et al., 2010). Abdominal circumference was additionally examined (measured below the lowest rib and just anterior to the pelvis; to the nearest hundredth) at the end of the six-week feeding protocol to compare abdominal adiposity between groups.

Lipopolysaccharide Assay: Undiluted plasma lipopolysaccharide concentrations were quantified in duplicate on samples that were collected from the same animals for which morphometric data were available (n=6/group). A commercially-available kit was used (Pierce LAL Chromogenic Endotoxin Kit, Thermo Scientific, Rockford, IL, USA) per the manufacturer's protocol to examine systemic circulation of endotoxins (levels as low as 0.1 EU/mL) after the six-week feeding protocol. Intra-assay coefficient of variation less than 10% were considered acceptable.

Cecal Stool Analyses: Microbial genomic DNA was extracted from samples collected from the cecum of the same animals for which morphometric data were obtained following the 6-week diet (n=6/group). This was done using a commercially available kit per the manufacturer's instructions (PowerSoil® DNA Isolation Kit, MoBio Laboratories, Inc., Carlsbad, CA, USA). DNA concentration was quantified in micro-liter volumes (2µl of DNA sample was compared to 2µl of eluent) using a µDrop™ plate adaptor (Catalog #N12391, ThermoFisher Scientific, Waltham, MA, USA) and Multiskan ™ GO Microplate spectrophotometer (Catalog # 5119300, ThermoFisher Scientific Waltham, MA, USA). Sample purity was determined at OD_{260}.

Illumina Sequencing: Microbial taxonomy was analyzed with the following protocol proposed by the Earth Microbiome Project using QIIME analyses. Amplicon sequencing of the V4 region of the 16SrRNA gene was performed with the barcoded primer set 515f/806r designed by Caporaso et al. (2010). Triplicate PCR amplifications for each sample were performed and quantified using Quant-iTTM PicoGreen® dsDNA Assay Kit (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA). A total of 240 ng of DNA per sample was pooled and then cleaned using QIA quick PCR purification kit (QIAGEN, Valencia, CA, USA). Subsequently, the pooled DNA concentration was determined with the following equation: $\frac{concentration\ in \frac{ng}{ul}}{\frac{660g}{mol} * average\ library\ size} * 10^6$ and diluted with 0.2 N NaOH to a final concentration of 4 nM. Afterwards, the DNA was denatured and diluted to a final concentration of 4 pM with a 30% PhiX (PhiX Control V3, Illumina, Inc.). DNA was loaded in the MiSeq Illumina Sequencer (Illumina, San Diego, CA, USA) using the chemistry version 2 (2 x 150 paired-end) as per manufacturer protocol. All samples were sequenced at the Microbiome Analysis Laboratory at Arizona State University (Tempe, AZ, USA).

Microbial Sequence Analyses: Sequence data were assigned to operational taxonomic units (OTU) with a 97% similarity threshold using QIIME's uclust-based open-reference OTU picking protocol against SILVA and/or Greengenes reference databases (Caporaso et al., 2010). Sequences not matching the reference databases were clustered de novo, so all sequences were included in the analysis. Data were initially analyzed with plugins

implemented in QIIME and differential microbial abundance differences between diet groups were identified using linear discriminant analysis of effect size (LEfSe) analyses via the online Galaxy module (http://huttenhower.sph.harvard.edu/galaxy/) as previously described (Segata et al., 2011).

Western Blot Analyses: Liver and cecum samples collected from a randomly-selected subset of animals fed either chow or HFD were used for western blot analyses (n=6/group). Western blots were performed to examine the expression of proteins associated with inflammation (NF-kB and IL-1β) in the cecal wall (n=4 samples/group). Moreover, expression of proteins related to insulin signaling (p-PI3K; p85/p55 subunits), gluconeogenesis (PEPCK-C) and inflammation (TLR-4, XBP1, iNOS, IL-1β) were measured in liver samples (n=5-6/group). Frozen cecal and liver samples were cleaned with ice-cold PBS, pH 7.4, and then homogenized in ice-cold Tris-HCL homogenization buffer containing 10 mM Tris (pH 7.6), 1 mM EDTA, 1% triton X-100, 0.1% Na-deoxycholate, 0.03% protease phosphatase inhibitor cocktail (Halt Protease and Phosphatase Inhibitor Cocktail (100X), ThermoFisher Scientific, Waltham, MA, USA) and 1mM phenylmethanesulfonyl fluoride (PMSF) using the Beadbug Microtube Homogenizer for three minutes (3.00 mm zirconium beads, Benchmark Scientific, Edison, NJ, USA). Cecum and liver homogenates were centrifuged at 4000 g for 10 minutes at 4 °C to remove insoluble debris. The protein concentration of the supernatants was determined using the Bradford method (Bio-Rad, Hercules, CA). Cecal supernatants (25 μg total protein/lane) were resolved by 7.5% Tris-HCL sodium dodecyl sulfate polyacrylamide gel electrophoresis at 200 mV for 35 minutes (SDS-PAGE) (Bio-Rad,

Hercules, CA) then transferred at 100 mV for 90 min onto polyvinylidene difluoride membranes (PVDF) pre-wetted with Tris-buffered saline (Bio-Rad, #170-6453, Hercules, CA) and 0.1% Tween 20 (Bio-Rad, #170-6531, Hercules, CA, USA), TTBS. Protein molecular weight for phosphorylated NF-kB in the cecum as well as insulin signaling intermediates, markers of gluconeogenesis and inflammation in the liver were determined with SeeBlue Plus2 Pre-stained standard (ThermoFisher Scientific, Cat. No. LC5925). Protein molecular weight for IL-1β was determined with Colorburst Pre-stained standard (ThermoFisher Scientific, Cat. No. LC592).

A. Protein Expression in the Cecum: To prevent non-specific binding of antibodies, the membranes were incubated overnight at 4 °C in blocking buffer; TTBS containing 3% bovine serum albumin fraction V (BSA) and 5% nonfat dry milk. The PVDF membranes (Bio-Rad, #160-0174), Hercules, CA, USA) were then washed with TTBS (3 washes, 5 minutes each) and incubated for 4 hours at room temperature with 1:1000 anti-NF-kB p65 rabbit polyclonal antibody (GeneTex, Inc., #GTX11742) or anti-IL-1β 3A6 mouse monoclonal antibody (Cell Signaling Technologies, #12242). Subsequently, membranes were washed in TTBS (5 washes, 5 minutes each) and then incubated with either 1:1000 anti-rabbit IgG, HRP-linked secondary antibody (Cell Signaling Technologies, #70745) or 1:5000 anti-mouse IgG, HRP-linked secondary antibody (Vector Lab, #PI-200), as appropriate, prepared in TTBS for 1 hour at room temperature. Membranes were washed with Tris-buffered saline (TBS, BioRad, 170-6435) after incubation with secondary antibody then exposed to Pierce enhanced chemiluminescence (ECL) western blotting substrate for one minute (Catalog # 32106,

Thermo Scientific, Rockford, IL, USA). The immunoreactive bands were visualized by exposure to x-ray film (Kodak X-OMAT, Thermo Fisher Scientific, Pittsburgh, PA). Total protein concentration was normalized and quantified by staining the PVDF membrane with Coomassie Brilliant Blue R-250 (BioRad, 161-0400, Hercules, CA, USA) prepared in 50% methanol, 42.5% deionized water and 7.5% acetic acid. Densitometry was determined using NIH ImageJ software (Schneider et al., 2012).

B. Protein Expression in Liver: Anti-rabbit beta-actin primary antibody (1:1000; Cell Signaling Technologies, Cat. No. 12620S) was used as a loading control for all samples except IL-1β and was added to the same PVDF membranes at the following concentrations for each protein of interest: PEPCK-C and iNOS, 1: 10,000; p-PI3K, 1:1000; XBP1 and TLR-4, 1:2000. For IL-1β (1:1000), coomassie total protein stain was used as a loading control since IL1β and beta-actin are similar molecular weights. Subsequently, membranes were washed in TTBS and then incubated with anti-rabbit (Cat. No. 70745, Cell Signaling Technologies, USA) or anti-mouse (Cat. No. PI-200, Vector Laboratories, Burlingame, CA, USA) secondary antibody for 1 hour at room temperature (PEPCK-C, iNOS and IL-1β, 1:5000; p-PI3K, TLR-4 and XBP1, 1:2000) then exposed to Pierce enhanced chemiluminescence western blotting substrate for one minute (Catalog # 32106, Thermo Scientific, Rockford, IL, USA). The immunoreactive bands were visualized by exposure to x-ray film (Kodak X-OMAT, Thermo Fisher Scientific, Pittsburgh, PA, USA) and densitometry analyzed using NIH ImageJ software (Schneider et al., 2012).

Liver Oil-Red-O: Oil-Red-O staining was performed to evaluate lipid infiltration of the liver from the same study animals used for liver western blot analyses and plasma alanine aminotransferase (ALT) activity assays (n=4/group). Frozen OCT-embedded liver samples were sectioned at 12-µm using a cryostat (Leica Biosystems CM1950; Buffalo Grove, IL, USA) and sections were collected onto poly-lysine coated microscope slides, stained with Oil-Red-O and counterstained with hematoxylin using a commercially available kit as per the manufacturer's protocol (Cat No. ORK-2-IFU; ScyTek Laboratories Inc., Logan, UT, USA). Coverslips were mounted using heated glycerin jelly created by mixing 1.25 g gelatin and 7.5 mL distilled water with gentle heat until dissolved. Next, 8.75 mL glycerin and 0.125 mL phenol were added and allowed to mix with gentle heat until dissolved. The resultant glycerin jelly was stored at 4°C until use. Tissue sections were viewed immediately after collection at 200X magnification with an Olympus BX50 microscope and images collected using an Olympus DP70 camera (Melville, NY, USA).

Liver Triacylglycerol Assay: Liver triacylglycerol (n=5/group) was measured in samples collected from the same study animals used to assess protein expression in the liver via Western blot analyses. Liver triacylglycerol was measured in duplicate according to the methods of Jouihan (2012).

Alanine Aminotransferase (ALT) Activity Assay: Plasma alanine aminotransferase activity was quantified in duplicate (9-10/group) with a commercially available kit (Cat No. MAK052, Sigma Aldrich, St. Louis, MO, USA) per the manufacturer's protocol to

examine hepatocellular injury after the six-week feeding protocol. Samples were obtained from a randomly selected subset of animals fed either chow or HFD.

STATISTICS ANALYSES

Data are expressed as mean ± SEM. Data were analyzed using SigmaPlot (Systat Software Version 10.0, San Jose, CA, USA). Phyla were expressed as relative abundance of total gut microbiota and abundances were compared by Mann-Whitney U-tests where $p<0.05$ was considered statistically significant. Spearman correlations were performed to compare relationships between relative abundance of each phyla, genera and body mass, waist circumference and epididymal fat pad mass. Differences in the *Firmicutes:Bacteriodetes* (F:B) ratio, genera and ALT activity were calculated and compared between chow and HFD groups by Mann-Whitney U test. Data for F:B ratio from one chow-fed and one HFD-fed rats were excluded because *Bacteriodetes* were not detected in these samples. Morphometric, western blot and lipopolysaccharide data were analyzed by Student's t-tests. Statistical significance was concluded with a $p<0.05$ for all analyses. LEfSe analysis was conducted using a series of different tests including, Kruskal-Wallis sum-rank test, Wilcoxon rank-sum and linear discriminant analysis as previously described (Segata et al., 2011).

RESULTS

Changes in Morphology and Inflammation Following HFD: Following the six-week diet, epididymal fat pad mass (Mann-Whitney, W=40.0, *p=0.002*) and waist circumference (Student's t-test, t=-4.281, df=11, *p=0.003*) were greater in HFD-fed rats

in comparison to rats fed standard chow (Table 1). HFD-fed rats also had a significant increase in plasma endotoxins (Student's t-test, t=-2.602, df=8, *p=0.032*) after the six-week feeding protocol (Fig. 1). Inflammation in the cecal wall of the HFD rats was indicated by increased total protein expression of the inflammatory transcription factor NF-kB (Student's t-test, t=-2.670, df=6, *p=0.037*) and the inflammatory cytokine IL-1β (Student's t-test, t=-2.572, df=6, *p=0.042*) (Fig. 2).

Gut Microbiome Analysis: Consistent biological and statistically significant taxonomic differences in cecal samples between diet groups can be seen in Figure 3. Moreover, a total of 25 operational taxonomic units (OTU) were identified as differentially abundant features between the HFD and chow diet-fed animals (Fig. 4A). Red bars indicate the taxa significantly more abundant in chow fed rats while the green bars show the taxa significantly more abundant in the HFD group (LDA score >2, $p<0.05$, Wilcoxon rank-sum test). Cecal samples from chow-fed rats were more enriched in the following gram-negative genera *Prevotella* and *Prevotellaceae* (phylum: *Bacteroidetes*). Additionally, an array of gram-positive bacteria (*Lachnospira, Anaerostipes, Coprabacillus, Anaerofustis, Eubacteriaceae, Candidatus Arthromitus* and *Chrsistensenella)* from the *Firmicutes* phylum were significantly more abundant in chow-fed rats. LEfSe analysis of the 6-week cecal samples for HFD-fed rats showed significantly greater presence of gram-positive bacteria which include four subclasses of *Firmicutes* (*Clostridiales, Streptococcaceae, Erysipelotrichales* and *Eryspelotrichia*) as well as the phyla *Actinobacteria*. In HFD-fed rats, a significant positive correlation was observed between body mass and *Actinobacteria* (S=6.591, p=0.049, r=0.812). However, no relationship with other

bacterial species were observed in chow and HFD-fed rats. Finally, *Firmicutes:Bacteriodetes* ratio was calculated as a biomarker of gut dysbiosis (Fig. 4B), however, no significant differences were observed between groups (n=5, Mann-Whitney U=12, p=1.000).

Liver Oil-Red-O: Simple steatosis was observed in HFD-fed rats as evidenced by Oil-Red-O staining of liver sections (Fig 5A, Fig. 5B).

Liver Triacylglycerol Assay*:* Livers from HFD-fed rats had significantly greater triacylglycerol concentrations than livers from chow-fed rats (Fig. 5C, Student's t-test, t=-5.102, p<0.001).

Alanine Aminotransferase Activity Assay: Serum samples from HFD-fed rats had significantly higher ALT activity than serum samples from chow-fed rats (Fig. 6, Mann-Whitney, U=99.000, p<0.001).

Insulin Signaling and Inflammation in the Liver: Protein expression of PEPCK-C (Student's t-test, t=-0.234, p=0.819), two phosphorylated regions of PI3K (p55; Student's t-test, t=-0.575, *p=0.576* and p85; t= 0.149, *p=0.884*) and the inflammatory cytokines TLR-4 (Student's t-test, t=0.507, p=0.623), XBP-1 (Mann Whitney, U=16.000, *p=0.622*), iNOS (Mann Whitney, U=10.000, p=0.240) were not significantly altered following 6-weeks of HFD (Fig. 7).

DISCUSSION

Obesity and type 2 diabetes are characterized by low-grade inflammation, which may develop as a result of a fat-enriched diet (Cani et al., 2008). Others have previously shown that changes in the gut microbiome increase the production of pro-inflammatory cytokines such as IL-1β and bacterial LPS which induces metabolic endotoxemia, thereby increasing the risk of insulin resistance (Cani et al., 2008; Hawkesworth et al., 2013). In the current study, the rise in endotoxins could be explained by the increased expression of pro-inflammatory cytokines in the gut and the breakdown of beneficial gram-negative bacteria. This response can be explained by the activation of NF-kB from intestinal Paneth cells which can initiate the production of pro-inflammatory cytokines, thereby, stimulating cell shedding and increasing intestinal permeability (Bischoff et al., 2014). An increase in intestinal permeability may allow pro-inflammatory cytokines and LPS to cross the lamina propria and enter systemic circulation (Hanson et al., 2015; Netto Candida et al., 2018; Oliveira et al., 2018), and so also increase the risk of inflammation of other tissues in the HFD rats.

It is possible that pair-housing animals in the present study may be a limitation in regard to the cecal microbial analyses. Previous research examining the microbiome of fresh fecal samples collected from Zucker rats housed together have shown similar fecal microbial populations despite obesity status (Hakkak et al., 2017), although none of the animals in the current study developed obesity. Additionally, a 16-week HFD study conducted by Lecomte et al. (2015) found that pair-housing rats showed no variation in the gut microbiota and Bray-Curtis similarities (comparing similarities of two samples) were comparable between diets at all taxonomic levels.

LEfSe analyses were used to identify the taxonomic structure of the gut microbiome. Results of these analyses showed differences between rats fed a chow or HFD for 6 weeks. The cecal samples of chow-fed rats were more abundant in *Bacteroidetes* (*Prevotellaceae, Prevotella), Firmicutes* (*Eubacteriaceae*, *Lachnospira, Anaerostipes, Coprobacillus*, *Anaerofustis, Coprobacillus), Candidatus Arthomitus* and *Christensenella*. The abundance of the *Bacteroidetes* and *Firmicutes* has been previously reported to be representative of a "healthy" gut microbiota commonly found in rats (Clarke et al., 2012). Conversely, gram-positive *Firmicutes* are typically more enriched in HFD-fed rats. The present study revealed that 6-weeks of HFD feeding increased the abundance of *Firmicutes, Clostridiales, Proteobacteria* and *Streptococcaceae*, which may be influenced by consumption of energy dense diets. These data are consistent with prior research demonstrating that a short-term (7-day) HFD increased different subclasses *of Firmicutes* such as *Erysipelotrichale* (Vaughn et al., 2017), which was consistent with findings from the present study. Likewise, a prior study of female mice fed a western diet high in carbohydrates and saturated fat for 14 days resulted in increased abundance of *Firmicutes* (Yin et al., 2018).

The consumption of a HFD has been reported to decrease *Bacteroidetes* while increasing *Firmicutes* and *Proteobacteria.* Many classes of *Firmicutes* found in the 6-week HFD cecal samples have also been reported to be enriched in human and animal models of metabolic and gastrointestinal diseases such as colorectal cancer and irritable bowel disease (Kaakoush, 2015). Gram-positive bacteria (*Firmicutes, Actinobacteria, Clostridiales, Proteobacteria* and *Streptococcaceae)* are also associated with the onset of NAFLD in clinical studies (Jiang et al., 2015) and literature suggests that *Erysipelotrichi*

*(*Phylum: *Firmicutes)* is positively correlated with an increase in pro-inflammatory cytokines and HFD consumption (Turnbaugh et al., 2009). Additionally, *Dorea (*Phylum*: Firmicutes),* a species commonly abundant in patients with irritable bowel syndrome and non-alcoholic fatty liver disease (Rajilic-Stojanovic et al., 2011), was found to be more enriched in HFD rats in the current study.

Interestingly, *Actinobacteria (subclass: Rothia, Micrococcaceae, Corynebacterium, Corynebacteriaceae and Actinomyocytes) and Bacteroidetes (subclass: Bacteroidales)*, common phyla associated with a "healthy gut" (Clarke et al., 2012), were also significantly abundant in the cecal contents of HFD-fed rats. This is consistent with research that has shown the abundance of *Actinobacteria* and *Bacteroidetes* is high in the large intestine of rats (Li et al., 2017). Moreover, rats in the current study were fed a 60% HFD which contained 6.5% of a dietary fiber called Solka Floc (FCC200). Solka Floc is a powdered cellulose added to dry pet food for shape and texture. Cellulose acts as an energy source for *Actinobacteria* which produces short chain fatty acids responsible for anti-inflammatory properties (Nagao-Kitamoto & Kamada, 2017). This amount of fiber was greater than the cellulose and lignin content reported for the chow diet (3.5%). Decreased consumption of cellulose can lead to gastrointestinal complications such as inflammatory bowel disease (Nagy-Szakal et al., 2013). Literature suggests that supplementation with cellulose has been associated with a beneficial shift in the microbiome of rats, specifically increasing *Actinobacteria* and other commensal microbes (Nagy-Szakal et al., 2013). Therefore, the addition of cellulose in the HFD given to the rats in the present study may have contributed to the increase in the abundance of *Actinobacteria* and *Bacteroidetes* in the cecum. These results suggest a

compensatory response to the 6-week HFD consumption but may also demonstrate the complexity of the microbiome.

In addition to an increase of inflammatory markers in the intestine and plasma LPS in HFD rats, the present study shows that high-fat intake for 6 weeks increases hepatic lipids and plasma ALT activity in young rats in the absence of measurable variations in proteins associated with insulin signaling, gluconeogenesis or inflammation. Elevated ALT activity is indicative of liver damage which is consistent with prior studies that have shown an increase in ALT during a 6-week HFD (Tan et al., 2013). Interestingly, the increase in liver triacylglycerol concentrations occurred independently of any changes in fasting plasma triacylglycerol, as plasma levels were previously found to be normal (Sweazea et al., 2010). These findings are in contrast with studies that administered adult rats a mixed high carbohydrate and fat diet for longer durations (Auberval et al., 2014).

Prior studies in our laboratory have shown rats fed HFD develop impaired glucose tolerance and fasting as well as fed-state hyperglycemia (Sweazea et al., 2010). Therefore, we hypothesized that the livers of these animals would have decreased protein expression of the tyrosine phosphorylated regulatory subunits of PI3K, p85 (Tyr 458) and p55 (Tyr 199) as this protein is an important signaling intermediate for insulin-mediated cellular metabolism and gluconeogenesis (Huang et al., 2007). Similarly, we had anticipated a rise in the hepatic protein expression of PEPCK-C suggestive of elevated hepatic gluconeogenesis in HFD animals. In contrast to our hypothesis, the data suggest that a 6-week HFD is likely not sufficient to induce measurable changes in the expression of proteins involved in insulin signaling or gluconeogenesis.

Previous research from our laboratory has shown that 6 weeks of HFD increases circulating TNF-α and impairs acetylcholine-mediated vasodilation through the actions of cyclooxygenase, iNOS, and oxidative stress (Sweazea et al., 2010; Sweazea & Walker, 2011). Thus, we had predicted that hepatic protein expression of TLR-4 and iNOS would increase, thereby contributing to the chronic inflammatory nature of high fat intake in these rats. However, contrary to our hypothesis, no significant differences were observed in the expression of these proteins between the dietary groups. Similarly, the hepatic protein expression of XBP1, which has a protective role in the pathogenesis of nonalcoholic steatohepatitis (Liu et al., 2015), was not significantly different.

Alterations in the gut microbiome have been associated with the onset of metabolic syndrome. Additionally, the overgrowth of pathogenic bacteria and an increase in plasma LPS has been shown in patients diagnosed with NAFLD. The consumption of a high-fat diet can increase intestinal permeability by promoting the production of pro-inflammatory cytokines which degrade gram-negative bacteria and promote the translocation of bacterial endotoxins into the portal vein; thereby increasing the risk of NAFLD through the activation of hepatic inflammatory cells. Bacterial endotoxins are recognized by toll-like receptors on hepatocytes and initiate an immune response subsequently activating NF-kB and the release of pro-inflammatory cytokines (Mokhtari et al., 2017).

Few studies have examined the impact of a high-fat diet on the fecal taxonomy of rats and the subsequent physiological consequences on the liver. However, prior research in older rats has provided important information about the effects of longer duration HFD. For example, Ainslie et al. (2000) evaluated 20-22-week-old female Wistar rats

placed on a moderately HFD (36% fat, 51% carbohydrates and 13% protein) for 14 weeks; at 4 weeks HFD-fed rats had a 38% increase in abdominal fat mass compared to controls, whereas by 14 weeks, HFD rats gained twice as much weight and consumed more energy (32 kj/d) than the control group (Ainslie et al., 2000). Similarly, findings from the current study showed that consuming HFD for a period of 6 weeks increased adiposity. Moreover, others have shown that feeding rats a combined Westernized diet containing high concentrations of both fat and carbohydrates for longer durations (10-32 weeks) results in nonalcoholic steatohepatitis marked by the infiltration of immune cells and increased expression of inflammatory markers in the liver of 8-9-week-old male Wistar rats (Ainslie et al., 2000; Roth et al., 2012).

Studies have shown that prolonged consumption of energy dense foods increases the risk of metabolic syndrome and consequently NAFLD (Roth et al., 2012). Similar to humans, prior studies from our group have shown that this 6-week feeding protocol promotes increased adiposity, endothelial dysfunction, systolic hypertension and impaired glucose tolerance (Sweazea et al., 2010). Diet-induced dysbiosis of the gut microbiome has been proposed to play a causal role in the onset of adult obesity thereby leading to the exacerbation of metabolic diseases.

In summary, the prevalence of obesity continues to increase in the United States. This trend can be attributed to a lack of physical activity and the consumption of inexpensive energy-dense foods (Browning et al., 2004). Diet is a critical variable in understanding the progression of weight gain. However, there is a clear difference in the susceptibility to weight gain in people (Browning et al., 2004). While some individuals are able to maintain their weight while consuming a high fat diet (obesity-resistant),

others are not and thus are more prone to weight gain in a similar obesogenic environment (Browning et al., 2004). A previous study showed that obesity-resistant male rats have better control of food intake and increased total energy expenditure following the introduction of an *ad libitum* HFD (Schmidt et al., 2012). Additionally, a recent study showed that a 7-week HFD (60% kcal) can promote minor increases in epididymal fat tissue and weight gain in obesity-resistant rats (Jackman et al., 2010). This is consistent with our study demonstrating that a 6-week HFD increases epididymal fat pad mass with small increases in body weight. Although previous research from our laboratory has shown significant weight gain in rats during a 6-week HFD, rats in the present study may be obesity-resistant, resulting in minor changes in adiposity.

The current study additionally demonstrates that 6 weeks of HFD promotes cecal inflammation, systemic endotoxemia and simple steatosis in male Sprague-Dawley rats. The findings also suggest that prolonged consumption increases the abundance of gram-positive bacteria associated with metabolic disease but does not affect the abundance of commensal bacteria commonly abundant in a healthy gut flora. Investigating the progression of poor diet-induced metabolic syndrome and intestinal dysbiosis is thus important for the development of appropriate treatments and preventative measures of metabolic diseases in the future.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest.

REFERENCES

Ainslie DA, Proietto J, Fam BC, Thorburn AW. (2000) Short-term, high-fat diets lower circulating leptin concentrations in rats. *American Journal of Clinical Nutrition* **7**:438–442 DOI 10.1093/ajcn/71.2.438.

Angelakis E, Raoult D. (2018) Gut microbiota modifications and weight gain in early life. *Human Microbiome Journal* 7-8:10-14. DOI 10.1016/j.humic.2018.01.002.

Auberval N, Dal S, Bietiger W, Pinget M, Jeandidier N, Maillard-Pedracini E. (2014) Metabolic and oxidative stress markers in Wistar rats after 2 months on a high-fat diet. *Diabetology and Metabolic Syndrome* **6**:130 DOI 10.1186/1758-5996-6-130.

Bischoff SC, Barbara G, Buurman W, Ockhuizen T, Schulzke JD, Serino M, Wells JM. (2014) Intestinal permeability – a new target for disease prevention and therapy. *BMC Gastroenterology* **14**:189 DOI 10.1186/s12876-014-0189-7.

Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH. (2004) Prevalence of hepatic steatosis in an urban population in the United States: Impact of ethnicity. *Hepatology* **40**:1387–1395 DOI 10.1002/hep.20466.

Cani PD, Bibiloni R, Knauf C, Neyrinck AM, Delzenne NM. (2008) Changes in gut microbiota control metabolic diet–induced obesity and diabetes in mice. *Diabetes* **57**: 1470–1481 DOI 10.2337/db07-1403.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Knight R. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*. **7**:335–336 DOI 10.1038/nmeth.f.303.

Clarke SF, Murphy EF, Nilaweera K, Ross PR, Shanahan F, O'Toole PW, Cotter PD. (2012) The gut microbiota and its relationship to diet and obesity: new insights. *Gut Microbes* **3**:186–202 DOI 10.4161/gmic.20168.

Cox AJ, West NP, Cripps AW. 2015. Obesity, inflammation, and the gut microbiota. *The Lancet Diabetes and Endocrinology* **3**:207–215 DOI 10.1016/S2213-8587(14)70134-2.

Hakkak R, Korourian S, Foley SL, Erickson BD. 2017. Assessment of gut microbiota populations in lean and obese Zucker rats. *PLoS ONE* **12**: e0181451 DOI 10.1371/journal.pone.0181451.

Hansen TH, Gøbel RJ, Hansen T, Pedersen O. 2015. The gut microbiome in cardio-metabolic health. *Genome Medicine* **7**:33 DOI 10.1186/s13073-015-0157-z.

Harley IT, Karp CL. 2012. Obesity and the gut microbiome: Striving for causality. *Molecular Metabolism* **1**:21–31 DOI 10.1016/j.molmet.2012.07.002.

Hawkesworth S, Moore SE, Fulford AJC, Barclay GR, Darboe AA, Mark H, Prentice AM. 2013. Evidence for metabolic endotoxemia in obese and diabetic Gambian women. *Nutrition and Diabetes* **3**:e83 DOI 10.1038/nutd.2013.24.

Huang W, Dedousis N, O'Doherty RM. 2007. Hepatic steatosis and plasma dyslipidemia induced by a high-sucrose diet are corrected by an acute leptin infusion. *Journal of Applied Physiology* **102**:2260–2265 DOI 10.1152/japplphysiol.01449.2006.

Jackman MR, MacLean PS, Bessesen DH. (2010) Energy expenditure in obesity-prone and obesity-resistant rats before and after the introduction of a high-fat diet. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **299**: R1097–R1105 DOI 10.1152/ajpregu.00549.2009.

Jiang W, Wu N, Wang X, Chi Y, Zhang Y, Qiu X, Hu Y, Li J, Liu Y. (2015) Dysbiosis gut microbiota associated with inflammation and impaired mucosal immune function in intestine of humans with non-alcoholic fatty liver disease. *Scientific Reports* **5**:1–7 DOI 10.1038/srep08096.

Jouihan H. (2012) Measurement of liver triglyceride content. *Bio-protocol* **2**: e223 DOI 10.21769/BioProtoc.223.

Kaakoush NO. (2015) Insights into the role of *Erysipelotrichaceae* in the human host. *Frontiers in Cellular and Infection Microbiology* **5**:84 DOI 10.3389/fcimb.2015.00084.

Kim KA, Gu W, Lee IA, Joh EH, Kim DH, Faulds M, Anderson N. 2012. High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. *PLoS ONE* **7**:e47713 DOI 10.1371/journal.pone.0047713.

Lecomte V, Kaakoush NO, Maloney CA, Raipuria M, Huinao KD, Mitchell HM, Morris MJ. (2015) Changes in gut microbiota in rats fed a high fat diet correlate with obesity-associated metabolic parameters. *PLoS ONE* **10**:1–22 DOI 10.1371/journal.pone.0126931.

Li D, Chen H, Mao B, Yang Q, Zhao J, Gu Z, Zhang H, Chen W. (2017) Microbial biogeography and core microbiota of the rat digestive tract. *Scientific Reports* **8**: 45840 DOI 10.1038/srep45840.

Liu X, Henkel AS, LeCuyer BE, Schipma MJ, Anderson KA, Green RM. (2015) Hepatocyte X-box binding protein 1 deficiency increases liver injury in mice fed a

high-fat/sugar diet. *American Journal of Physiology – Gastrointestinal and Liver Physiology* **309**: G965–974 DOI 10.1152/ajpgi.00132.2015.

Mokhtari Z, Gibson DL, Hekmatdoost A. (2017) Nonalcoholic fatty liver disease, the gut microbiome, and diet. *Advances in Nutrition* **8**:240–252 DOI 10.3945/an.116.013151.

Moore XJ, Chaudhary N, Akinyemiju T. (2017) Metabolic syndrome prevalence by race/ethnicity and sex in the United States, National Health and Nutrition Examination Survey, 1988-2012. *Preventing Chronic Disease* **14**:1–16 DOI 10.5888/pcd14.160287.

Nagao-Kitamoto H, Kamada N. (2017) Host-microbial cross-talk in inflammatory bowel disease. *Immune Network* **17**:1–12 DOI 10.4110/in.2017.17.1.1.

Nagy-Szakal D, Hollister EB, Luna RA, Szigeti R, Tatevian N, Smith CW, Versalovic J, Kellermayer R. (2013) Cellulose supplementation early in life ameliorates colitis in adult mice. *PLoS ONE* **8**: e56685 DOI 10.1371/journal.pone.0056685.

Netto Candido TL, Bressan J, Alfenas RCG. (2018) Dysbiosis and metabolic endotoxemia induced by high-fat diet. *Nutricion Hospitalaria* **35**: 1432-1440. DOI 10.20960/nh.1792.

Oliveira RB, Canuto LP, Collares-Buzato CB. [Epub ahead of print] Intestinal lumina; content from high-fat-fed prediabetic mice changes epithelial barrier function in vitro. *Life Science*. DOI 10.1016/j.lfs.2018.11.012.

Rajilić-Stojanović M, Biagi E, Heilig HGHJ, Kajander K, Kekkonen RA, Tims S, De Vos WM. (2011) Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. *Gastroenterology* **141**:1792–1801 DOI 10.1053/j.gastro.2011.07.043.

Roth CL, Elfers CT, Figlewicz DP, Melhorn SJ, Morton GJ, Hoofnagle A, Yeh MM, Nelson JE, Kowdley KV. (2012) Vitamin D deficiency in obese rats exacerbates nonalcoholic fatty liver disease and increases hepatic resistin and Toll-like receptor activation. *Hepatology* **55**:1103–1111 DOI 10.1002/hep.24737.

Schmidt SL, Harmon KA, Sharp TA, Kealey EH, Bessesen DH. (2012) The effects of overfeeding on spontaneous physical activity in obesity prone and obesity resistant humans. *Obesity* **20**:2186–2193 DOI 10.1038/oby.2012.103.

Schneider CA, Rasband WS, Eliceiri KW. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9**:671–675 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5554542/.

Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. (2011) Metagenomic biomarker discovery and explanation. *Genome Biology* **12**: R60. DOI 10.1186/gb-2011-12-6-r60.

Sweazea KL, Lekic M, Walker BR. (2010) Comparison of mechanisms involved in impaired vascular reactivity between high sucrose and high fat diets in rats. *Nutrition and Metabolism* **7**:48 DOI 10.1186/1743-7075-7-48.

Sweazea KL, Walker BR. (2011) High fat feeding impairs endothelin-1 mediated vasoconstriction through increased iNOS-derived nitric oxide. *Hormone and Metabolic Research* **43**:470–476 DOI 10.1055/s-0031-1273763.

Tan Y, Lao W, Xiao L, Wang Z, Xiao W, Kamal M, Paul Seale J, Qu X. (2013) Managing the combination of nonalcoholic fatty liver disease and metabolic syndrome with Chinese herbal extracts in high-fat-diet fed rats. *Evidence-Based Complementary and Alternative Medicine* **2013**:306738 DOI 10.1155/2013/306738.

Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. (2009) The effect of diet on the human gut microbiome: A metagenomic analysis in humanized gnotobiotic mice. *Science Translational Medicine* **1**:6RA14 DOI 10.1126/scitranslmed.3000322.

Vaughn AC, Cooper EM, Dilorenzo PM, O'Loughlin LJ, Konkel ME, Peters JH, Hajnal A, Sen T, Lee SH, de la Serre CB, Czaja K. (2017) Energy-dense diet triggers changes in gut microbiota, reorganization of gut-brain vagal communication and increases body fat accumulation. *Acta Neurobiologiae Experimentalis* **77**:18–30 https://www.ncbi.nlm.nih.gov/pubmed/28379213.

Yin J, Li Y, Han H, Chen S, Gao J, Liu G, Wu X, Deng J, Yu O, Huang X, Fang R, Li T, Reiter RJ, Zhang D, Zhu C, Zhu G, Ren W, Yin Y. (2018) Melatonin reprogramming of gut microbiota improves lipid dysmetabolism in high-fat-diet-fed mice. *Journal of Pineal Research*. **65**:e12524.

Younossi Z, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, George J, Bugianesi E. (2018) Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol.* 15:11-20 DOI 10.1038/nrgastro.2017.109

Zhu L, Baker RD, Baker SS. (2015) Gut microbiome and nonalcoholic fatty liver diseases. *Pediatric Research* **77**:245–251 DOI 10.1038/pr.2014.157.

Table 1: Morphometric Parameters Following 6-week Feeding Protocol

Parameters	Chow	HFD	p
Body Mass (g)	357.3±4.22	368.9±9.31	0.345
Epididymal Fat Pad Mass (g)	3.10±0.17	*5.59±0.47	0.002
Abdominal Circumference (cm)	17.22±0.08	*18.34±0.20	0.003

Data is expressed as mean ±SEM, *n*=5-8 per group. Data were analyzed using the non-parametric Mann-Whitney U test.

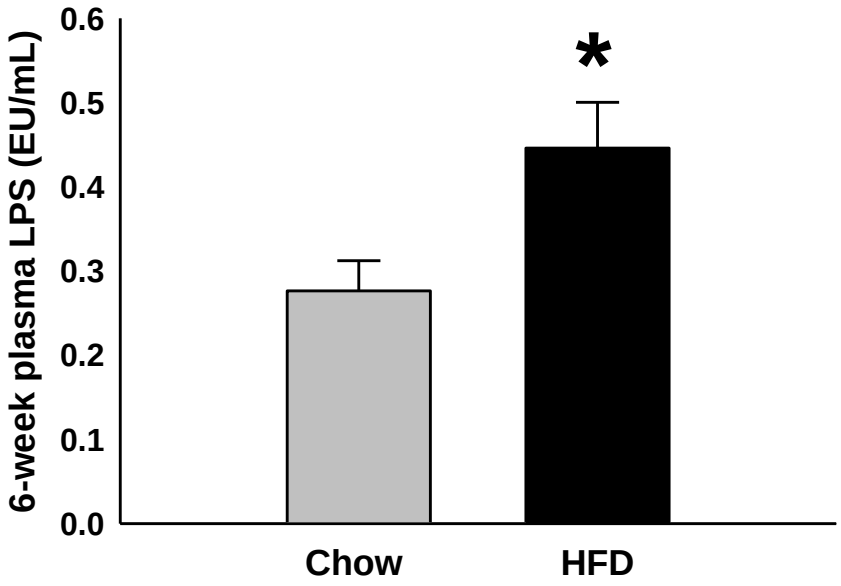


Figure 1: Plasma lipopolysaccharide concentrations in Chow and HFD rats

Plasma LPS concentrations were significantly higher in male Sprague-Dawley rats fed HFD for 6 weeks as compared to chow-fed animals (*$p=0.032$). Data are expressed as mean ±SEM, n=5 per group.

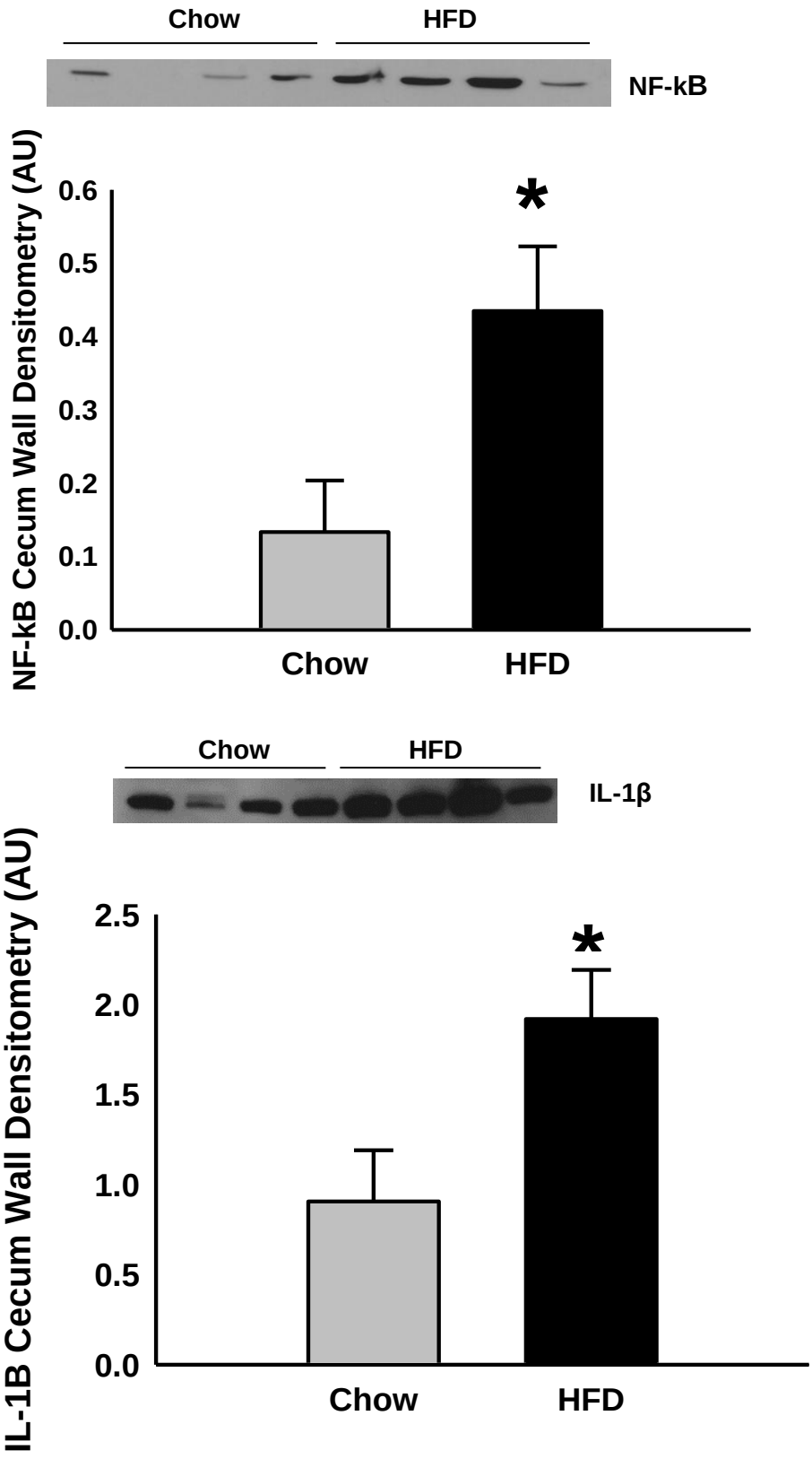

Chow
HFD
NF-kB
NF-kB Cecum Wall Densitometry (AU)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
Chow
HFD
Chow
HFD
IL-1β
IL-1B Cecum Wall Densitometry (AU)
2.5
2.0
1.5
1.0
0.5
0.0
*
Chow
HFD

Figure 2: Western blot analysis of NF-kB and IL-1β in the cecum

Western blots were used to detect protein expression of NF-kB in ceca homogenates from male Sprague-Dawley rats fed either Chow or HFD for 6 weeks. Densitometry of the bands of interest were normalized to total protein expression as determined by Coomassie Brilliant Blue and are expressed as a ratio (± SEM). Inset: Representative blot of NF-kB (64 kDa) in ceca homogenates from rats in both treatment groups (n=4 per group). NF-kB concentration was increased in the cecal wall of HFD-fed rats compared to chow-fed rats (Student's t-test, df=6, t=-2.670, *p=0.037). Inset: Image of IL-1β (31 kDa) in ceca homogenates from rats in all both treatment groups (n=4 per group). HFD rats had a significant increase in IL-1β concentrations in the cecal wall (Student's t-test, df= 6, t=-2.572, *p=0.042).

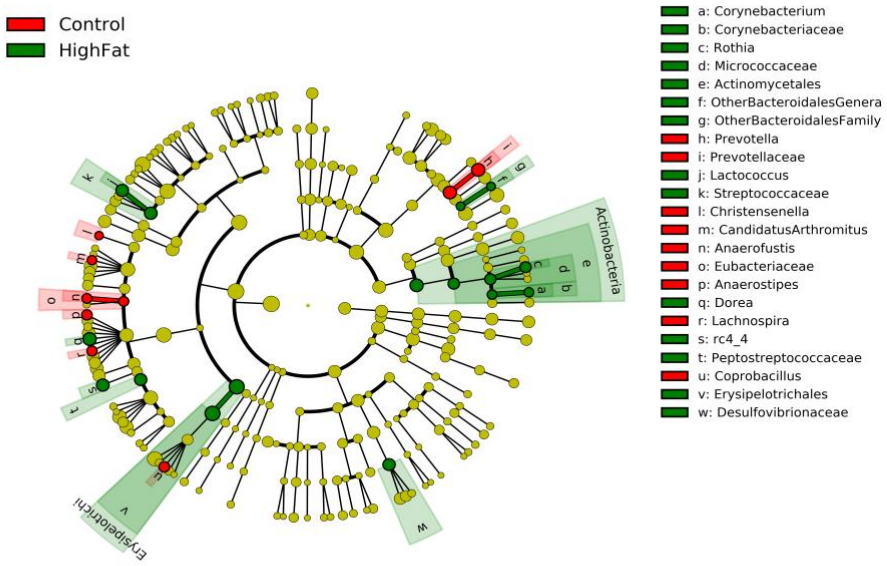


Figure 3: Hierarchical structure of differentially abundant taxa

Cladogram depicting differentially abundant microbial taxa in cecal samples collected from male Sprague-Dawley rats fed either Chow (n=6) or HFD (n=6) for 6 weeks. Microbial taxa enriched in HFD rats are shown in green and those enriched in chow-fed animals are shown in red.

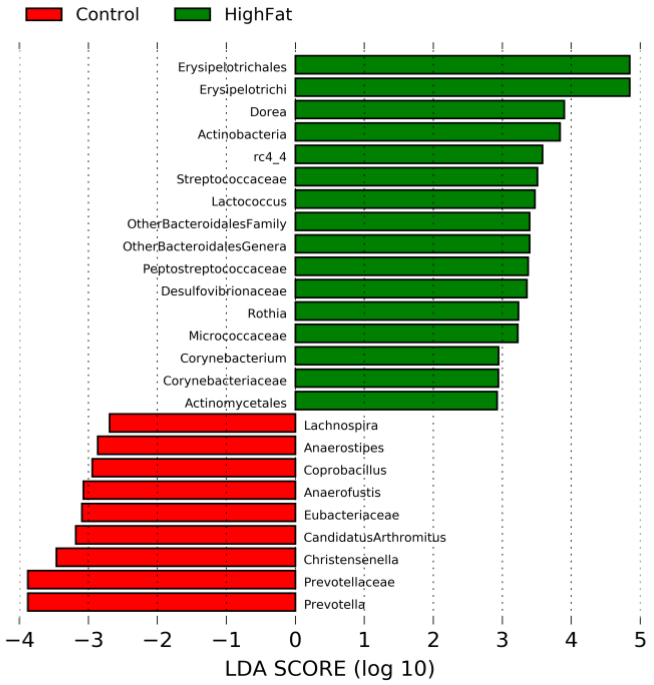
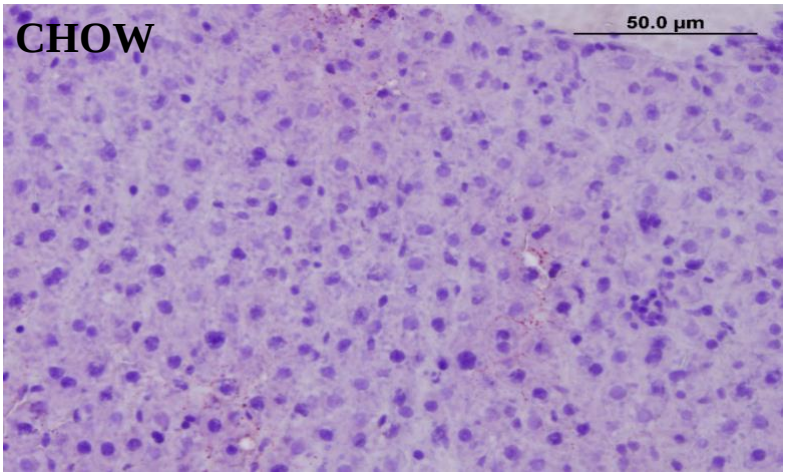


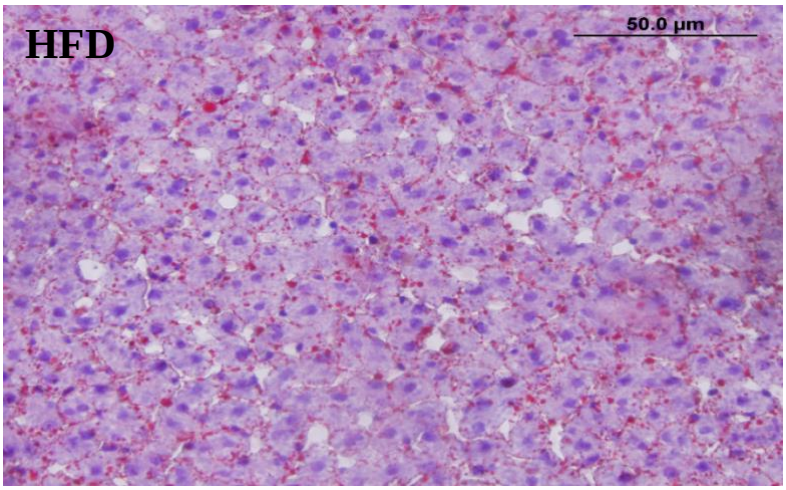
Figure 4: Effect sizes for differentially abundant taxa

A) Linear discriminate analysis of effect size (LEfSe) results depicting different microbial taxonomic signatures in samples collected from the cecum of male Sprague-Dawley rats fed either HFD (n=6) or chow (n=6) for 6 weeks. Cecal samples collected from chow-fed animals (red, negative PDA score bars) are more enriched in *Prevotella* and *Prevotellaceae* while cecal samples collected from HFD-fed animals (green, positive LDA score bars) are more abundant in *Erysipelotrichales* and *Erysipelotrichi.* B) F:B ratio for cecal samples from chow (n=5) versus HFD (n=5) rats. *Bacteriodetes* were not detected in n=1 chow and n=1 HFD rat. Therefore, these samples were excluded from this analysis.

A


CHOW
50.0 µm

B


HFD
50.0 µm

C

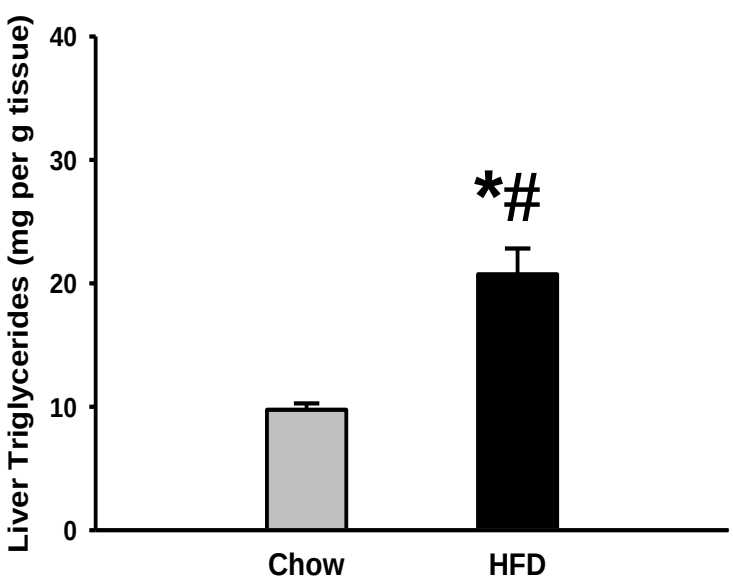

Liver Triglycerides (mg per g tissue)
40
30
20
10
0
*#
Chow
HFD

Figure 5: Hepatic lipid accumulation

Representative images of Oil-Red-O stained hepatic tissues collected from male Sprague-Dawley rats fed either chow or high-fat diet (HFD) for 6 weeks. Frozen tissue sections were stained with Oil-Red-O to show lipid content and counterstained with hematoxylin to show nuclei (blue). Livers from HFD rats showed evidence of simple steatosis after 6 weeks (*p<0.001). Images taken at 200X.

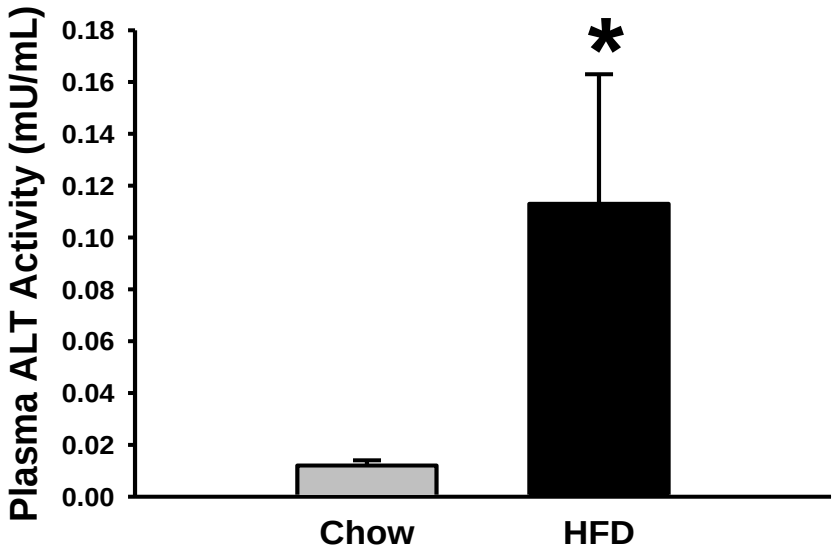


Figure 6: Plasma ALT activity in Chow and HFD rats

Plasma ALT activity was significantly higher in male Sprague-Dawley rats fed HFD as compared to chow diet for 6 weeks (*$p<0.001$). Data are expressed as mean ± SEM, n=10 per group.

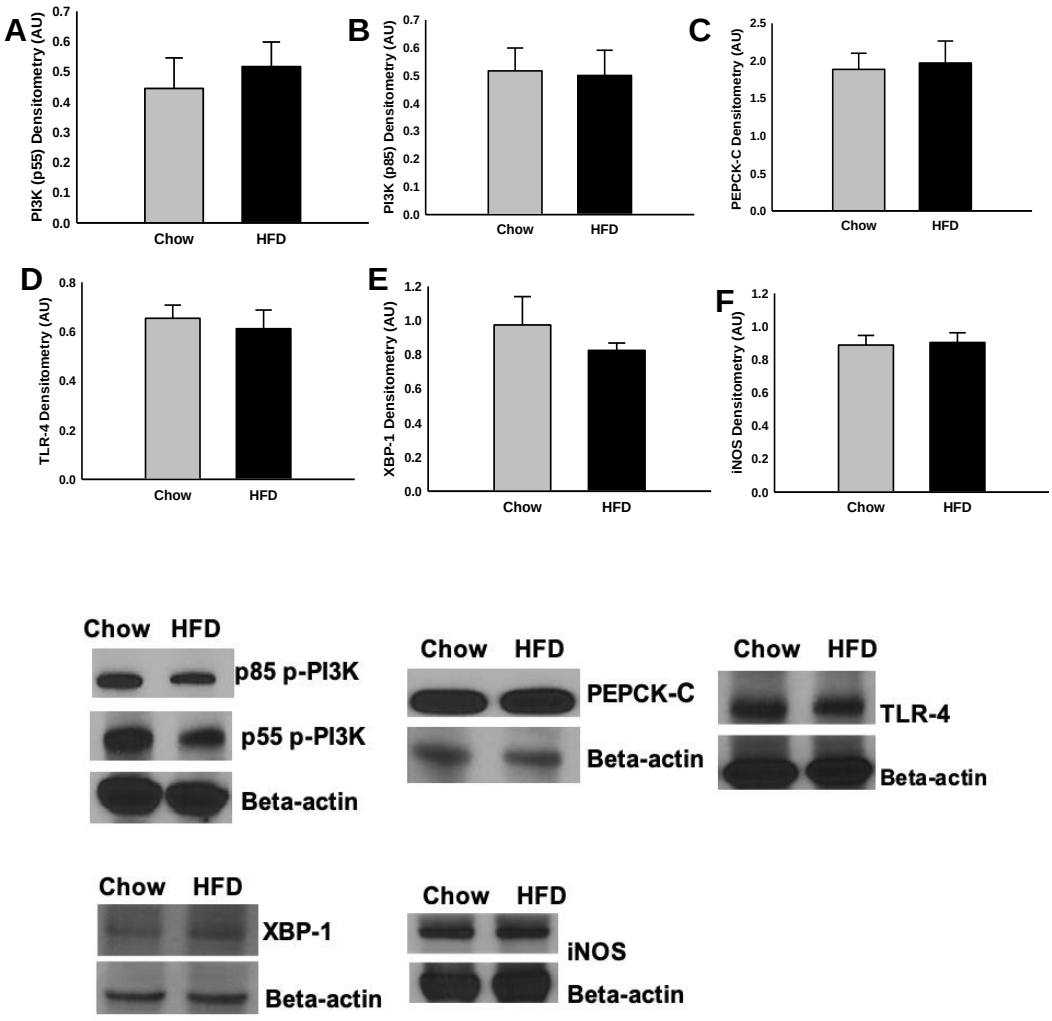


Figure 7: Western blot analyses of PI3K, PEPCK-C, TLR-4, X$BP-1 and iNOS in liver tissue Protein expression of several insulin signaling intermediates and inflammatory cytokines in liver samples collected from male Sprague-Dawley rats fed either HFD or chow for 6 weeks were analyzed by Western blot. Values are expressed as a ratio (± SEM). (A, B) Representative blot of p-PI3K (p85 (Tyr 458)/p55 (Tyr 199)) in liver homogenate from rats in all three treatment groups (n=6/group), *p=0.884* and *p=0.576* respectively. (C) Representative blot of PEPCK-C in liver homogenate from rats in all three treatment groups (n=5-6/group) *p=0.819.* (D) Representative blot of TLR-4 in

liver homogenate from rats in all three treatment groups (n=6/group), *p=0.623*. (E) Representative blot of iNOS liver homogenate from rats in all three treatment groups (n=6/group), *p=0.240.* (F) Representative blot of XBP1 (n=6/group, *p=0.622*).

CHAPTER THREE

A NOVEL ORGANOMETALLIC COMPLEX ATTENUATES RISK FACTORS IN A RODENT MODEL OF METABOLIC SYNDROME

(In Review for publication)

ABSTRACT

The prevalence of metabolic syndrome has risen 35% since 2012 and over two-thirds of Americans exhibit features characterizing this condition (obesity, dyslipidemia, hyperglycemia, insulin resistance and/or endothelial dysfunction). The aim of this study was to evaluate the effects of a novel dietary supplemental organometallic complex (OMC) on these risk factors in a rodent model of metabolic syndrome-like complications. Six-week old male Sprague-Dawley rats were fed either standard chow or a high-fat diet (HFD) composed of 60% kcal from fat for 10 weeks. Rats were also treated with OMC in their drinking water at either 0 mg/mL (control), 0.6 mg/mL, or 3.0 mg/mL. The HFD-treated rats exhibited significantly increased body mass ($p<0.05$), epididymal fat pad mass ($p<0.001$), abdominal circumference ($p=0.010$), in addition to elevations in plasma lipopolysaccharide ($p<0.001$), hepatocyte injury (plasma ALT activity; $p<0.05$), fasting serum glucose ($p=0.025$) and insulin concentrations ($p=0.009$). OMC did not affect body weight or adiposity induced by the HFD. At the higher dose OMC significantly blunted HFD-induced hyperglycemia ($p=0.021$), whereas both low and high doses of OMC prevented HFD-induced increases in LPS ($p=0.002$ and <0.001, respectively) and hepatocyte injury (ALT activity, $p<0.05$). Despite evidence of oxidative stress (elevated urinary H_2O_2 $p=0.032$) in HFD-treated rats, OMC exhibited no demonstrable antioxidative effect. Consistent with prior studies, HFD rats developed impaired

endothelium-dependent vasodilation and the high dose of OMC abrogated this impairment ($p<0.05$). These findings suggest that the OMC supplement, particularly at the higher dose, ameliorated several risk factors associated with metabolic syndrome via a non-antioxidant-dependent mechanism.

INTRODUCTION

Current estimates predict that approximately 20-25% of all US adults have metabolic syndrome (MetSyn), a constellation of symptoms characterized by abdominal obesity, dyslipidemia, hypertension, insulin resistance, and a pro-inflammatory or thrombotic state [1]. Several interrelated environmental and lifestyle factors contribute toward development of MetSyn and culminate in an increased risk for developing cardiovascular disease and type 2 diabetes [2]. The pathogenesis and underlying mechanisms responsible for developing MetSyn are still subject to intensive investigation; however, excessive caloric intake and lack of physical activity are well established contributory factors. As a consequence of the effects of these factors, metabolic derangements including glucose and lipid dysregulation, insulin resistance, and endothelial dysfunction may be further promoted by oxidative stress and activation of various inflammatory cascades characterized by stimulation of interleukin-6 and tumor necrosis factor alpha (TNF-α) synthesis within adipocytes and immune cells [3], and c-reactive protein (CRP) within the liver [4].

Because central obesity and visceral adiposity are two common hallmarks of MetSyn, management of the condition inevitably involves pharmacological and/or dietary-induced weight loss and weight loss maintenance [5], as well as increased

physical activity [6]. Other recommendations for MetSyn management include reductions in dietary sodium, simple carbohydrates, trans-fatty acids, cholesterol, and saturated fatty acids as exemplified by individuals adhering to the Mediterranean or Dietary Approaches to Stop Hypertension (DASH) diets [7, 8]. In addition to these overall comprehensive modifications to diet and lifestyle, several dietary components or bioactives have been reported to positively influence one or more MetSyn risk factors. Included here are turmeric, polyphenols, cinnamon, garlic, omega-3 fatty acids, and cruciferous vegetables [9, 10]. Ultimately, management and greater understanding of the underlying pathophysiology and biochemical mechanisms will result in greater potential therapeutic modalities in treating and preventing MetSyn.

As part of our longstanding interest in elucidating the effects of metabolic dysfunctions associated with vascular and endothelial derangements, we have utilized a high-fat diet (HFD)-induced rodent model of MetSyn. Previously we observed that rats fed HFD for 6 weeks exhibited all the hallmarks of MetSyn including weight gain, increased oxidative stress, visceral adiposity, inflammation, and glucose and lipid dysregulation [11, 12]. Moreover, we previously demonstrated that HFD impairs nitric oxide (NO)-dependent and independent vasodilation in isolated small resistance mesenteric arteries [11]. Interestingly, these effects were reversed by antioxidants or anti-inflammatory interventions [11] thereby providing potential avenues for MetSyn management.

Although diet and exercise are recommended for the treatment of MetSyn, natural health products are a popular alternative for humans who are resistant to dietary change. The purpose of the current study was to characterize the effect of a novel and unique

dietary supplemental organometallic complex (OMC) on the pathophysiological and biochemical disturbances observed in this HFD-induced model of MetSyn. OMC is a complex derived from plant and soil fractions. A prior study has shown that supplementation with OMC for 31 days decreased weight gain, blood glucose concentrations and hemoglobin A1c in genetically diabetic mice [Deneau et al., 2010]. In the current study, we observed similar effects where OMC significantly attenuated several risk factors associated with MetSyn.

MATERIALS AND METHODS

Animal Model

Six-week old male Sprague-Dawley rats (157.5 ± 1.32 g body mass; n=42) were purchased from Envigo (formerly Harlad Teklad) and randomly divided into two groups: either a standard chow maintenance diet (n=10, 3.1 kcal/g; Envigo, Madison, WI) or a 60% kcal from fat diet (n= 8, 5.21 kcal/g; Cat. No. D12492; Research Diets Inc, New Brunswick, NJ) for 10 weeks. Female rats were excluded as estrogen exerts cardioprotective effects in young animals. The calorie composition of the chow diet is 24% from protein, 58% from carbohydrates, and 18% from fat whereas the high fat diet was comprised of 20% protein, 20% carbohydrates and 60% fat (mainly saturated fat – lard). Rats in each dietary group (n=6/group) were administered 0 (control), 0.6, or 3.0 mg/mL OMC (provided by Isagenix International, LLC; Gilbert, Arizona) in their drinking water throughout the study. The dosage of OMC chosen for this study was based upon previous work by Deneau et al. [13] who evaluated a very similar ingredient in a mouse model of genetically-induced diabetes. Rats were exposed to 12:12 h light: dark cycle and were housed singly to avoid coprophagic cross-contamination as separate

studies were designed to evaluate the gut microbiome. Food and OMC-treated water was replaced every 2-3 days to prevent spoiling. Animals were allowed free access to water and food *ad libitum*. Study animals were euthanized (sodium pentobarbital, 200 mg/kg, i.p.) at the end of the 10 weeks. All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee.

OMC Supplement

Organometallic complex (OMC) is a proprietary nutritional ingredient marketed by the study sponsor (Isagenix International, LLC, Gilbert, AZ) and is a constituent of their trademarked ingredient "Ionic Alfalfa™". The ingredient is organically extracted and isolated for use as a dietary supplement by Mineral Biosciences, LLC (Goodyear, AZ). OMC is an ancient plant and soil-derived material containing several trace elements, minerals and organic compounds. Further chemical and biological characteristics are detailed in Table 1.

Morphometrics

Body mass was measured weekly to assess changes in response to the diet and OMC treatments. Nasoanal length, tail length, and abdominal circumference (immediately anterior to the hindleg) were measured using a flexible tape measure at the end of the 10-week trial. Gerbaix et al. [14] compared measurements of abdominal circumference in rats with values obtained by dual-energy x-ray absorptiometry (DXA) and found a strong significant correlation showing that abdominal circumference can be used to predict the accumulation of visceral fat in these animals. Following euthanasia, blood was collected by cardiac puncture and the plasma isolated and frozen at -80°C until

use. The epididymal fat pad was extracted from each animal to assess adiposity as previously described [11]. Lee's Index of Obesity was calculated as cube root of body mass (g) / nasoanal length (cm)) * 1000 [15]. Body mass index (BMI) was calculated as body mass (g) / nasoanal length $(cm)^2$ [16].

Glucoregulatory Variables

Rats were food-restricted by providing an aliquot of food (2g/rat at baseline and 4g/rat at weeks 6 and 10) at 6:00 pm the night prior to the fasting blood draws. The following morning fasting blood samples (~1mL) were collected from the tail vein at baseline, weeks 6, and 10. Serum was then separated from whole blood and stored at -80°C until analyses. Fasting serum glucose concentrations were measured via the glucose oxidase method using a commercially available kit (Cat. No. 10009582, Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol. Fasting serum insulin concentrations were measured using a commercially available kit (Cat. No. 90060, Crystal Chem, Elk Grove Village, IL). Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated from the fasting serum glucose and insulin concentrations using the following equation: (fasting serum glucose (mM/L) x fasting serum insulin (mU/L)) / 22.5. Studies found that HOMA-IR is correlated with hyperinsulinemic-isoglycemic clamps in rats [17]. HOMA-IR is also correlated with insulin tolerance tests and can thus be used as a surrogate marker of insulin resistance in rats fed a high fat diet with 90% sensitivity [18].

Biomarkers of Oxidative Stress and Hepatocyte Injury

Plasma superoxide dismutase (SOD) activity was measured at the end of the study using commercially available kits (Cat. No. 706002, Cayman Chemical, Ann Arbor, Michigan). Urine hydrogen peroxide and creatinine concentrations were measured using commercially available kits (Cat. No. ab102500, Abcam, Cambridge, MA; Cat. No. CR01, Oxford Biochemical Research, Rochester Hilla, MI). Activity of plasma ALT and AST were also measured at the end of the 10-week feeding protocol using commercially available kits (Cat. No. MAK052 and MAK055, respectively; Sigma Aldrich, St. Louis, MO).

Quantification of Plasma Lipopolysaccharide

Plasma lipopolysaccharide concentrations were quantified with a commercially available kit (Cat. No. 88282, Thermo Fisher Scientific Rockford, IL) per the manufacturer's protocol.

Endothelium-dependent Vasodilation

Stock solutions of acetylcholine (ACh, 1.0 M, Sigma Aldrich) and phenylephrine (PE, 1.0M, Sigma Aldrich) were prepared in deionized water, aliquoted, and stored at -20°C until use. Following euthanasia, a midline laparotomy was performed to remove the mesenteric arcade, which was immediately transferred to ice-cold HEPES-buffered saline (in mM: 134.4 NaCl, 6 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, and 10 glucose, pH 7.4 with NaOH). The arcade was pinned out in a Silastic-coated dissection dish and mesenteric resistance arteries (~1mm length; 126 ± 3 μm, i.d.) were isolated and transferred to a vessel chamber (Cat. No. CH-1, Living Systems Instrumentation, St. Albans, VT)

containing HEPES-buffered saline. Isolated arteries were then cannulated with glass pipettes, secured with silk ligatures, and stretched longitudinally to approximate *in situ* length. Vessels were then pressurized to 60 mmHg using a servo-controlled peristaltic pump (Living Systems Instrumentation; St. Albans, VT) and the vessel chamber transferred to the stage of an inverted Nikon microscope for analysis. Vessels were continuously superfused with warm (37°C) physiological saline solution (PSS, in mM: 129.8 NaCl, 5.4 KCl, 0.5 NaH_2PO_4, 0.83 $MgSO_4$, 19 $NaHCO_3$, 1.8 $CaCl_2$, and 5.5 glucose) at a rate of 10 mL/min. PSS was aerated with a gas mixture containing 21% O_2, 6% CO_2, balance N_2 to maintain pH and oxygenation.

Following a 30-minute equilibration of isolated arteries in PSS, vessels were pre-constricted with increasing concentrations of PE in the superfusate until they reached 50% of their resting inner diameter. Endothelium-dependent vasodilation was assessed by exposing pre-constricted arteries to stepwise increases of the endothelium-dependent vasodilator ACh (10^{-9} to 10^{-5} M, 3 min per step) in the superfusate followed by a calcium-free PSS solution (in mM: 129.8 NaCl, 5.4 KCl, 0.5 NaH_2PO_4, 19.0 $NaHCO_3$, 5.5 glucose, and 3 EDTA) to measure the passive inner diameter. Intraluminal diameter (i.d.) was continuously monitored from video microscopy of bright field images using an edge-detection Vessel Diameter System (IonOptix, Milton, MA, USA). Vasodilation was calculated as the percent reversal of PE-mediated vasoconstriction.

STATISTICAL METHODS

All data are expressed as means ± SEM. Data collected at multiple time points (body mass, glucose and insulin) were analyzed by two-way repeated measures ANOVA.

Percent data were arcsine-transformed to approximate normal distribution prior to analyses. Epididymal fat pad mass data were log transformed prior to analysis by two-way ANOVA to approximate normal distribution. All other data were analyzed by two-way ANOVA with Tukey posthoc analyses (SigmaPlot 10.0, Systat Software, San Jose, CA). A probability of ≤ 0.05 was accepted as statistically significant.

RESULTS

Morphometrics

HFD rats gained significantly more weight than chow-fed controls (two-way RM ANOVA, df=5, F=7.056, $p<0.001$, Fig 1). This effect of HFD was lost at week 10 for animals supplemented with the high dose of OMC (Table 2). Similarly, epididymal fat pad mass (one-way ANOVA followed by Tukey, H=26.129, df=5, $p<0.001$) abdominal circumference (one-way ANOVA followed by Tukey, H=14.267, df=5, $p<0.001$), and naso-anal length (one-way ANOVA, Student-Newman-Keuls, df=5, F=5.801, $p<0.02$) were all significantly increased in HFD rats compared to controls (Table 2). Since tail length was not different between groups, the change in naso-anal length may be attributed to the increase in abdominal circumference in the HFD animals. Neither Lee's Index of Obesity nor BMI were significantly different between HFD and chow fed rats indicating that the rats were simply overweight as opposed to obese (Table 2). OMC did not affect any morphometric variable (Fig 1, Table 2). While attempts were made to measure water intake (and hence intake of the OMC), the rodent water bottles tended to leak if disturbed allowing for only estimations of intake. The HFD is highly palatable. Thus, food intake

was not measured as the high fat content of the food makes it difficult to separate from the bedding material for accurate measurement.

Glucoregulatory Variables

Rats developed significant hyperglycemia following 6 and 10 weeks of HFD (two-way ANOVA, $p<0.001$ and $p=0.006$, respectively) compared to chow-fed controls (Table 3). While administration of low dose OMC (0.6 mg/mL) tended to reduce HFD-induced hyperglycemia (two-way ANOVA, $p=0.067$), high dose OMC (3 mg/mL) significantly prevented hyperglycemia at 10 weeks (two-way ANOVA, $p=0.021$). OMC had no effect on fasting serum glucose concentrations in chow-fed animals. After 6 weeks of high fat intake rats trended towards increased fasting serum insulin concentrations compared to chow controls (two-way ANOVA, $p=0.079$). By 10 weeks differences in fasting serum insulin were significant between diets (two-way RM ANOVA, $p=0.007$). Similarly, fasting serum insulin concentrations tended to be higher after 6 weeks in HFD rats treated with 0.6 mg/mL OMC compared to the respective chow controls (two-way ANOVA, $p=0.029$). Consistent with the glucose data, HOMA-IR was greater in HFD rats after 6 and 10 weeks compared to chow controls ($p=0.009$ and 0.005, respectively). HFD rats supplemented with 0.6 mg/mL OMC also tended to have higher HOMA-IR than chow animals at week 6 (two-way ANOVA, $p=0.057$). Although HOMA-IR was increased with HFD it was not affected by OMC.

Biomarkers of Oxidative Stress and Hepatocyte Injury

Plasma SOD activity was significantly higher in chow rats treated with 0.6 mg/mL OMC compared to HFD-fed rats treated with the same dose (two-way ANOVA followed by Student-Newman-Keuls test, p=0.047; Fig 2A). This difference was likely driven by the slight elevation in SOD activity in the chow animals. No other changes in SOD activity were observed. HFD-fed rats had significantly elevated urinary concentrations of H_2O_2 compared to chow fed animals (two-way ANOVA, df=1, F=13.725, p=0.001), which were not affected by OMC (Fig 2B).

Plasma ALT activity was significantly greater in HFD rats compared to chow-fed animals (two-way ANOVA, df=1, F=45.13, p<0.001, Fig 3A). The high dose of OMC significantly prevented the increase in HFD-induced ALT activity compared to both the control (two-way ANOVA followed by Tukey test, p=0.005) and low-dose (two-way ANOVA followed by Tukey test, p=0.014) treated animals. In fact, there were no differences in ALT activity between the HFD and Chow rats treated with the high dose of OMC (two-way ANOVA followed by Tukey test, p=0.113; Fig 3A). Additionally, the low dose of OMC significantly reduced HFD-induced elevations in ALT activity (two-way ANOVA followed by Tukey test, p<0.001). In contrast, there were no significant differences in AST activity between or within the groups (Fig 3B).

Quantification of Plasma Lipopolysaccharide (LPS)

Plasma LPS were significantly elevated in the HFD rats compared to chow control (two-way ANOVA, df=5, F=4.886, p<0.05; Fig 4). However, treatment with OMC significantly blunted HFD-induced increases in plasma LPS at both 0.6 and 3.0

mg/mL OMC (two-way ANOVA by Student-Newman-Keuls Method, $p<0.05$; Fig. 2C). No effect of OMC toward plasma LPS was observed in control chow-fed rats.

Endothelium-dependent Vasodilation

Endothelium-dependent vasodilation of *ex vivo* arteries from HFD animals was significantly impaired in comparison to arteries isolated the chow-fed controls (two-way RM ANOVA, F=0.582, $p=0.030$ Fig 5). OMC was effective at both doses at preventing the HFD-induced impaired vasodilation (two-way RM ANOVA, F=3.008, $p<0.001$, Fig 5B). In contrast, the low dose of OMC slightly impaired vasodilation of *ex vivo* arteries from chow rats, although responses to the higher doses of ACh were normal (Fig 5A). The high dose of OMC slightly increased vasodilation compared to the low dose in arteries from chow-fed animals (Fig 5A).

DISCUSSION

The present study evaluated and characterized the effect of OMC supplementation on MetSyn-associated biochemical and pathological events in a HFD rat model. The OMC supplement is a soil-derived complex primarily consisting of a combination of minerals, trace elements, organic acids, particularly fulvic acid, and various other microbial degradation products from plant and animal origins. Results from this study demonstrated that OMC attenuated several features associated with MetSyn and warrants further investigation.

Metabolic syndrome is a cluster of risk factors that collectively are associated with increased prevalence of cardiovascular disease [19]. Diagnostically, the presence of three or more of these risk factors - abdominal obesity, elevated fasting glucose, dyslipidemias (reduced HDL and increased triglycerides), inflammation, and/or

hypertension – define MetSyn. Previous research from our laboratory have confirmed HFD feeding promotes symptoms consistent with MetSyn-associated sequelae [11,12]. For example, HFD feeding to 6-week old male Sprague Dawley rats increased body mass and abdominal adiposity, promoted oxidative stress, impaired endothelium-dependent vasodilation, and elevated fasting glucose. Similar findings have been reported in the Wistar rat [20, 21], thereby supporting a strain-independent, pathological similarity in this model of MetSyn-like complications.

Protection against several MetSyn-associated pathological changes were afforded by OMC whereas others were unaffected. For example, OMC-treated animals were protected against HFD-induced hyperglycemia. This protection may be attributed to several biological or chemical properties exhibited by the materials' primary components. First, OMC is rich in minerals and trace elements and this mineral profile may be favorable against several HFD-mediated pathophysiological endpoints including glucose and insulin regulation. In this context, increased intake of several dietary minerals has been associated with a reduced risk for developing MetSyn [22]. Additionally, fulvic acid was recently reported to stimulate insulin secretion in pigs without affecting glucose concentrations [23]. We did not observe any influence of OMC on either serum glucose or insulin levels in Chow-fed rats, but a pronounced glycemia-moderating effect was observed in HFD-treated rats. Using a similar soil-derived mineraloid compound, leonardite, Deneau et al. [13] reported reductions in blood glucose and glycated hemoglobin in a genetically-modified mouse model of diabetes. Mechanistically, these authors speculated some of their observed effects on glucose status were associated with increased gene expression of mitochondrial and energy-regulating enzymes. Interestingly,

these researchers also reported less weight gain with their ingredient compared to control-fed animals. Likewise, the high dose of OMC prevented the augmented weight gain in animals fed HFD for 10 weeks. Although rats fed the HFD had significantly greater naso-anal length than those fed a chow diet, this was likely attributed to the increase in abdominal circumference in the HFD animals as tail length (a measure of skeletal growth) was not significantly different.

Overweight and obesity - central features of MetSyn - are also associated with endotoxemia, insulin resistance, hyperglycemia, and endothelial dysfunction perhaps proceeding increased generation of inflammatory cytokines and oxidative stress [24, 25]. Once initiated, oxidative stress can further impair endothelium-dependent vasodilation by increasing vascular levels of superoxide anion ($O_2^{\cdot -}$) and a concomitant reduction in the bioavailability of the endogenous vasodilator nitric oxide (NO) [11, 26]. Indeed, this endothelial dysfunction has been reported in obese patients [27]. Multiple factors contribute to chronic oxidative stress and inflammation in individuals with MetSyn, including chronic hyperglycemia, endotoxemia, and diets high in saturated fats. The association between HFD and saturated fat intake was described recently by Lopez-Moreno et al. (2017) who noted elevated postprandial plasma LPS purportedly due to HFD promotion of LPS intestinal absorption [28]. These endotoxins, once released from the lysis of gram-negative bacteria in the small intestine [29], induce Rac/NADPH oxidase-dependent $O_2^{\cdot -}$ generation by stimulating macrophage release of TNF-a and other proinflammatory cytokines resulting in further propagation of reactive oxygen species (ROS) generation via TNF-a attenuation of macrophage scavenging activity [24]. Thus, despite no observed antioxidant activity by OMC, the complex prevented increases

in plasma LPS concentrations. Several potential, non-antioxidant-based mechanisms may explain this protection: 1) fulvic acid, a component of OMC, has shown anti-inflammatory activity through inhibition of ERK/JNK and COX-II expression [30], 2) the compound also provides anti-microbial activity [31] and 3) shilajit, another earth-based complex frequently utilized in traditional medicine and rich in fulvic acid, also possesses a potent anti-ulcerative effect [32]. In addition, other multi-mineral rich natural products have demonstrated considerable hepato- and/or gastrointestinal-protection. In this context, Aslam et al. [33, 34] reported significant reductions in liver injury and gastrointestinal inflammation from mice fed HFD but supplemented with a mineral-rich seaweed-derived preparation. Although, again, we did not observe any antioxidant protection by OMC, other studies with similar mineral-rich natural products have been found to reduce NF-kB signaling [35], consistent with an anti-inflammatory effect such as that observed with the amelioration of plasma LPS concentrations in OMC-treated animals. Collectively, these reports hypothesize mechanisms by which OMC may alter intestinal permeability and protect against endotoxemic damage to the gastrointestinal tract. Prior research in our laboratory has shown that urinary increases in H_2O_2 were not evident following 6 weeks of HFD [36]. Data from the present study show that an additional 4 weeks of HFD resulted in increased urinary H_2O_2. However, our data do not support an antioxidant role of OMC as these levels were unaffected by the supplement. Intriguingly, oxidative stress was not attenuated by OMC, an observation that may suggest OMC ameliorated pathophysiological disturbances of HFD-induced metabolic disturbances via non-antioxidant-dependent mechanisms. Although fulvic acid itself has

demonstrated *in vitro* antioxidant properties [37] our analysis of OMC revealed a low overall oxygen radical absorbance capacity (ORAC) score.

Elevated levels of ROS such as H_2O_2 have been linked to the development of hepatic steatosis by free fatty acid peroxisomal beta-oxidation [8]. The generation of ROS can induce apoptosis of hepatocytes, promote an inflammatory response and increase ALT activity, a key indicator of liver injury [38]. Moreover, a relationship between liver injury and endotoxemia has been reported by others. For example, Kai et al (2017) observed LPS injections exacerbated liver injury in HFD-fed rats via mechanisms implicating elevated peroxisome proliferator-activated receptors (PPARs) and beta-oxidation enzymes [39]. Furthermore, inflammatory genes are activated in adipocytes through the generation of free radicals [40]. Inflammatory cytokines, such as TNF-α, can also promote insulin resistance and glucose intolerance by reducing tyrosine phosphorylation and instead promoting serine phosphorylation of insulin receptor substrate-1 (IRS-1), thereby inhibiting the cellular effects of insulin [41]. The hepatoprotective and gastrointestinal-modifying effects of OMC observed in the current study suggest multi-faceted mechanisms contributing to its overall benefits in this model of MetSyn-like complications.

In summary, the present study demonstrated OMC, a novel and unique soil-derived product, prevented several of the complications associated with HFD-induced metabolic dysfunction. Although further biochemical and chemical characterization of OMC is required (and currently underway), we hypothesize that the combination of organic acids, trace elements, and mineral composition of the supplement function collectively in this protection via non-antioxidant mechanisms.

REFERENCES

Beltran-Sanchez H, Harhay MO, Harhay, MM, McElligott, S. Prevalence and trends of Metabolic Syndrome in the adult US population, 1999–2010. J Am Coll Cardiol (2013); 62(8): 697-703. doi:10.1016/j.jacc.2013.05.064.

Rochlani, Y, Pothineni, NV, Kovelamudi, S, Mehta, JL. Metabolic syndrome: Pathophysiology, management, and modulation by natural compounds. Ther. Adv. Cardiovasc. Dis (2017); 11(8):215–225. doi: 10.1177/1753944717711379.

Bastard JP, Jardel C, Bruckert E, Blondy P, Capeau J, Laville M, et al. Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. J Clin Endocrinol Metab (2000); 85(9): 3338-42. doi:10.1210/jcem.85.9.6839.

Tsigos C, Kyrou I, Chala E, Tsapogas P, Stavridis JC, Raptis SA, et al. Circulating tumor necrosis factor alpha concentrations are higher in abdominal versus peripheral obesity. Metabolism (1999); 48(10):1332-1335. doi: 10.1016/S0026-0495(99(90277-9.

Almeda-Valdes P, Herrera-Mercadillo RJ, Aguilar-Salinas CA, Uribe M, Méndez-Sánchez N. The Role of Diet in Patients with Metabolic Syndrome. Curr Med Chem (2017). doi: 10.2174/0929867324666170518095316.

Ostman C, Smart NA, Morcos D, Duller A, Ridley W, Jewiss D. The effect of exercise training on clinical outcomes in patients with the metabolic syndrome: a systematic review and meta-analysis. Cardiovasc Diabetol (2017); 16(1):110, 2017. doi: 10.1186/s12933-017-0590-y.

Azadbakht L, Mirmiran P, Esmaillzadeh A, Azizi T, Azizi F. Beneficial effects of a Dietary Approaches to Stop Hypertension eating plan on features of the metabolic syndrome. Diabetes Care (2005); 28(12): 2823-31. https://doi.org/10.2337/diacare.28.12.2833.

Gusdon AM, Song K, Qu S. Nonalcoholic Fatty Liver Disease: Pathogenesis and Therapeutics from a Mitochondria-Centric Perspective. Oxidative Medicine and Cellular Longevity (2014); 1–20. https://doi.org/10.1155/2014/637027.

Moss JWE, Williams, JO, Ramji DP. Nutraceuticals as therapeutic agents for atherosclerosis. Biochim Biophys Acta (2018); 1864:1562-72. doi: 10.1016/j.bbadis.2018.02.006.

Ríos-Hoyo A, Cortés MJ, Ríos-Ontiveros H, Meaney E, Ceballos G, Gutiérrez-Salmeán G. Obesity, Metabolic Syndrome, and Dietary Therapeutical Approaches with a

Special Focus on Nutraceuticals (Polyphenols): A Mini-Review. Int J Vitam Nutr Res (2014); 84(3-4):113-23. doi: 10.1024/0300-9831/a000198.

Sweazea KL, Lekic M, Walker BR. Comparison of mechanisms involved in impaired vascular reactivity between high sucrose and high fat diets in rats. Nutr Metab (Lond) (2010); 7:48. doi:10.1186/1743-7075-7-48.

Sweazea KL, Walker BR. High fat feeding impairs endothelin-1 mediated vasodilation through increased iNOS-derived nitric oxide. Horm Metab Res (2011); 43(7): 470-476. doi: 10.1055/s-0031-1273763.

Deneau J, Ahmed T, Blotsky R, Bojanowski K. Anti-diabetic activity of a mineraloid isolate, in vitro and in genetically diabetic mice. Int J Vitam Nutr Res (2011); 81(1): 34-42. doi: 10.1024/0300-9831/a000048.

Gerbaix M, Metz L, Ringot E, Courteix D. Visceral fat mass determination in rodent: Validation of dual-energy x-ray absorptiometry and anthropometric techniques in fat and lean rats. Lipids Health Dis (2010); 9:140. doi:10.1186/1476-511X-9-140.

Bernardis LL. Prediction of carcass fat, water and lean body mass from Lee's "nutritive ratio" in rats with hypothalamic obesity. Experientia (1970); 26(7): 789-790. doi: 10.1007/BF02232553.

Novelli EL, Diniz YS, Galhardi CM, Ebaid GM, Rodrigues HG, Mani F, et al. Anthropometrical parameters and markers of obesity in rats. Lab Anim (2007); 41(1): 111-119. doi: 10.1258/002367707779399518.

Cacho J, Sevillano J, de Castro J, Herrera E, Ramos MP. Validation of simple indexes to assess insulin sensitivity during pregnancy in Wistar and Sprague-Dawley rats. Am J Physiol Endocrinol Metab (2008). 295(5):E1269-E1276.

Antunes LC, Elkfury JL, Jordana MN, Foletto KC, Bertoluci MC. Validation of HOMA-IR in a model of insulin resistance induced by a high-fat diet in Wistar rats. Arch Endocrinol Metab (2016) 60(2): 138-142.

Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman, JI, Donato, KA, et al. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation (2009); 120(16): 1640-5. doi:10.1161/CIRCULATIONAHA.109.192644.

Auberval N, Dal S, Bietiger W, Pinget M, Jeandidier N, Maillard-Pedracini E, et al. Metabolic and oxidative stress markers in Wistar rats after 2 months on a high-fat

diet. Diabetol Metab Syndr (2014); 6:130. https://doi.org/10.1186/1758-5996-6-130.

Panchal SK, Poudyal H, Iyer A, Nazer R, Alam MA, Diwan V, et al. High-carbohydrate, high-fat diet-induced metabolic syndrome and cardiovascular remodeling in rats. Journal of Cardiovascular Pharmacology (2011); 57(1): 611–624. https://doi.org/10.1097/FJC.0b013e3181feb90a

Robberecht, H, De Bruyne T, Hermans N. Biomarkers of the metabolic syndrome: Influence of minerals, oligo- and trace elements. J Trace Elem Med Biol (2017); 43:23-28. doi: 10.1016/j.jtemb.2016.10.005.

Chang Q, Lu Z, He M, Gao, R, Bai, H, Shi, B, et al. Effects of dietary supplementation of fulvic acid on lipid metabolism of finishing pigs. J Anim Sci (2014); 92(11): 4921-6. doi:10.2527/jas/2014-8137.

Hsu H-Y, Wen M-H. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. J Biol Chem (2002); 277:22131–9. https://doi.org/10.1074/jbc.M111883200.

McMurray F, Patten DA, Harper ME. Reactive Oxygen Species and Oxidative Stress in Obesity—Recent Findings and Empirical Approaches. Obesity (2016); 24(11): 2301-2310. doi: 10.1002/oby.21654

Taniyama Y, Griendling KK. Reactive oxygen species in the vasculature: Molecular and cellular mechanisms. Hypertens (2003); 42: 1075-1081. doi: 10.1161/01.HYP.0000100443.09293.4F.

Van Guilder GP, Hoetzer GL, Dengel DR, Stauffer BL, DeSouza CA. Impaired endothelium-dependent vasodilation in normotensive and normoglycemic obese adult humans. J Cardiovasc Pharmacol (2006); 47(2): 310–313. https://doi.org/10.1097/01.fjc.0000205097.29946.d3.

Lopez-Moreno J, Garcia-Carpintero S, Jimenez-Lucena R, Haro C, Rangel-Zuniga OA, Blanco-Rojo R, et al. Effect of dietary lipids on endotoxemia influences postprandial inflammatory response. Journal of Agriculture and Food Chemistry (2017); 65(35):7756–7763. doi: 10.1021/acs.jafc.7b01909.

Wang, X., & Quinn, P. Endotoxins: Lipopolysaccharides of Gram-Negative Bacteria. Sub-cellular biochemistry (2010); 53:3-25. https://doi.org/10.1007/978-90-481-9078-2_1.

Chien SJ, Chen TC, Kuo HC, Chen, CN, Chang, SF. Fulvic acid attenuates homocysteine-induced cyclooxygenase-2 expression in human monocytes. BMC Complement Altern Med (2015); 15: 61. doi: 10.1186/s12905-015-0583-x.

Van Rensburg CE, van Straten A, Dekker J. An *in vitro* investigation of the antimicrobial activity of oxifulvic acid. J Antiomicrob Chemother (2000); 46(5):853. https://doi.org/10/1093/jac/46.5.853.

Ghosal S, Singh SK, Kumar Y, Srivastava R, Goel RK, Dey R, Bhattacharya SK. Anti-ulcerogenic activity of fulvic acids and 4'-methoxy-6-carbomethoxybiphenyl isolated from Shilajit. Phytother Res (1988); 2(4): 187-191. doi:10.1002/ptr.2650020408.

Aslam MN, Paruchuri T, Bhagavathula N, Varani J. A mineral-rich red algae extract inhibits polyp formation and inflammation in the gastrointestinal tract of mice on a high-fat diet. Integr Cancer Ther (2010); 9(1): 93-9. doi:10.1177/1534735409360360.

Aslam MN, Bergin I, Naik M, Paruchuri T, Hampton A, Rehman M, et al. A multi-mineral natural product inhibits liver tumor formation in C57BL/6 mice. Biol Trace Elem Res (2012);64(7):1020-8. doi:10.1080/01635581.2012.713160.

O'Gorman DM, O'Carroll C, Carmody RJ. Evidence that marine-derived, multi-mineral, Aquamin inhibits the NF-κB signaling pathway in vitro. Phytother Res (2012); 26(4):630-2. doi: 10.1002/ptr.3601.

Crinigan C, Calhoun M, Sweazea KL. Short-term high fat intake does not significantly alter markers of renal function or inflammation in young male Sprague-Dawley rats. J Nutr Metab (2015). 157520. doi: 10.1155/2015/157520.

Rodriguez NC, Urrutia EC, Gertrudis BH, Chaverri JP, Mejia GB. Antioxidant activity of fulvic acid: A living matter-derived bioactive compound. Journal of Food, Agriculture & Environment (2011); 9(3-4), 123-127. https://doi.org/10.1155/2018/5391014.

Li S, Tan HY, Wang N, Zhang, ZJ, Lao, L, Wong, CW, et al. The Role of Oxidative Stress and Antioxidants in Liver Diseases. International Journal of Molecular Sciences (2015); 16(11): 26087–26124. https://doi.org/10.3390/ijms161125942.

Kai M, Miyoshi M, Fujiwara M, Nishiyama Y, Inoue T, Maeshige N, et al. A lard-rich high-fat diet increases hepatic peroxisome proliferator-activated receptors in endotoxemic rats. J Surg Res (2017); 212:22-32. doi: 10.1016/j.jss.2016.11.048.

Han CY. Roles of Reactive Oxygen Species on Insulin Resistance in Adipose Tissue. Diabetes Metab J (2016); 40(4): 272–279. https://doi.org/10.4093/dmj.2016.40.4.272.

Kanety H, Feinstein R, Papa MZ, Hemi R, Karasik A. Tumor necrosis Factor alpha-induced phosphorylation of insulin receptor substrate-1 (IRS-1). Possible mechanism for suppression of insulin-stimulated tyrosine phosphorylation of IRS-1. J Biol Chem (1995); 270(40): 23780-23784. doi: 10.1074/jbc.270.40.23780.

Lamar RT, OLK DC, Mayhew L, Bloom PR. A new standardized method for quantification of humic and fulvic acids in humic ores and commercial product. Journal of AOAC International (2014); 97(3): 721–730. doi: https://doi.org/10.5740/jaoacint.13-393.

Table 1: Physical, Chemical, and Functional Characteristics of OMC

Component Measured	Concentration or Value	Analytical Methodology or Source
Total Minerals	142391 ppm	ICP
Calcium	49610 ppm	ICP
Sulfur	28040 ppm	ICP
Potassium	15420 ppm	ICP
Sodium	14990 ppm	ICP
Magnesium	12630 ppm	ICP
Fulvic Acids	14.9%	Lamar et al., 2014 [42]
Humic Acids	<0.1%	Lamar et al., 2014 [42]
Protein	23 mg/g	CLG-PRO4 determination by combustion
Nucleic Acids	ND	DAPI (4',6-Diamidino-2-phenylindole)-staining
Total Polyphenols	0.24%	Folin-Ciocalteu
ORAC Score[a]-Hydrophilic	24.92	Brunswick Laboratories
ORAC Score[a]-Hydrophobic	5.44	Brunswick Laboratories

[a]ORAC: oxygen radical absorbance capacity, [b]ICP: Inductively coupled plasma mass spectrometry.

Table 2: Morphometrics at ten weeks

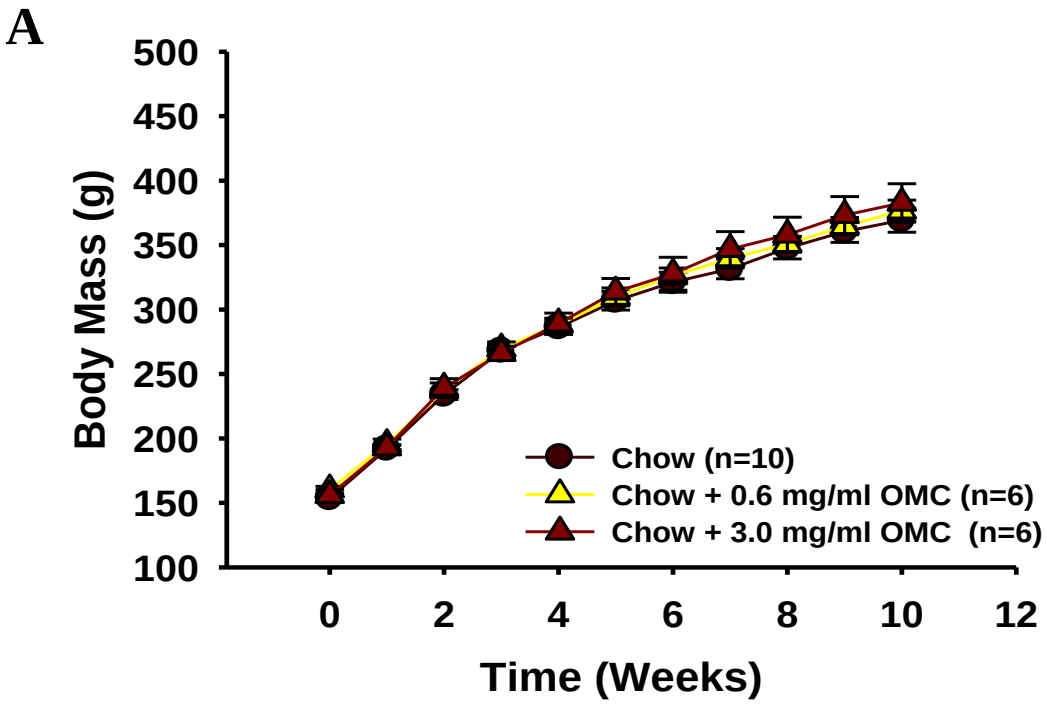
	Control	0.6 mg/ml OMC	3.0 mg/ml OMC
Body Mass (g)			
Chow	369 ± 9.33 (10)	377 ± 8.10 (6)	383 ± 14.8 (6)
HFD	**411 ± 9.84 (8)#**	**425 ± 16.8 (6)#**	417 ± 12.7 (6)
Epididymal fat pad mass (g)[a]			
Chow	3.70 ± 0.16 (10)	4.14 ± 0.24 (6)	3.89 ± 0.41 (6)
HFD	**6.09 ± 0.32 (8)#**	**7.44 ± 1.09 (6)#**	**6.67 ± 0.65 (6)#**
Epididymal fat pad mass (% body mass)			
Chow	1.00 ± 0.03 (10)	1.10 ± 0.07 (6)	1.01 ± 0.08 (6)
HFD	**1.48 ± 0.05 (8)#**	**1.73 ± 0.20 (6)#**	**1.59 ± 0.12 (6)#**
Abdominal circumference (cm)			
Chow	16.8 ± 0.28 (10)	17.5 ± 0.17 (6)	17.3 ± 0.20 (6)
HFD	**17.9 ± 0.25 (8)#**	**18.9 ± 0.62 (6)#**	**18.3 ± 0.24 (6)#**
Tail length (cm)			
Chow	21.3 ± 0.27 (10)	21.1 ± 0.29 (6)	21.4 ± 0.51 (6)
HFD	21.3 ± 0.22 (8)	21.5 ± 0.26 (6)	21.5 ± 0.31 (6)
Naso-anal length (cm)			
Chow	22.3 ± 0.15 (10)	22.1 ± 0.14 (6)	22.5 ± 0.24 (6)
HFD	**23.1 ± 0.14 (8)#**	**23.1 ± 0.20 (6)#**	**23.2 ± 0.30 (6)#**
Lee's Index of Obesity			
Chow	321.1 ± 1.7 (10)	327.3 ± 3.3 (6)	322.9 ± 2.5 (6)
HFD	321.9 ± 1.7 (8)	324.7 ± 2.8 (6)	322.0 ± 1.8 (6)
BMI			
Chow	0.74 ± 0.01 (10)	0.77 ± 0.02 (6)	0.76 ± 0.02 (6)
HFD	0.77 ± 0.01 (8)	0.79 ± 0.02 (6)	0.77 ± 0.01 (6)

Data expressed as mean ± SEM (n). Data analyzed by two-way ANOVA. #$p<0.02$ vs respective chow treated animal. [a]Data was log transformed prior to statistical analyses to approximate normality. Lee's Index of Obesity = (cube root of body mass (g) / nasoanal length (cm)) * 1000 (Bernardis, 1970). Body mass index (BMI) = body mass (g) / nasoanal length $(cm)^2$ (Novelli et al., 2007).

Table 3: Fasting Biochemical Parameters

	Week 6 Mean ± SEM (n)	**Week 10 Mean ± SEM (n)**
Serum glucose (mM/L)		
Chow	6.88 ± 0.28 (10)	7.08 ± 0.17 (10)
Chow + 0.6 mg/ml OMC	7.43 ± 0.36 (6)	7.34 ± 0.30 (6)
Chow + 3 mg/ml OMC	6.73 ± 0.45 (6)	7.01 ± 0.17 (6)
HFD	**8.83 ± 0.41 (8)***	**8.32 ± 0.33 (8)***
HFD + 0.6 mg/ml OMC	8.01 ± 0.34 (6)	7.30 ± 0.04 (6)
HFD + 3 mg/ml OMC	7.91 ± 0.29 (6)	**7.07 ± 0.19 (6)#**
Serum insulin (mU/L)		
Chow	12.9 ± 2.73 (10)	12.2 ± 1.27 (10)
Chow + 0.6 mg/ml OMC	14.7 ± 3.28 (6)	16.3 ± 2.99 (6)
Chow + 3 mg/ml OMC	12.4 ± 2.11 (6)	15.2 ± 1.69 (6)
HFD	**29.2 ± 5.52 (8)***	**32.8 ± 3.80 (8)***
HFD + 0.6 mg/ml OMC	**34.6 ± 7.41 (6)***	34.3 ± 6.55 (6)
HFD + 3 mg/ml OMC	30.3 ± 9.18 (6)	25.8 ± 5.36 (6)
HOMA-IR		
Chow	3.92 ± 0.76 (10)	3.86 ± 0.42 (10)
Chow + 0.6 mg/ml OMC	5.09 ± 1.48 (6)	5.51 ± 1.22 (6)
Chow + 3 mg/ml OMC	3.88 ± 0.87 (6)	4.72 ± 0.51 (6)
HFD	**11.7 ± 2.47 (8)***	**12.1 ± 1.38 (8)***
HFD + 0.6 mg/ml OMC	12.8 ± 3.35 (6)	11.1 ± 2.14 (6)
HFD + 3 mg/ml OMC	10.7 ± 3.25 (6)	8.07 ± 1.06 (6)

Data expressed as mean ± SEM (n). Data analyzed by two-way RM ANOVA. †$p<0.05$ vs week 0 within subjects; §$p<0.05$ vs week 6 within subjects; #$p<0.05$ vs HFD control at the same time point, *$p<0.05$ vs chow control at the same time point.


A
Body Mass (g)
500
450
400
350
300
250
200
150
100
0
2
4
6
8
10
12
Time (Weeks)
Chow (n=10)
Chow + 0.6 mg/ml OMC (n=6)
Chow + 3.0 mg/ml OMC (n=6)

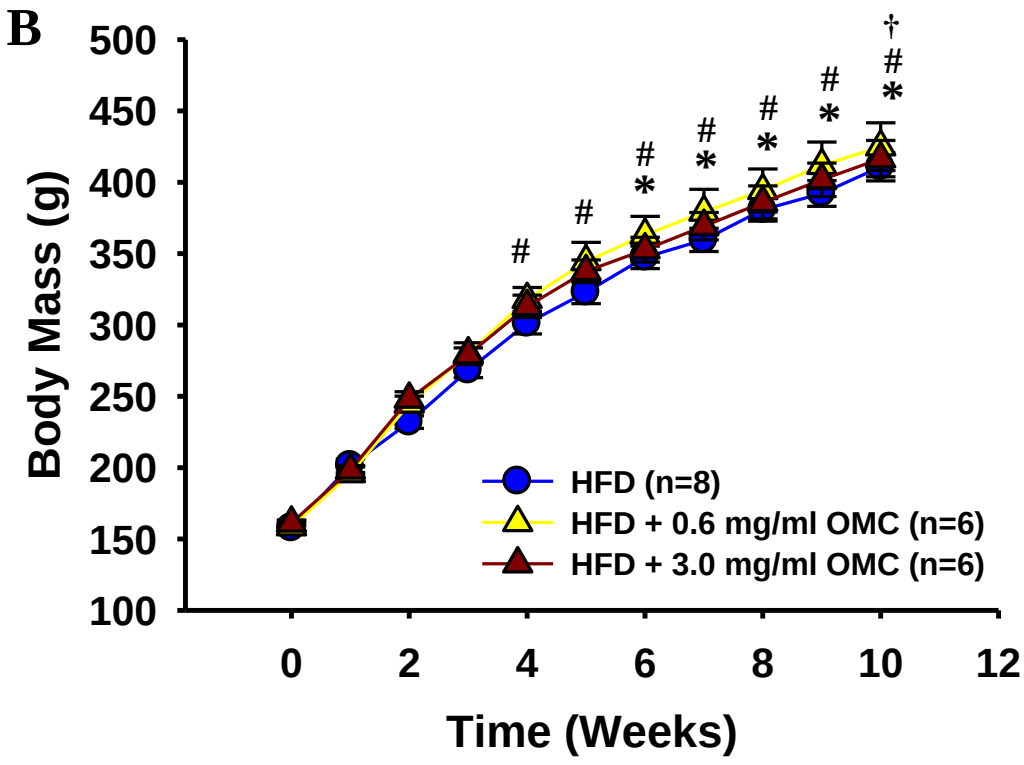

B
Body Mass (g)
500
450
400
350
300
250
200
150
100
0
2
4
6
8
10
12
Time (Weeks)
HFD (n=8)
HFD + 0.6 mg/ml OMC (n=6)
HFD + 3.0 mg/ml OMC (n=6)
#
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Figure 1: Effects of OMC treatment on body mass of rats fed either (A) standard rodent chow or (B) a 60% kcal from fat high fat diet (HFD) for 10 weeks. Data are expressed as mean ± SEM. Data were analyzed by two-way RM ANOVA (SigmaStat 3.0, Systat Software, San Jose, CA). *****$p<0.05$ HFD vs Chow; #$p<0.05$ HFD + 0.6 mg/ml OMC vs Chow + 0.6 mg/ml OMC, †$p<0.05$ HFD + 3 mg/ml OMC vs Chow + 3 mg/ml OMC; n=6-10 per group.

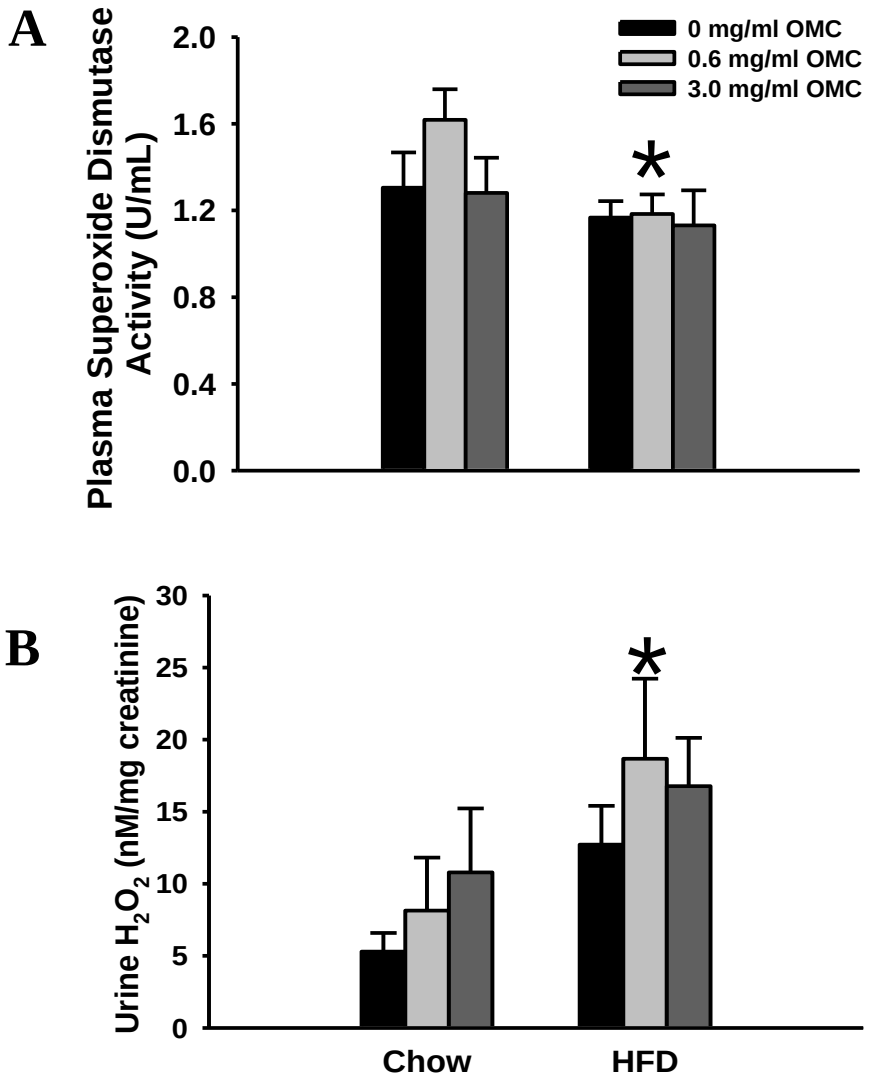
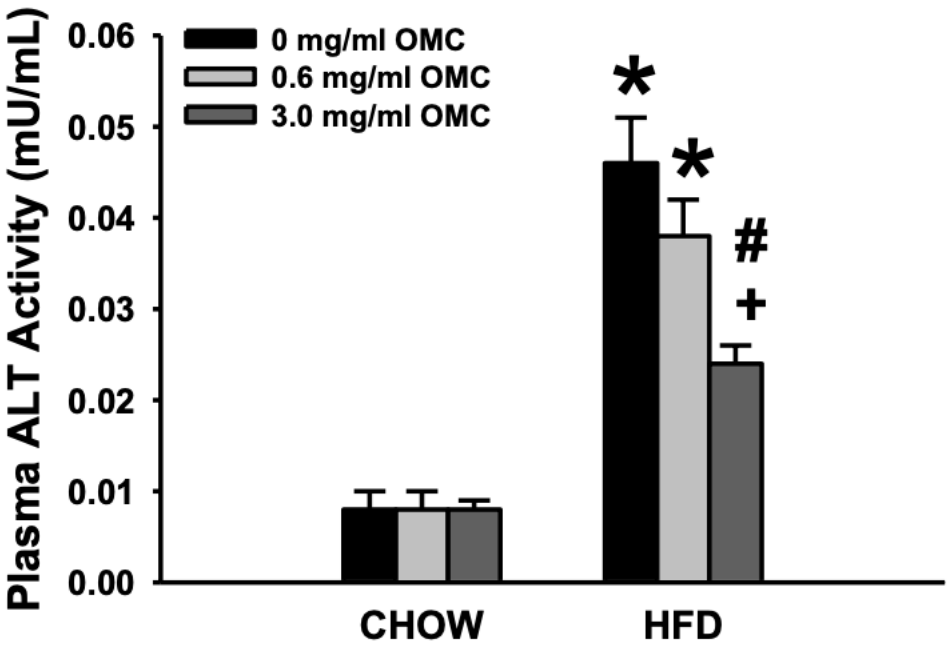


Figure 2: (A) Plasma superoxide dismutase (SOD) activity and (B) urine hydrogen peroxide (H_2O_2) concentrations normalized to urine creatinine. Data are expressed as mean ±SEM and analyzed by two-way ANOVA, *n*=6-10 per group for plasma and *n*=4-8 per group for urine. *****$p<0.05$ HFD vs Chow.

A


0 mg/ml OMC
0.6 mg/ml OMC
3.0 mg/ml OMC
Plasma ALT Activity (mU/mL)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
*
*
#
+
CHOW
HFD

B

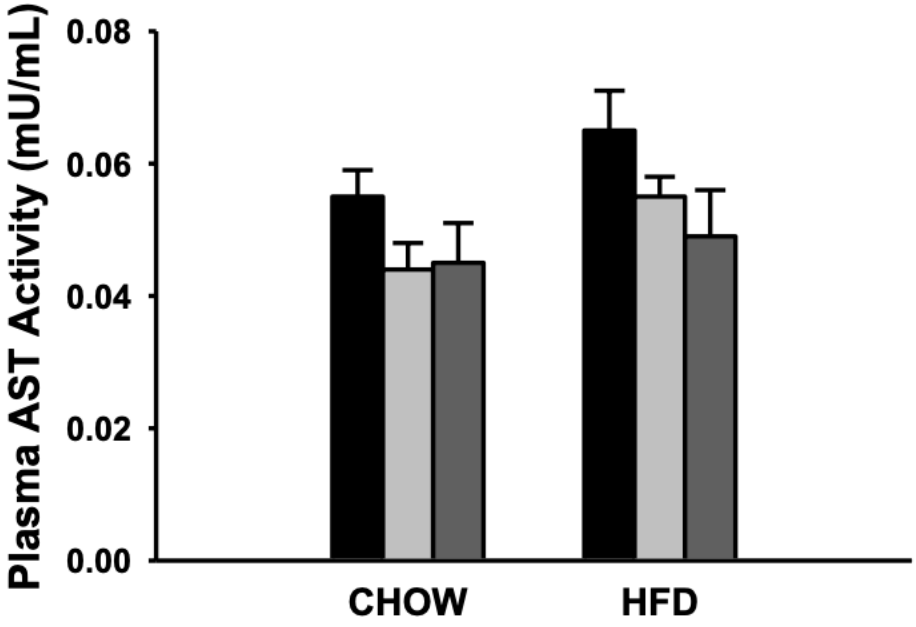

Plasma AST Activity (mU/mL)
0.08
0.06
0.04
0.02
0.00
CHOW
HFD

Figure 3: Plasma ALT (A) and AST (B) activity after the 10-week diet. Data are expressed as mean ± SEM, n=6-10 per group. Data were analyzed by two-way ANOVA. #p<0.05 vs. HFD control, *p<0.05 vs chow control, †p<0.05 vs HFD 0.6 mg/ml OMC.

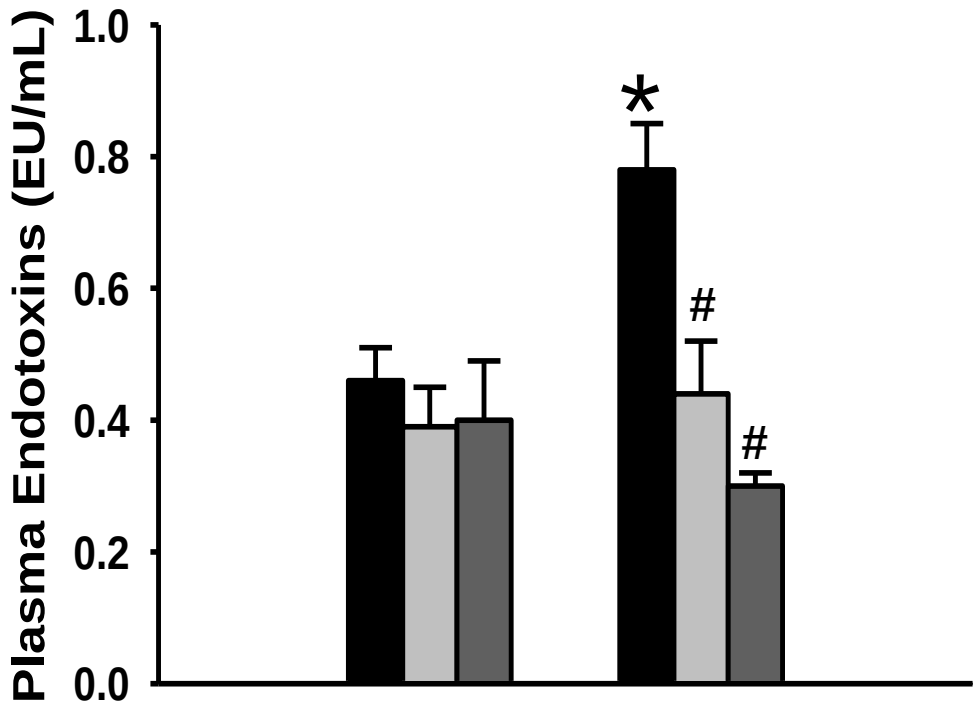


Figure 4: Plasma lipopolysaccharide (LPS) concentrations. Data are expressed as mean ±SEM, *n*=6-10 per group. *****p<0.05 HFD vs Chow; #p<0.05 HFD + 0.6 mg/ml OMC vs Chow + 0.6 mg/ml OMC.

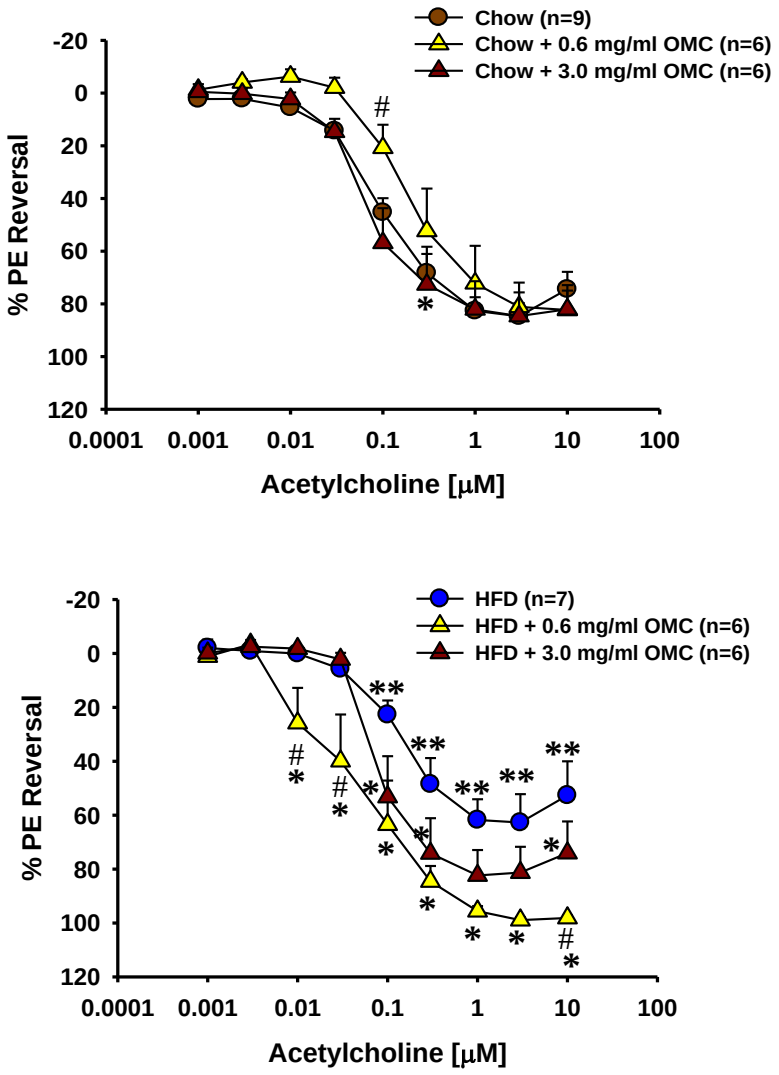


Figure 5: Endothelium-dependent vasodilation of arteries from (A) chow and (B) HFD-fed rats treated with OMC. Isolated mesenteric resistance arteries were pre-constricted to 50% of resting inner diameter then exposed to increasing concentrations of ACh in the superfusate to obtain percent reversal of phenylephrine mediated vasodilation. Data are

expressed as mean ± SEM. Data were arcsine transformed prior to analysis by two-way repeated measures ANOVA to approximate normal distribution. *****$p<0.05$ vs respective control; **$p<0.05$ vs chow control; #$p<0.05$ 0.6 vs 3.0 mg/mL OMC.

CHAPTER FOUR:

EFFECTS OF A 10-WEEK HIGH FAT DIET ON THE GUT MICROBIOME OF MALE SPRAGUE-DAWLEY RATS

ABSTRACT

The pathological causes of diet-induced obesity and metabolic complications continue to be under investigation in westernized societies. Several studies have shown that the gut microbiome is a critical factor in the development and treatment of metabolic disease. In this study, we used linear discriminant analysis of effect size (LEfSe) analyses to examine the effects of a 10-week high fat diet (HFD) on gut microbial abundance in male Sprague-Dawley rats. At baseline, all study animals were fed a standard rodent chow before randomization but showed differences in microbial composition. In order to analyze gut microbial differences at baseline, data was divided into two groups, "Control" and "ControlB" (linear discriminant analysis (LDA) score >2, $p<0.05$, Wilcoxon rank-sum test; Control: *Streptococcaceae*, *Prevotellaceae*, *Streptococcus*, *CF231*, *Mogibacteriaceae*, *Actinomycetales*, *Coriobacteriales*, *Coriobacteriia*, *Aldercreutzia*, *Ruminococcus*, *Bifidobacterium*, *Bifidobacteriaceae*, *Bifidobacteriales* and *Actinobacteria;* ControlB: *rc4_4*, *Porphyromonadaceae* and *Parabacteroides*). Variations in microbial abundance may be due to coprophagic activity during transport. At the end of the 10-week feeding protocol, chow-fed rats were enriched in *Oceanospirillales*, *Erysipelotrichaceae*, *Alistipes* and *Rikenellaceae* (LDA score >2, $p<0.05$, Wilcoxon rank-sum test). The microbiome of HFD-fed rats was less diverse but more enriched in *Rickettsiales* and bacteria identified as "other" (LDA score >2, $p<0.05$,

Wilcoxon rank-sum). These findings are consistent with the results of other studies that indicate a HFD alters microbial communities in rats. Further research is needed to specify the mechanism of action for each microbial species and their contribution towards the development of metabolic diseases.

INTRODUCTION

The microbiome is composed of bacteria, fungi, protozoa and viruses present from the skin to the gastrointestinal tract of all organisms [Liang et al., 2018]. The gut microbiota is associated with the modulation of host metabolism and the maturation of host immunity [Parseus et al., 2017]. The gut microbiota is considered to be an environmental factor that can contribute to the onset of many metabolic and gastrointestinal diseases if there are significant alterations in the microbial communities [Backhed et al., 2004]. These changes can result in obesity and associated comorbidities, with profound effects on metabolic health in westernized societies [Nagpal et al., 2018]. More specifically, the prolonged intake of fatty foods promotes intestinal dysbiosis and the subsequent production of pro-inflammatory cytokines [He et al., 2018] associated with the development of metabolic disease.

In non-human studies, a healthy gut microbiome is balanced with gram-positive and negative bacteria which include the major phyla; *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Actinobacteria* [Lecomte et al., 2015]. However, the consumption of energy dense foods can have a profound effect on microbial abundance, thereby increasing risk factors of metabolic syndrome [Sonnenburg et al., 2018]. Elevated levels of gram-negative bacteria have been linked to obesity in human and animal microbiome

research [Conlon et al., 2015]. An increase of gram-negative bacteria or pathogens in the gut lumen initiates a response from the intestinal mucosal immune system which secretes antimicrobial peptides (AMPs) from Peyer's patches in the small intestine [Jung et al., 2010]. AMPs can cross the intestinal epithelium and destroy gram-negative bacteria subsequently releasing lipopolysaccharides (LPS; endotoxins). Endotoxins promote the production of pro-inflammatory cytokines that increase intestinal permeability and systemic inflammation marked by plasma endotoxemia [Boutagy et al., 2017].

Diet is a key factor in the development of metabolic complications and gut microbial alteration in mammals [Martinez et al., 2017]. In animal models, a high fat diet (HFD) results in altered abundance of *Bacteroidetes* and *Firmicutes* [Lecomte et al., 2015]. Previous research from our laboratory has found that consumption of a HFD for six weeks promotes hyperglycemia, increases hepatic lipid accumulation and alters microbial abundance and composition in rats [Crawford, 2019]. Similarly, in 2015, Lecomte et al. demonstrated that a HFD (43% fat, 17% protein, 40 % carbohydrates) consumed by 6-week old male Sprague-Dawley rats for 16 weeks resulted in differences in microbial composition across all taxonomic levels [Lecomte et al., 2015].

The current study investigates the effect of a 10-week HFD on the development of metabolic diseases in male Sprague-Dawley rats. This study thereby highlights the importance of the gut microbiome as a potential therapeutic target to treat metabolic diseases. Although gut microbiome research has been exhaustive in animal models, identifying individual bacterial species associated with poor dietary habits continues to provide information about diet composition and intake on a developing microbiome. Therefore, the goal of the present study is to determine how consumption of a HFD for

10 weeks by male Sprague-Dawley rats affects their gut microbial composition. We hypothesize that high-fat, high-caloric intake for 10 weeks increases the abundance of bacteria associated with metabolic disease.

METHODS AND MATERIALS:

Animal Models: Six-week old male Sprague-Dawley rats (140-160 g, n=14, Envigo, Madison, WI) were randomly divided into two groups that received either a standard chow diet (n=8; 24% protein, 58% carbohydrates and 18% kcal fat) (Teklad Global, Indianapolis, IN) or a diet including 60% kcal from fat (n=6; Cat. No. D12492; Research Diets Inc, New Brunswick, NJ) for 10 weeks. To properly examine characteristics of the gut microbiome, rats were singly housed to prevent coprophagy. Animals had *ad libitum* access to water and food. Fecal samples were collected weekly, placed in sterile tubes and stored at -80° C until analysis. All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee.

Fecal Microbial DNA Preparation: Microbial genomic DNA was extracted from fecal samples collected from all study animals at baseline and week 10 using a commercially available kit per the manufacturer's instructions (PowerSoil® DNA Isolation Kit, MoBio Laboratories, Inc., Carlsbad, CA, USA). DNA concentration was quantified in micro-liter volumes (2μl of DNA sample was compared to 2μl of eluent) using a μDrop™ plate adaptor (Catalog #N12391, ThermoFisher Scientific, Waltham, MA, USA) and Multiskan ™ GO Microplate spectrophotometer (Catalog # 5119300, ThermoFisher Scientific Waltham, MA, USA). Sample purity was determined at OD_{260}.

Illumina and Microbial Analysis: Fecal contents were sequenced at the Microbiome Analysis Laboratory at Arizona State University with the protocol developed by the Earth Microbiome Project using QIIME analyses. To distinguish microbial differences between groups and identify genomic features, QIIME taxonomy data were formatted and uploaded into the online Galaxy module, linear discriminant analysis of effect size (LEfSe) for analysis.

STATISTICAL ANALYSES

Data are expressed as means ± SEM. Data were analyzed using SigmaPlot (Systat Software Version 10.0, San Jose, CA, USA). LEfSe analysis was conducted using Kruskal-Wallis sum-rank tests, Wilcoxon rank-sum tests, and linear discriminant analysis as previously described [Segata et al., 2011].

RESULTS:

***Fecal Contents Microbial* Analysis:** Baseline LEfSe results showed significant differences in taxonomy between individuals before they divided into two experimental groups (Figure 1) with a total of 21 operational taxonomic units (OTU) identified as differentially abundant features. At baseline, all rats received chow diet. However, due to microbial differences in abundance before randomization, we divided them into two groups for LEfSe analysis: “Control” and “ControlB”. Red bars indicate the taxa significantly abundant in the Control group while the green bars show the taxa significantly more abundant in the ControlB group (LDA score >2, $p<0.05$, Wilcoxon

rank-sum test). Baseline fecal samples from the Control rats were more enriched in the following bacteria: *Streptococcaceae, Prevotellaceae, Streptococcus, CF231, Mogibacteriaceae, Actinomycetales, Coriobacteriales, Coriobacteriia, Aldercreutzia, Ruminococcus, Bifidobacterium, Bifidobacteriaceae, Bifidobacteriales* and *Actinobacteria*. However, ControlB rats were more abundant in rc4_4, *Porphyromonadaceae* and *Parabacteroides*. Study animals were randomized, and fecal samples were collected at 10 weeks following euthanasia. LEfSe results identified 10 OTUs and showed that chow-fed rats had more *Oceanospirillales, Erysipelotrichaceae, Alistipes* and *Rikenellaceae* than HFD-fed rats. Fecal samples of rats fed HFD were more abundant in mitochondria, *Rickettsiales* and bacteria identified as "other" (LDA score >2, $p<0.05$, Wilcoxon rank-sum test, Figure 2) than samples from control rats.

DISCUSSION:

The mammalian digestive tract contains trillions of symbiotic microorganisms that provide many functions separate from the host genome [Gill et al., 2006]. These microbes, collectively known at the gut microbiome, maintain physiological homeostasis of the host [Dethlesen et al., 2007]. The gut microbiota in particular is considered to be a causative factor in the development of metabolic complications [Sanz et al., 2010] such as increasing risk factors for obesity and type 2 diabetes [Sharma et al., 2019]. Human and animal studies suggest that obesity is characterized by a decrease of gut microbial diversity and the presence of intestinal dysbiosis indicated by the *Bacteroidetes* to *Firmicutes* ratio [Valdes et al., 2018]. Previous research has found that rats fed a HFD for 16 weeks demonstrated significant differences in bacterial diversity in comparison to rats

fed a standard rodent chow [Lecomte et al., 2015]. Additionally, previous work in our laboratory has shown that just 6 weeks of HFD can significantly alter gut microbial diversity [Crawford et al., 2019].

The current study examined the effects of HFD consumption for 10 weeks on gut microbial abundance and composition in male Sprague-Dawley rats. LefSe results indicated significant differences between study animals at baseline. During the acclimation period (one week), all study animals received standard rodent chow until randomization. At this time, rats had a gut microbiome enriched with gram-positive bacteria which included: *Streptococcaceae*, *Streptococcus*, *Mogibacteriaceae*, *Actinomycetales*, *Coriobacteriales*, *Coriobacteriia*, *Adlercreutzia*, *Ruminococcus*, *Bifidobacterium*, *Bifidobacteriaceae*, *Bifidobacteriales* and *Actinobacteria.* We also identified two species which are a part of the *Bacteroidetes* family: *Prevotellaceae* and *CF231*.

However, some rats at baseline had a microbiome that was significantly different from that of other rats. During baseline, the gut of these rats was more enriched with the genus *rc4_4* which belongs to the phyla *Firmicutes* and *Porphyromonadaceae* and *Parabacteroides* which belong to *Bacteroidetes*. Differences in baseline microbial abundance could be the result of rats sharing the same food/hydration source, housing conditions at Envigo and/or coprophagic activity by rats during transport to the study facility. Study animals were shipped in pairs then housed separately upon arrival. Studies have shown that coprophagic activity can affect microbial composition of rats when housed together [Hakkak et al., 2017]. However, these differences were not seen in chow-fed rats at the end of the 10-week feeding protocol. Overall, the presence of these

species in all study animals are common in rats as indicated by previous murine microbiome studies [Lecomte et al., 2015; Vaughn et al., 2017].

Fecal samples were collected at the end of the 10-week feeding protocol and compared to baseline results. The 10-week LEfSe results indicated that the microbiome of chow-fed rats was more enriched in *Oceanospirillales*, *Erysipelotrichaceae*, *Alistipes* and *Rikenellaceae*. These microbes belong to the *Proteobacteria*, *Firmicutes* and *Bacteroidetes* phyla, respectively. However, the results showed less bacterial diversity in chow-fed rats in comparison to their baseline data. Although not a model of aging, the current model was validated by previous microbiome/aging studies that demonstrated humans and rats show a decrease in gut microbial diversity possibly driven by a weaken immune system. [Nagpal et al., 2018; Flemer et al., 2017].

Conversely, HFD-fed animals had a microbiota more enriched with mitochondria, *Rickettsiales* and microbes specified as "other". The presence of mitochondrial DNA in the gut microbiome is not uncommon. Recent studies report that mitochondrial DNA is released as a result of stress and cell death subsequently inducing an inflammatory response [Hu et al., 2018]. The presence of mitochondrial DNA is also linked to the onset of intestinal ischemia and an increase in intestinal permeability [Hu et al., 2018]. Equally, the gut microbiota aids in nutrition metabolism alongside mitochondria and produces metabolites to influence mitochondrial function [Franco-Obreygon et al., 2017]. However, the increased abundance of mitochondria may be indicative of disease due to mitochondria being a major producer of reactive oxygen species [Chunchai et al., 2018]. Additionally, bacterium of the order *Rickettsiales*, an obligate intracellular parasite [Kang et al., 2014], were more enriched in HFD fed rats. *Rickettsiales* is a member of the

Alphaproteobacteria family which shares a common ancestor with mitochondria [Thrash et al., 2011].

Prior research from our laboratory has shown that 6 weeks of HFD consumption increased the abundance of *Firmicutes*, *Clostridales*, *Proteobacteria* and *Steptocaccaceae*. Gut microbial diversity also decreased in comparison to chow-fed rats [Crawford et al., 2019]. Lecomte et al. (2015) demonstrated that a 16-week HFD consumption increased microbial diversity in rats but decreased the relative abundance of *Firmicutes*, a phylum highly associated with obesity and metabolic disease. Interestingly, in the present study, consumption of both the 10-week chow diet and the HFD lowered microbial diversity, which may be a result of normal aging as indicated by Flemer et al. (2017).

Gut microbiome studies suggest that changes in the *Bacteroidetes* to *Firmicutes* ratio increases the risk of metabolic disease in humans and rodents [Ley et al., 2006]. The relative abundance of *Bacteroidetes* has been reported to decrease in obese patients in comparison to "lean" individuals [Ley et al., 2006]. Conversely, the abundance of *Firmicutes* increases in the microbiota of obese individuals. Literature suggests that *Firmicutes* plays a role in elevating the capacity to harvest energy from a diet, thereby promoting calorie absorption and thus weight gain [Turnbaugh et al., 2006]. In the current study, the microbiota of HFD-fed rats did not show a significant increase in the relative abundance of *Firmicutes*. Based on these data, it appears that differences in microbial diversity and abundance may vary across species and genetic predisposition to obesity, as well as the duration of HFD consumption, diet composition and environment.

In summary, consumption of HFD for ten weeks can lower microbial diversity in male Sprague-Dawley rats. Microbial diversity decreased during a standard chow diet, suggesting the effects of age on microbial abundance in a healthy gut microbiome. Consumption of HFD also increased mitochondrial DNA which may be the result of cellular death or stress of the intestinal epithelium. Mitochondrial DNA may also serve as a mediator for the production of reactive oxygen species, thereby subsequently increasing the risk of systemic inflammation and metabolic syndrome. Further investigation is needed to examine microbial abundance and the correlation between the development of obesity, hyperglycemia and gastrointestinal diseases during poor dietary consumption.

REFERENCES:

Liang D, Leung RK, Guan W, Au WW. Involvement of gut microbiome in human health and disease: brief overview, knowledge gaps and research opportunities. *Gut Pathog*. 2018; 10:3. Published 2018 Jan 25. doi:10.1186/s13099-018-0230-4

Parséus A, Sommer N, Sommer F, et al. Microbiota-induced obesity requires farnesoid X receptor. *Gut*. 2016;66(3):429-437.

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V, Koh, G. Y., Nagy, A., … Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(44), 15718 LP-15723. https://doi.org/10.1073/pnas.0407076101

Nagpal, R., Newman, T. M., Wang, S., Jain, S., Lovato, J. F., & Yadav, H. (2018). Obesity-Linked Gut Microbiome Dysbiosis Associated with Derangements in Gut Permeability and Intestinal Cellular Homeostasis Independent of Diet, *2018*.

He, C., Cheng, D., Peng, C., Li, Y., Zhu, Y., & Lu, N. (2018). High-Fat Diet Induces Dysbiosis of Gastric Microbiota Prior to Gut Microbiota in Association With Metabolic Disorders in Mice. *Frontiers in microbiology*, *9*, 639. doi:10.3389/fmicb.2018.00639

Lecomte V, Kaakoush NO, Maloney CA, Raipuria M, Huinao KD, Mitchell HM, et al. (2015) Changes in Gut Microbiota in Rats Fed a High Fat Diet Correlate with Obesity-Associated Metabolic Parameters. PLoS ONE 10(5): e0126931. https://doi.org/10.1371/journal.pone.0126931

Sonnenburg, J. L., & Academy, S. (2018). HHS Public Access, *535*(7610), 56–64. https://doi.org/10.1038/nature18846.Diet

Conlon, M. A., & Bird, A. R. (2015). The impact of diet and lifestyle on gut microbiota and human health. *Nutrients*, *7*(1), 17–44. https://doi.org/10.3390/nu7010017

Jung, C., & Hugot, J. (2010). Peyer ' s Patches : The Immune Sensors of the Intestine, *2010*. https://doi.org/10.4061/2010/823710

Boutagy, N. E., McMillan, R. P., Frisard, M. I., & Hulver, M. W. (2015). Metabolic endotoxemia with obesity: Is it real and is it relevant?. *Biochimie*, *124*, 11-20.

Martinez, K. B., Leone, V., & Chang, E. B. (2017). Western diets, gut dysbiosis, and metabolic diseases: Are they linked? *Gut Microbes*, *8*(2), 130–142. https://doi.org/10.1080/19490976.2016.1270811

Crawford, M., Whisner, C., Al-Nakkash, L., Sweazea KL. (2019). Six-week high fat diet alter the gut microbiome and promotes cecal inflammation, endoxtin production and simple steatosis without obesity in male rats. *Lipids.*

Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. (2011) Metagenomic biomarker discovery and explanation. *Genome Biology* **12**: R60. DOI 10.1186/gb-2011-12-6-r60.

Gill, S. R., Pop, M., Deboy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., Gordon, J. I., Relman, D. A., Fraser-Liggett, C. M., Nelson, K. E. (2006). Metagenomic analysis of the human distal gut microbiome. *Science (New York, N.Y.), 312*(5778), 1355-9.

Dethlefsen, L., McFall-Ngai, M., & Relman, D. A. (2007). An ecological and evolutionary perspective on human–microbe mutualism and disease. *Nature, 449*, 811. Retrieved from https://doi.org/10.1038/nature06245

Sanz, Y., Santacruz, A., & Gauffin, P. (2019). 3rd International Immunonutrition Workshop Session 8 : Probiotics in the defence and metabolic balance of the organism Gut microbiota in obesity and metabolic disorders Proceedings of the Nutrition Society Proceedings of the Nutrition Society, (October 2009), 434–441. https://doi.org/10.1017/S0029665110001813

Sharma, S., & Tripathi, P. (2019). ScienceDirect. *The Journal of Nutritional Biochemistry*, *63*, 101–108. https://doi.org/10.1016/j.jnutbio.2018.10.003

Franco-Obregón A, Gilbert JA. The Microbiome-Mitochondrion Connection: Common Ancestries, Common Mechanisms, Common Goals. *mSystems*. 2017;2(3):e00018-17. Published 2017 May 9. doi:10.1128/mSystems.00018-17

Vaughn AC, Cooper EM, DiLorenzo PM, et al. Energy-dense diet triggers changes in gut microbiota, reorganization of gut-brain vagal communication and increases body fat accumulation. *Acta Neurobiol Exp (Wars)*. 2017;77(1):18-30.

Nagpal R, Mainali R, Ahmadi S, et al. Gut microbiome and aging: Physiological and mechanistic insights. *Nutr Healthy Aging*. 2018;4(4):267-285. Published 2018 Jun 15. doi:10.3233/NHA-170030

Flemer B, Gaci N, Borrel G, et al. Fecal microbiota variation across the lifespan of the healthy laboratory rat. *Gut Microbes*. 2017;8(5):428-439

Hu, Q., Ren, H., Ren, J., Liu, Q., Wu, J., Wu, X., … Li, J. (2018). Released Mitochondrial DNA Following Intestinal Ischemia Reperfusion Induces the Inflammatory Response and Gut Barrier Dysfunction. *Scientific Reports*, *8*(1), 7350. https://doi.org/10.1038/s41598-018-25387-8

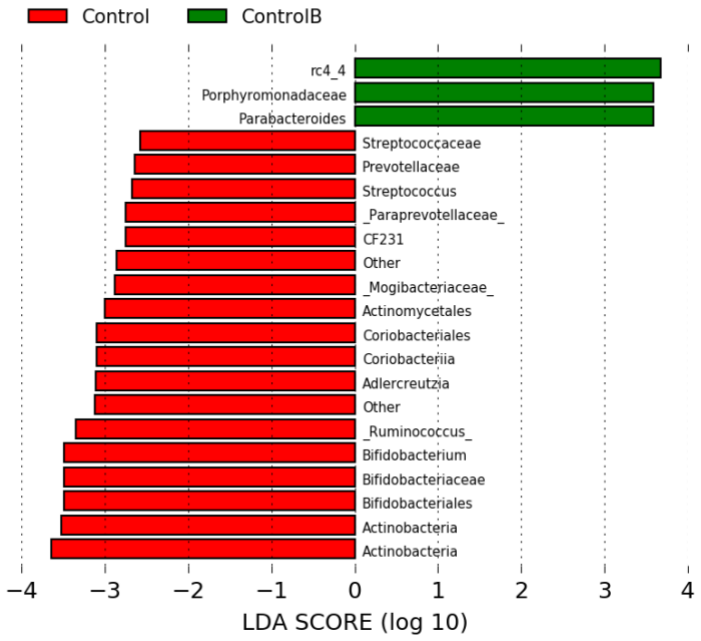
Chunchai T, Thunapong W, Yasom S, et al. Decreased microglial activation through gut-brain axis by prebiotics, probiotics, or synbiotics effectively restored cognitive function in obese-insulin resistant rats. *J Neuroinflammation*. 2018;15(1):11. Published 2018 Jan 9. doi:10.1186/s12974-018-1055-2

Kang YJ, Diao XN, Zhao GY, et al. Extensive diversity of Rickettsiales bacteria in two species of ticks from China and the evolution of the Rickettsiales. *BMC Evol Biol*. 2014;14:167. Published 2014 Jul 30. doi:10.1186/s12862-014-0167-2

Hakkak R, Korourian S, Foley SL, Erickson BD. Assessment of gut microbiota populations in lean and obese Zucker rats. *PLoS One*. 2017;12(7):e0181451. Published 2017 Jul 13. doi:10.1371/journal.pone.0181451

Ley, R. E., Turnbaugh, P. J., Klein, S., & Gordon, J. I. (2006). Human gut microbes associated with obesity. *Nature*, *444*, 1022. Retrieved from https://doi.org/10.1038/4441022a

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., & Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*, *444*, 1027. Retrieved from https://doi.org/10.1038/nature0541


Control
ControlB
rc4_4
Porphyromonadaceae
Parabacteroides
Streptococcaceae
Prevotellaceae
Streptococcus
Paraprevotellaceae
CF231
Other
Mogibacteriaceae
Actinomycetales
Coriobacteriales
Coriobacteriia
Adlercreutzia
Other
Ruminococcus
Bifidobacterium
Bifidobacteriaceae
Bifidobacteriales
Actinobacteria
Actinobacteria
−4
−3
−2
−1
0
1
2
3
4
LDA SCORE (log 10)

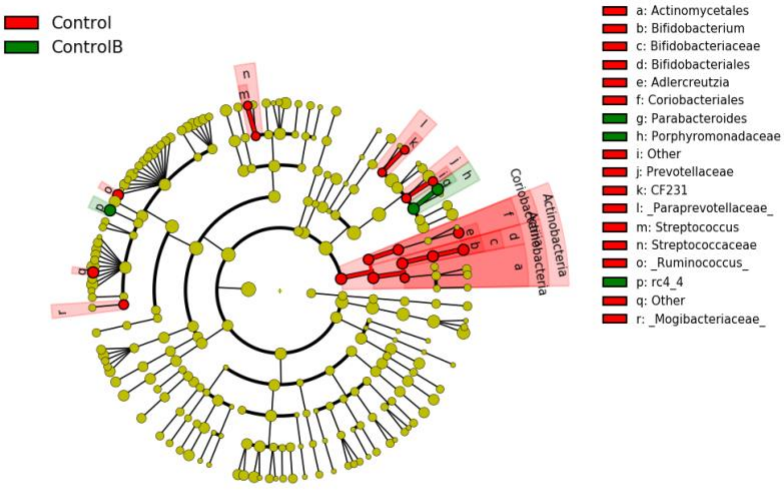
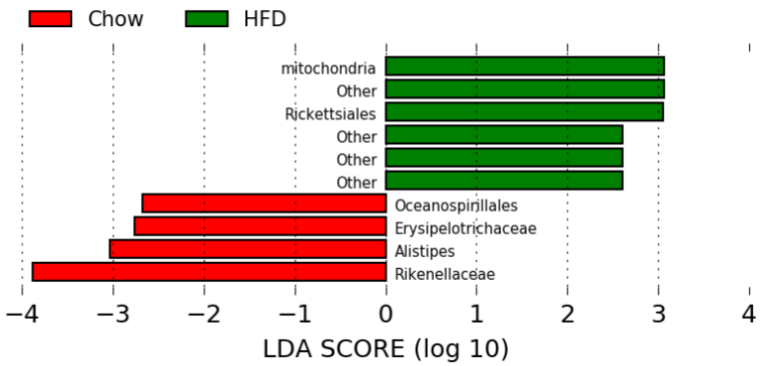

Control
ControlB
a: Actinomycetales
b: Bifidobacterium
c: Bifidobacteriaceae
d: Bifidobacteriales
e: Adlercreutzia
f: Coriobacteriales
g: Parabacteroides
h: Porphyromonadaceae
i: Other
j: Prevotellaceae
k: CF231
l: _Paraprevotellaceae_
m: Streptococcus
n: Streptococcaceae
o: _Ruminococcus_
p: rc4_4
q: Other
r: _Mogibacteriaceae_
Actinobacteria
Coriobacteriia

Figure 1. Week 0 effect sizes and hierarchical structure of differentially abundant taxa. Linear discriminate analysis of effect size (LEfSe) results depicting different microbial taxonomic signatures in samples collected from fecal samples of male Sprague-Dawley rats fed from chow at baseline (n=8, red, negative PDA score bars). Fecal samples collected from chow-fed animals at baseline are more enriched in *Streptococcaceae*, *Prevotellaceae*, *Streptococcus*, *CF231*, *Mogibacteriaceae*, *Actinomycetales*, *Coriobacteriales*, *Coriobacteriia*, *Aldercreutzia*, *Ruminococcus*, *Bifidobacterium*, *Bifidobacteriaceae*, *Bifidobacteriales* and *Actinobacteria.* However, a subsection of chow-fed rats (ControlB, green, positive LDA score bars) at baseline are more abundant in *rc4_4*, *Porphyromonadaceae* and *Parabacteroides*. (B) Cladogram depicting differentially abundant microbial taxa in fecal samples collected at baseline from male Sprague-Dawley rats fed a Chow diet (n=8). Microbial taxa enriched in Control rats are shown in red and those enriched in ControlB animals are shown in green.



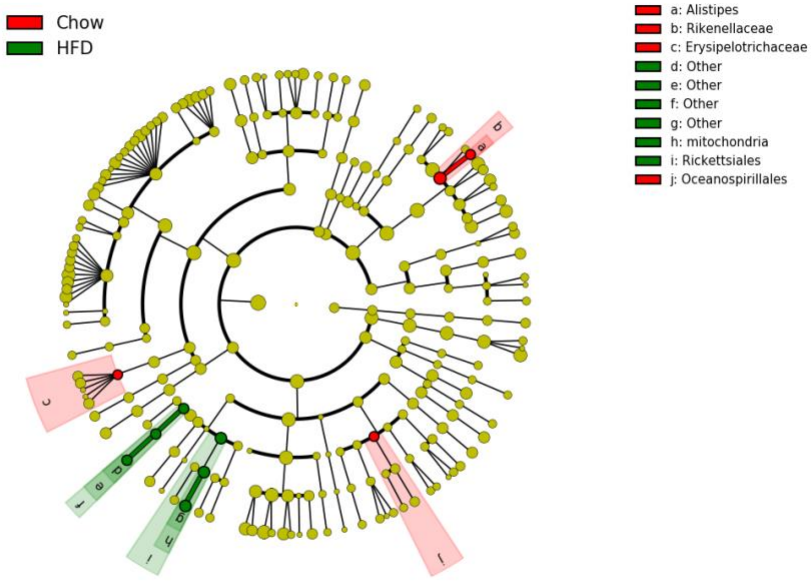


Figure 2: Week 10 effect sizes and hierarchical structure of differentially abundant taxa. LEfSe results depicting different microbial taxonomic signatures in samples collected from fecal samples of male Sprague-Dawley rats fed either HFD (n=8) or chow (n=8) for 10 weeks. Fecal samples collected from chow-fed animals (red, negative PDA

score bars) are more enriched in *Oceanospirillales*, *Erysipelotrichaceae*, *Alistipes* and *Rikenellaceae* while fecal samples collected from HFD-fed animals (green, positive LDA score bars) are more abundant in mitochondria, *Rickettsiales* and bacteria identified as "other. B) Cladogram depicting differentially abundant microbial taxa from fecal samples collected from male Sprague-Dawley rats fed either Chow (n=8) or HFD (n=8) for 10 weeks. Microbial taxa enriched in HFD rats are shown in green and those enriched in chow-fed animals are shown in red.

CHAPTER FIVE

EFFECTS OF A NOVEL ORGANOMETALLIC COMPLEX ON MARKERS OF GUT AND LIVER INFLAMMATION IN MALE SPRAGUE-DAWLEY RATS FED A HIGH FAT DIET FOR 10 WEEKS

ABSTRACT

Diet-induced obesity and metabolic syndrome are associated with the onset of gastrointestinal diseases, such as hepatic steatosis and gut inflammation. Prior research shows that a proprietary soil-derived compound decreases weight gain and blood glucose in diabetic mice. The aim of this study was to examine the effects of a similar organometallic complex (OMC) on gastrointestinal inflammation in rats fed a high fat diet (HFD). Six-week-old male Sprague-Dawley rats (n=36) were divided into two dietary groups: chow (18.9% protein, 57.33% carbohydrates, 5% fat) or HFD (20% protein, 20% carbohydrates, 60% fat) for 10 weeks. Animals were further divided (n=6/group) and administered 0, 0.6 or 3.0 mg/mL OMC in their drinking water. HFD-consuming rats showed an increased in fat accumulation in the liver as indicated by elevated triglyceride concentrations ($p<0.05$) and further validated through Oil-Red- O staining. Western blot analyses of the cecum and small intestine showed no changes in protein expression of the inflammatory cytokines NF-kB and IL-1B (cecum: Two-Way ANOVA, $p=0.362$, $p=0.702$, respectively; small Intestine: Two-Way ANOVA, $p=0.746$, $p=0.603$, respectively) in rats fed HFD. Likewise, protein expression of NF-kB and IL-1B were not altered (Cecum: Two-Way ANOVA, $p=0.402$ and $p=0.062$, respectively; Small Intestine: Two-Way ANOVA, $p=0.304$ and $p=0.574$) by OMC-treatment. Thus,

consumption of HFD for ten weeks increased levels of hepatic triglycerides, but gut inflammatory biomarkers were not significantly altered by diet or OMC treatment. Further studies are needed to determine the mechanism of action of OMC and whether it can alter intestinal permeability.

INTRODUCTION

Metabolic syndrome is characterized by a cluster of abnormalities consisting of cardiovascular disease, hypertension, hyperglycemia and obesity [Watanabe et al., 2017]. Obesity contributes to the development of many hepatic and gastrointestinal disorders which include nonalcoholic fatty liver disease (NAFLD), gastric cancer and irritable bowel disease [Camilleri et al., 2017]. Sixty to one hundred million people are affected by digestive and liver disorders in the United States [National Institutes of Health, 2009] with the leading cause being the consumption of a westernized diet.

Nonalcoholic fatty liver disease is the most prevalent chronic liver disorder in the world [Mokhtari et al., 2017]. NAFLD is characterized by steatosis (excessive buildup of fat), likely related to the concurrent rise of obesity and type 2 diabetes in westernized societies [Jensen et al., 2018]. NAFLD denotes a spectrum of liver pathologies including simple hepatic steatosis (where greater than 5% of hepatocytes have increased lipid accumulation) to a more serious diagnosis, nonalcoholic steatohepatitis which is characterized by lobular inflammation [Jensen et al., 2018]. Research in animal models has shown that diet, more specifically, a high fat diet (HFD) is an important contributor to the onset of NAFLD. Current research from our laboratory has shown that just 6 weeks of HFD consumption promoted simple steatosis through an increase of hepatic lipid

accumulation and liver triglycerides [Crawford et al., 2019]. Additionally, a recent study indicated that 16 weeks of HFD (60% fat, 20% carbohydrate) consumption resulted in extensive steatosis and inflammation in male Sprague-Dawley rats [Jensen et al., 2018]. Moreover, hepatic dysfunction was confirmed in HFD-fed rats along with increased levels of liver triglycerides and inflammatory markers [Jensen et al., 2018]. Therefore, these studies confirm that dietary fat may be a primary factor of NAFLD development in male Sprague-Dawley rats as in humans [Pappachan et al., 2017].

The gastrointestinal (GI) tract is responsible for digestion, nutrient absorption and the regulation of food intake [Fu et al., 2014]. Gastrointestinal disorders like inflammatory bowel disease, Crohn's disease and ulcerative colitis are pathological conditions induced by a westernized lifestyle [Ng et al., 2013]. Many of the same factors that promote the development of these intestinal diseases also are thought to be responsible for gastrointestinal motility abnormalities [Ng et al., 2013].

Past literature has shown that changes in gastrointestinal motility in male Sprague-Dawley rats are associated with high fat diet intake for 6 weeks [Ng et al., 2013]. In addition to a significant weight gain during the 6-week HFD, study animals showed faster gastric emptying and changes in contraction and relaxation along the duodenum and ileum [Fu et al., 2014]. Current research from our laboratory has shown that 6 weeks of HFD can increase gut inflammation and plasma endotoxins, and alter the gut microbiome [Crawford et al., 2019]. Overall, both studies indicate that changes in gastrointestinal motility and the development of gut inflammation are effects of HFD intake.

Obesity is characterized by an imbalance of energy intake and energy expenditure [Kobyliak et al., 2016]. Therefore, a traditional treatment for obesity is dietary intervention. Many dietary approaches for weight loss such as low-fat, high-protein and low-carbohydrate diets are popular in the clinical community, however, many of these dietary approaches remain debatable [Thounaoiam et al., 2016]. Additionally, the success of weight loss treatments and products only produce effects characterized by a large weight loss in a short period of time [Thounaoiam et al., 2016]. Therefore, it is important to develop more effective strategies for weight control and the onset of metabolic syndrome.

The use of natural products and herbal medicines for the management of metabolic diseases continues to rise in popularity in westernized societies [Deneau et al., 2011]. Prior studies have examined the effects of an isolate named Totala- obtained from leonardite, a natural product of lignite [Yaligar et al., 2014]. In a diabetic animal model, treatment with Totala decreased weight gain, blood glucose and glycated hemoglobin [Yaligar et al., 2014]. The purpose of the present study is to examine a similar organometallic compound (OMC) in a diet-induced model of metabolic syndrome and gastrointestinal disease.

METHODS

Animal Models: Six-week old male Sprague-Dawley rats (140-160 g, n=36) were randomly divided into two groups that received either standard chow diet (Teklad Global 10% protein, Indianapolis, IN) or HFD containing 60% kcal from fat (Cat. No. D12492; Research Diets Inc, New Brunswick, NJ) for 10 weeks. Rats in each group were

administered 0 (control), 0.6, or 3.0 mg/mL OMC (provided by Isagenix International, LLC) in their drinking water throughout the study. Food and OMC-treated water were replaced every 2-3 days to prevent spoiling. Animals were allowed free access to water and food *ad libitum*. At the end of the 10-week study, animals were euthanized (sodium pentobarbital, 200 mg/kg, i.p.). All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee.

Western Blot Analysis: Pro-inflammatory cytokines (NF-kB (p65) and IL-1B) were examined in the cecum and proximal small intestine of all study animals via western blot analysis. Cecum and proximal small intestine samples were cleaned with ice-cold PBS, pH 7.4 then homogenized in ice-cold Tris-HCl buffer containing 10 mM Tris (pH 7.6), 1 mM EDTA, 1% triton X-100, 0.1% Na-deoxycholate, 0.03% protease phosphatase inhibitor cocktail (Halt Protease and Phosphatase Inhibitor Cocktail (100X), ThermoFisher Scientific, Waltham, MA, USA) and 1mM phenylmethanesulfonyl fluoride (PMSF) using the Beadbug Microtube Homogenizer for three minutes per the manufacturer's recommendation (3.00 mm zirconium beads, Benchmark Scientific, Edison, NJ, USA). Cecum and small intestine homogenates were centrifuged at 4000 g for 10 minutes at 4 ℃ to remove insoluble debris. To examine pro-inflammatory proteins, the protein concentrations of the supernatant were determined using the Bradford method (Bio-Rad, Hercules, CA). Cecal and proximal small intestine supernatants (50 μg/lane) were resolved by 7.5% Tris-HCL sodium dodecyl sulfate polyacrylamide gel electrophoresis at 200 mV for 35 minutes (SDS-PAGE) (Bio-Rad, Hercules, CA) then transferred at 100 mV for 90 min onto polyvinylidene difluoride

membranes (PVDF) pre-wetted with Tris-buffered saline (Bio-Rad, #170-6453, Hercules, CA) and 0.1% Tween 20 (Bio-Rad, #170-6531, Hercules, CA, USA), TTBS. Protein molecular weight for phosphorylated NF-kB (p65) was visualized with SeeBlue Plus2 Pre-stained standard (ThermoFisher Scientific, Cat. No. LC5925). Protein molecular weight for IL-1β was visualized with Colorburst Pre-stained standard (ThermoFisher Scientific, Cat. No. LC592).

A. ***Protein expression in the cecum and proximal small intestine*****:** To prevent non-specific binding of antibodies, the membranes were incubated overnight at 4 °C in blocking buffer; TTBS containing 3% bovine serum albumin fraction V (BSA) and 5% nonfat dry milk. The PVDF membranes (Bio-Rad, #160-0174), Hercules, CA, USA) were then washed with TTBS (3 washes, 5 minutes each) and incubated for 4 hours at room temperature with 1:1000 anti-NF-kB p65 rabbit polyclonal antibody (GeneTex, Inc., #GTX11742) and anti-IL-1β 3A6 mouse monoclonal antibody (Cell Signaling Technologies, #12242). Subsequently, membranes were washed in TTBS (5 washes, 5 minutes each) and then incubated with either 1:5000 anti-rabbit IgG, HRP-linked secondary antibody (NF-kB) or 1:5000 anti-mouse IgG, HRP-linked secondary antibody (IL-1B), as appropriate, prepared in TTBS for 1 hour at room temperature. Membranes were washed with Tris-buffered saline (TBS, BioRad, 170-6435) after incubation with secondary antibody then exposed to Pierce enhanced chemiluminescence (ECL) western blotting substrate for one minute (Catalog # 32106, Thermo Scientific, Rockford, IL, USA). The immunoreactive bands were visualized by exposure to x-ray film (Kodak X-OMAT, Thermo Fisher Scientific, Pittsburgh, PA). Total protein concentration was normalized and quantified by

staining the PVDF membrane with Coomassie Brilliant Blue R-250 (BioRad, 161-0400, Hercules, CA, USA) prepared in 50% methanol, 42.5% deionized water and 7.5% acetic acid. Densitometry was determined using NIH ImageJ software [Schneider et al., 2012].

Liver Triglyceride Assay: Liver triglycerides were measured by digesting 100-300 mg tissue (n=5 rats per group) in 350 µL ethanolic potassium hydroxide (1:2 ratio of 30% potassium hydroxide and ethanol) overnight at 55°C. The total volume was adjusted to 1000 µL with 50% ethanol and the mixture was vortexed then centrifuged at 13,000 rpm for 5 mins. The supernatant was transferred to a clean microcentrifuge tube and the volume adjusted to 1200 µL with 50% ethanol then vortexed. A 200 µL aliquot was transferred to a new tube to which 215 µL 1M magnesium chloride was added and the mixture was vortexed then placed on ice for 10 minutes. After centrifuging for 5 minutes at 13,000 rpm, the supernatant was transferred to a new tube to be used for the assay. Liver free glycerol concentrations were determined using a commercially available kit per the manufacturer's protocol (Sigma Aldrich, St. Louis, MO, USA) and triglyceride content was determined from the free glycerol concentrations using the following equation: Triglyceride (mg/g tissue) = [glycerol] (mg/dl) * (10/30) * (415/200) * 0.012(dL)/tissue mass (g).

Oil-Red-O: Samples of liver tissue were embedded in OCT compound, frozen in isopentane cooled by dry ice and stored at -80 °C until analysis. Lipid droplets and neutral fat were stained using a commercially available Oil Red O staining kit according

to the manufacturer's protocol (ScyTek Laboratories, Inc, Logan, UT, USA). Briefly, tissue sections (12 µm) were collected onto Colorfrost microscope slides (VWR, Radnor, PA, USA) using a cryostat (Leica Biosystems CM1950, Buffalo Grove, IL, USA). Sections were incubated with propylene glycol for 5 mins at room temperature followed by heated Oil Red O solution for 10 mins. Tissues sections were then treated with 85% propylene glycol for 1 min and rinsed with distilled water. Mayer's hematoxylin (Lillie's modification) was used to counterstain sections and slides were coverslipped using aqueous mounting medium (Cat No. AML060, ScyTek Laboratories, Inc) following washes in tap water and distilled water. Images of slides were collected at 40x magnification using an EVOS FL Auto Imaging System from Life Technologies (AMAFD1000, Carlsbad, CA, USA).

STATISTICAL ANALYSES

Data are expressed as means ± SEM. Data were analyzed using SigmaPlot (Systat Software Version 10.0, San Jose, CA, USA). Western blot and liver triglyceride/glycerol data were analyzed by two-way ANOVA. Statistical significance was concluded with a $p<0.05$ for all analyses. LEfSe analysis was conducted using Kruskal-Wallis sum-rank tests, Wilcoxon rank-sum tests, and linear discriminant analysis as previously described [Segata et al., 2011].

RESULTS

Pro-inflammatory Protein Expression in the Cecum: 10-week HFD consumption did not affect the expression of transcription factor NF-kB (two-way ANOVA, df= 1,

F=0.723, *p*=0.402) or pro-inflammatory cytokine IL-1B (two-way ANOVA, df=1, F=3.744, *p*=0.062). Additionally, OMC supplementation did not influence the expression of NF-kB (two-way ANOVA, df=2, F=1.050, *p*=0.362) or IL-1B (two-way ANOVA, df=2, F=0.358, *p*=0.702) (Fig. 1). Moreover, there were no significant interactions between HFD and OMC supplementation as indicated by two-way ANOVA (NF-kb, df=2, F=0.246, *p*=0.855; IL-1B, df=2, F=0.246, *p*=0.783) (Fig. 2).

Pro-inflammatory Protein Expression in the Proximal Small Intestine: 10-week HFD consumption did not have any effects on the expression of transcription factor NF-kB (two-way ANOVA, df=2, F=0.296, *p*=0.746) and pro-inflammatory cytokines IL-1B (two-way ANOVA , df=1, F=0.277, *p*=0.603). Additionally, OMC supplementation did not have any significant effects on the expression of NF-kB (two-way ANOVA, df= 1, F=1.096, *p*=0.304) and IL-1B (two-way ANOVA, df=2, F=565, *p*=0.574) (Fig. 3). Moreover, there were no significant interactions between HFD and OMC supplementation as indicated by two-way ANOVA (NF-kB, df=2, F=0.545, p=0.586; IL-1B, *p*=0.931) (Fig.4)

Liver Triglycerides**:** Liver free glycerol and triglyceride increased significantly following the consumption of HFD (two-way ANOVA, df=1, F=134.7, *p*<0.001). However, 10-week supplementation of 0.6mg/mL or 3.0mg/mL of OMC decreased liver glycerol and triglyceride concentrations in HFD-fed rats (two-way ANOVA followed by Tukey test, p<0.001). Additionally, Liver Oil-Red-O indicated the development of simple steatosis in rats fed a 10-week HFD (Fig. 5)

DISCUSSION

High fat diet intake is associated with the development of low-grade inflammation, a characteristic of obesity and type 2 diabetes. Inflammation induced by HFD-intake is linked to the development of gastrointestinal disorders including NAFLD and an increase in intestinal permeability [Bashiardes et al., 2016]. Natural health products are becoming popular alternative treatments for metabolic disease. In the current study, we examined the effects of an organometallic complex on the gastrointestinal system during the consumption of a HFD for ten weeks in male Sprague-Dawley rats.

A previous study in our laboratory showed that this treatment can increase plasma lipopolysaccharides (LPS) [Crawford et al., in review]. Circulating LPS is associated with the breakdown of intestinal epithelial cells through the degradation of tight junction proteins [Guo et al., 2015], which increases intestinal permeability [Lecomte et al., 2015] and promotes the activation of NF-kB in animal models [Sakai et al., 2017]. NF-kB is a transcription factor which regulates the activation of genes responsible for an immune and inflammatory response [Liu et al., 2017]. The expression of NF-kB is typically upregulated during inflammatory diseases linked to tissue damage [Guma et al., 2011]. Although the etiology of inflammatory diseases remains under investigation, it is understood that the activation of NF-kB is triggered by genetic and environmental factors [Guma et al., 2011].

In the present study, consumption of HFD for ten weeks did not increase the expression of inflammatory markers (NF-kB (p65) and IL-1B) in the cecum and proximal small intestine. In other animal models of diet-induced obesity and metabolic syndrome, the consumption of HFD for 2-16 weeks only increased tumor necrosis factor-alpha

(TNF-alpha) in the ileum of C57BL6 mice [Ding et al., 2010]. Tumor necrosis factor (TNF) is an important inducer of NF-kB (p65) [Hayden et al., 2014]. An earlier study indicated that LPS can activate intestinal NF-kB with the support of neutrophils and endogenous TNF [Plaen et al., 2000]. In the current study, we did not measure TNF expression, which may be a limitation. It is possible that HFD intake for 10 weeks did not increase the levels of intestinal TNF responsible for NF-kB (p65) activation, therefore, preventing the regulation of pro-inflammatory cytokines by intestinal NF-kB. However, previous studies have shown that diet-induced obesity and metabolic syndrome are marked by an increase in pro-inflammatory markers secreted from adipose tissue [Nishimura et al., 2009]. In addition, studies have shown that HFD consumption can increase LPS which is translocated from the gut and subsequently participates in the inflammatory responses in adipose tissue [Hersoug et al., 2018]. This evidence could be a reason for the lack of inflammation in the cecum and small intestine of HFD-fed rats after 10 weeks.

Rats consuming HFD for 10 weeks showed a significant increase in liver free glycerol and liver triglycerides compared to chow-fed rats. An increase in liver triglycerides was confirmed by examination of Oil Red-O-stained liver sections, in which HFD-fed rats showed larger lipid droplets than control rats However, 10-week supplementation of 0.6mg/mL or 3.0mg/mL of OMC did not prevent increases in liver free glycerol or triglyceride concentrations in HFD-fed rats.

Our work is validated by an earlier study which confirmed that the consumption of dietary saturated fats for 24 weeks can contribute to the development of NAFLD and has been linked to systemic inflammation [Yaligar et al., 2014]. Similarly, Jensen et al.

(2018) revealed that consumption of HFD for 16 weeks promoted steatosis, hepatic dysfunction, high levels of plasma triglycerides and inflammation in rats [Jensen et al., 2018]. Recent work in our laboratory has also shown that a similar HFD (60% fat) consumed for 6 weeks can increase plasma triglycerides as well as hepatic lipid accumulation in male Sprague-Dawley rats [Crawford et al., 2019].

The current work demonstrates that although OMC supplementation may play a vital role in decreasing symptoms of metabolic syndrome, it did not exhibit any significant effects on gut inflammation. OMC, a natural health product, is predominately composed of fulvic acid (FvA). Fulvic acid is derived from humic substances that are byproducts of organic degradation from microorganisms [Winkler et al., 2018]. The medicinal use of FvA in the form of Shilajit, has been recorded throughout history but being the most popular in traditional Indian medicine for 3000 years. Although few studies have examined the physiological mechanism of FvA in animal models, literature shows that FvA has anti-inflammatory properties. FvA has been shown to reduce the release of pro-inflammatory mediators from cells in addition to decreasing TNF-alpha expression after exposure to LPS in human monocytes [Junek et al., 2009].

It is well understood that poor gut health can lead to inflammation and disease [Winkler et al., 2018]. Recent studies have shown that FvA can alter the bacterial composition of soil microbiota by conjugating itself to various minerals, thereby aiding in nutrition uptake by plants [Petrovic et al., 1996]. In addition, it has also been shown that FvA can improve nutrient absorption in animal models [Mirza et al., 2011]. However, data from the current study does not show any significant effects of OMC supplementation on gut physiology.

In the liver, FvA can act as an antioxidant by uncoupling electron transport in liver mitochondria. This action is linked to a decrease in reactive oxygen species (ROS) production [Visser et al., 1987]. ROS can begin lipid peroxidation and cause damage to DNA [Li et al., 2015]. When levels of ROS increase, this can result in oxidative stress (imbalance between free radicals and antioxidants) [Li et al., 2012] and ultimately tissue damage [Casas-Grajales et al., 2015]. However, in the current study, OMC supplementation did not have an effect on liver triglycerides. This could be due to the small sample size which may prevent detection of subtle changes in protein expression.

In summary, the current study demonstrated that a 10-week HFD increases risk factors associated with hepatic steatosis but does not elevate markers of gut inflammation. In addition, OMC supplementation did not have any significant effects on gastrointestinal physiology and more research is needed to determine the exact mechanism of OMC in a pathogenic state induced by HFD-intake. Therefore, this study provides support for further investigation into therapeutic and preventive properties of natural health products on the alleviation of metabolic disease risk factors in the gastrointestinal system.

REFERENCES

Watanabe, M., Yokotsuka, M., Yamaoka, K., Adachi, M., Nemoto, A., & Tango, T. (2017). Effects of a lifestyle modification programme to reduce the number of risk factors for metabolic syndrome: a randomised controlled trial. *Public Health Nutrition*, *20*(1), 142–153. https://doi.org/10.1017/S1368980016001920

Camilleri, M., Malhi, H., & Acosta, A. (2017). Gastrointestinal Complications of Obesity. *Gastroenterology*, *152*(7), 1656–1670. https://doi.org/10.1053/j.gastro.2016.12.052

National Institutes of Health, U.S. Department of Health and Human Services. *Opportunities and Challenges in Digestive Diseases Research: Recommendations of the National Commission on Digestive Diseases.* Bethesda, MD: National Institutes of Health; 2009. NIH Publication 08–6514.

Mokhtari, Z., Gibson, D. L., & Hekmatdoost, A. (2017). Nonalcoholic Fatty Liver Disease, the Gut Microbiome, and Diet. *Advances in Nutrition: An International Review Journal*, *8*(2), 240–252. https://doi.org/10.3945/an.116.013151

Jensen, V. S., Hvid, H., Damgaard, J., Nygaard, H., Ingvorsen, C., Wulff, E. M., Lykkesfeldt, J., … Fledelius, C. (2018). Dietary fat stimulates development of NAFLD more potently than dietary fructose in Sprague-Dawley rats. *Diabetology & metabolic syndrome*, *10*, 4. doi:10.1186/s13098-018-0307-8

Pappachan, J. M., Babu, S., Krishnan, B., & Ravindran, N. C. (2017). Non-alcoholic Fatty Liver Disease: A Clinical Update. *Journal of Clinical and Translational Hepatology*, *5*(4), 384–393. https://doi.org/10.14218/JCTH.2017.00013

Crawford, M., Whisner, C., Al-Nakkash, A., Sweazea, KL. (2019). Six-week high fat diet promotes simple steatosis, intestinal inflammation and endotoxin production without obesity in male rats. *Lipids*.

Fu, X.-Y., Li, Z., Zhang, N., Yu, H.-T., Wang, S.-R., & Liu, J.-R. (2014). Effects of gastrointestinal motility on obesity. *Nutrition & Metabolism*, *11*(1), 3. https://doi.org/10.1186/1743-7075-11-3

Ng, S. C., Bernstein, C. N., Vatn, M. H., Lakatos, P. L., Loftus, E. V, … Tysk, C. (2013). Geographical variability and environmental risk factors in inflammatory bowel disease. *Gut*, *62*(4), 630 LP-649. https://doi.org/10.1136/gutjnl-2012-303661

Kobyliak, N., Conte, C., Cammarota, G., Haley, A. P., Styriak, I., Gaspar, L., … Kruzliak, P. (2016). Probiotics in prevention and treatment of obesity: a critical view. *Nutrition & Metabolism*, *13*(1), 14. https://doi.org/10.1186/s12986-016-0067-0

Thounaojam, M. C., Nammi, S., & Jadeja, R. (2016). Natural Products for the Treatment of Obesity, Metabolic Syndrome, and Type 2 Diabetes 2016. *Evidence-Based Complementary and Alternative Medicine : ECAM*, *2016*, 9072345. https://doi.org/10.1155/2016/9072345

Deneau, J. (2011). Anti-Diabetic Activity of a Mineraloid Isolate, in vitro and in Genetically Diabetic Mice. *International Journal for Vitamin and Nutrition Research*, *81*(1), 34–43.

Schneider CA, Rasband WS, Eliceiri KW. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9**:671–675

Bashiardes, S., Shapiro, H., Rozin, S., Shibolet, O., & Elinav, E. (2016). Non-alcoholic fatty liver and the gut microbiota. *Molecular Metabolism*, *5*(9), 782–794. https://doi.org/https://doi.org/10.1016/j.molmet.2016.06.003

Guo, S., Nighot, M., Al-Sadi, R., Alhmoud, T., Nighot, P., & Ma, T. Y. (2015). Lipopolysaccharide Regulation of Intestinal Tight Junction Permeability Is Mediated by TLR4 Signal Transduction Pathway Activation of FAK and MyD88. *Journal of Immunology (Baltimore, Md. : 1950)*, *195*(10), 4999–5010. https://doi.org/10.4049/jimmunol.1402598

Crawford, M., Gumpricht, E., Sweazea, KL. (2019). A Novel Organometallic Complex Attenuates Risk Factors in a Rodent Model of Metabolic Syndrome. In Review.

Lecomte, V., Kaakoush, N. O., Maloney, C. A., Raipuria, M., Huinao, K. D., Mitchell, H. M., & Morris, M. J. (2015). Changes in gut microbiota in rats fed a high fat diet correlate with obesity-associated metabolic parameters. *PLoS ONE*, *10*(5). https://doi.org/10.1371/journal.pone.0126931

Sakai, J., Cammarota, E., Wright, J. A., Cicuta, P., Gottschalk, R. A., Li, N., … Bryant, C. E. (2017). Lipopolysaccharide-induced NF-κB nuclear translocation is primarily dependent on MyD88, but TNFα expression requires TRIF and MyD88. *Scientific Reports*, *7*(1), 1428. https://doi.org/10.1038/s41598-017-01600-y

Liu, T., Zhang, L., Joo, D., & Sun, S.-C. (2017). NF-κB signaling in inflammation. *Signal Transduction And Targeted Therapy*, *2*, 17023. Retrieved from https://doi.org/10.1038/sigtrans.2017.23

Guma, M., Stepniak, D., Shaked, H., Spehlmann, M. E., Shenouda, S., Cheroutre, H., … Karin, M. (2011). Constitutive intestinal NF-κB does not trigger destructive inflammation unless accompanied by MAPK activation. *The Journal of Experimental Medicine*, *208*(9), 1889–1900. https://doi.org/10.1084/jem.20110242

Ding, S., Chi, M. M., Scull, B. P., Rigby, R., Schwerbrock, N. M. J., Magness, S., … Lund, P. K. (2010). High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse. *PloS One*, *5*(8), e12191–e12191. https://doi.org/10.1371/journal.pone.0012191

Hayden, M. S., & Ghosh, S. (2014). Regulation of NF-κB by TNF family cytokines. *Seminars in Immunology*, *26*(3), 253–266. https://doi.org/10.1016/j.smim.2014.05.004

De Plaen, I. G., Tan, X. D., Chang, H., Wang, L., Remick, D. G., & Hsueh, W. (2000). Lipopolysaccharide activates nuclear factor kappaB in rat intestine: role of endogenous platelet-activating factor and tumour necrosis factor. *British Journal of Pharmacology*, *129*(2), 307–314. https://doi.org/10.1038/sj.bjp.0703055

Nishimura, S., Manabe, I., & Nagai, R. (2009). Adipose tissue inflammation in obesity and metabolic syndrome. *Discovery Medicine*, *8*(41), 55-60.

Hersoug, L.-G., Møller, P., & Loft, S. (2018). Role of microbiota-derived lipopolysaccharide in adipose tissue inflammation, adipocyte size and pyroptosis during obesity. *Nutrition Research Reviews*, *31*(2), 153–163. https://doi.org/DOI: 10.1017/S0954422417000269

Yaligar, J., Gopalan, V., Kiat, O. W., Sugii, S., Shui, G., Lam, B. D., … Velan, S. S. (2014). Evaluation of dietary effects on hepatic lipids in high fat and placebo diet fed rats by in vivo MRS and LC-MS techniques. *PloS One*, *9*(3), e91436–e91436. https://doi.org/10.1371/journal.pone.0091436

Winkler, J., & Ghosh, S. (2018). Therapeutic Potential of Fulvic Acid in Chronic Inflammatory Diseases and Diabetes. *Journal of Diabetes Research*, *2018*, 1–7. https://doi.org/10.1155/2018/5391014

Junek, R., Morrow, R., Schoenherr, J. I., Schubert, R., Kallmeyer, R., Phull, S., & Klöcking, R. (2009). Bimodal effect of humic acids on the LPS-induced TNF-α release from differentiated U937 cells. *Phytomedicine*. https://doi.org/10.1016/j.phymed.2008.10.003

Petrovic, M., & Kastelan-Macan, M. (1996). The uptake of inorganic phosphorus by insoluble metal-humic complexes. *Water Science and Technology*, *34*(7–8), 253–258. https://doi.org/10.2166/wst.1996.0629

Mirza, M. A., Ahmad, N., Agarwal, S. P., Mahmood, D., Khalid Anwer, M., & Iqbal, Z. (2011). Comparative evaluation of humic substances in oral drug delivery. *Results in Pharma Sciences*, *1*(1), 16–26. https://doi.org/https://doi.org/10.1016/j.rinphs.2011.06.001

Visser, S. A. (1987). Effect of humic substances on mitochondrial respiration and oxidative phosphorylation. *Science of The Total Environment*, *62*, 347–354. https://doi.org/https://doi.org/10.1016/0048-9697(87)90521-3

Li, S., Tan, H.-Y., Wang, N., Zhang, Z.-J., Lao, L., Wong, C.-W., & Feng, Y. (2015). The Role of Oxidative Stress and Antioxidants in Liver Diseases. *International Journal of Molecular Sciences*, *16*(11), 26087–26124. https://doi.org/10.3390/ijms161125942

Casas-Grajales, S., & Muriel, P. (2015). Antioxidants in liver health. *World Journal of Gastrointestinal Pharmacology and Therapeutics*, *6*(3), 59–72. https://doi.org/10.4292/wjgpt.v6.i3.59

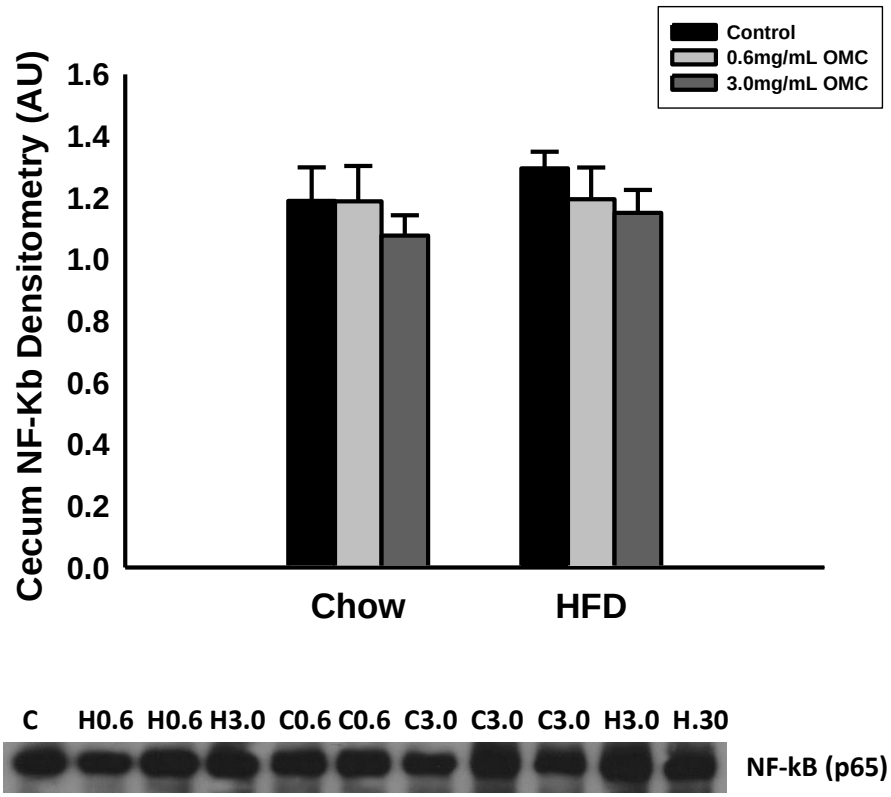


Figure 1: Western blot analyses of NF-kB in the cecum

Protein expression of inflammatory cytokines in cecum samples collected from male Sprague-Dawley rats from all treatment groups analyzed by Western blot. Densitometry values were normalized to Coomassie for all samples. Data are expressed as mean ±SEM and analyzed by two-way ANOVA, (A) Representative blot of NF-kB (p65) in cecum homogenate from rats in all treatment groups (n=6/group), *p=0.362*. Effects of OMC supplementation on NF-kB expression, *p=0.803*. HFD vs OMC interaction, *p*=0.855.

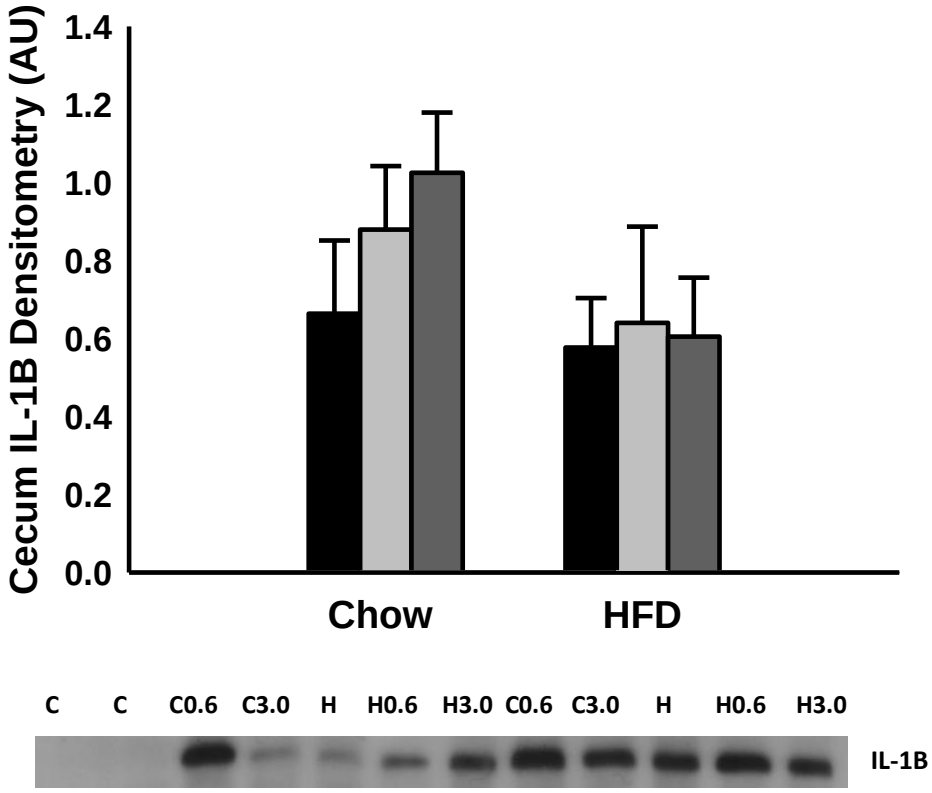


Figure 2. Western blot analyses of IL-1B in the cecum

Protein expression of inflammatory cytokines in cecum samples collected from male Sprague-Dawley rats from all treatment groups analyzed by Western blot. Densitometry values were normalized to Coomassie for all samples. Data are expressed as mean ±SEM and analyzed by Two-Way ANOVA. Representative blot of IL-1B in cecum homogenate from rats in all treatment groups (n=6/group) *p=0.702*. Effects of OMC supplementation on IL-1B expression, *p=0.602*. HFD vs. OMC interaction, *p=0.783*.

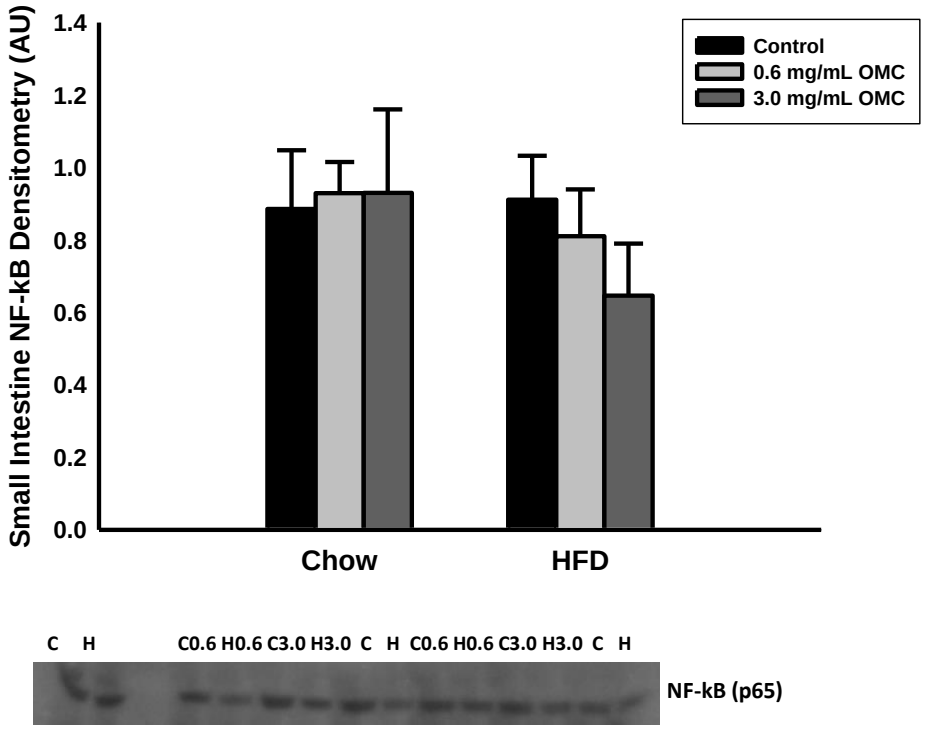


Figure 3: Western blot analyses of NF-kB in the small intestine

Protein expression of inflammatory cytokines in small intestine samples collected from male Sprague-Dawley rats from all treatment groups analyzed by Western blot. Densitometry values were normalized to Coomassie for all samples. Data are expressed as mean ±SEM and analyzed by two-way ANOVA. Representative blot of NF-kB (p65) in small intestine homogenate from rats in all treatment groups (n=6/group), *p=0.746*. Effects of OMC supplementation on NF-kB expression, *p=0.304*. HFD vs OMC interaction, *p*=0.586.

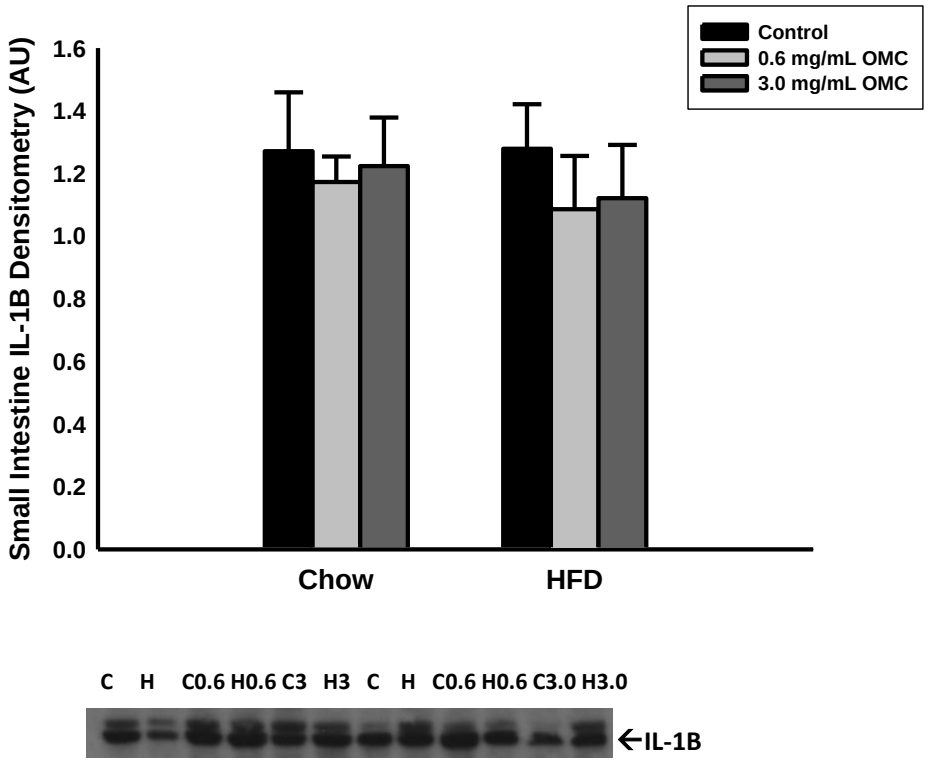
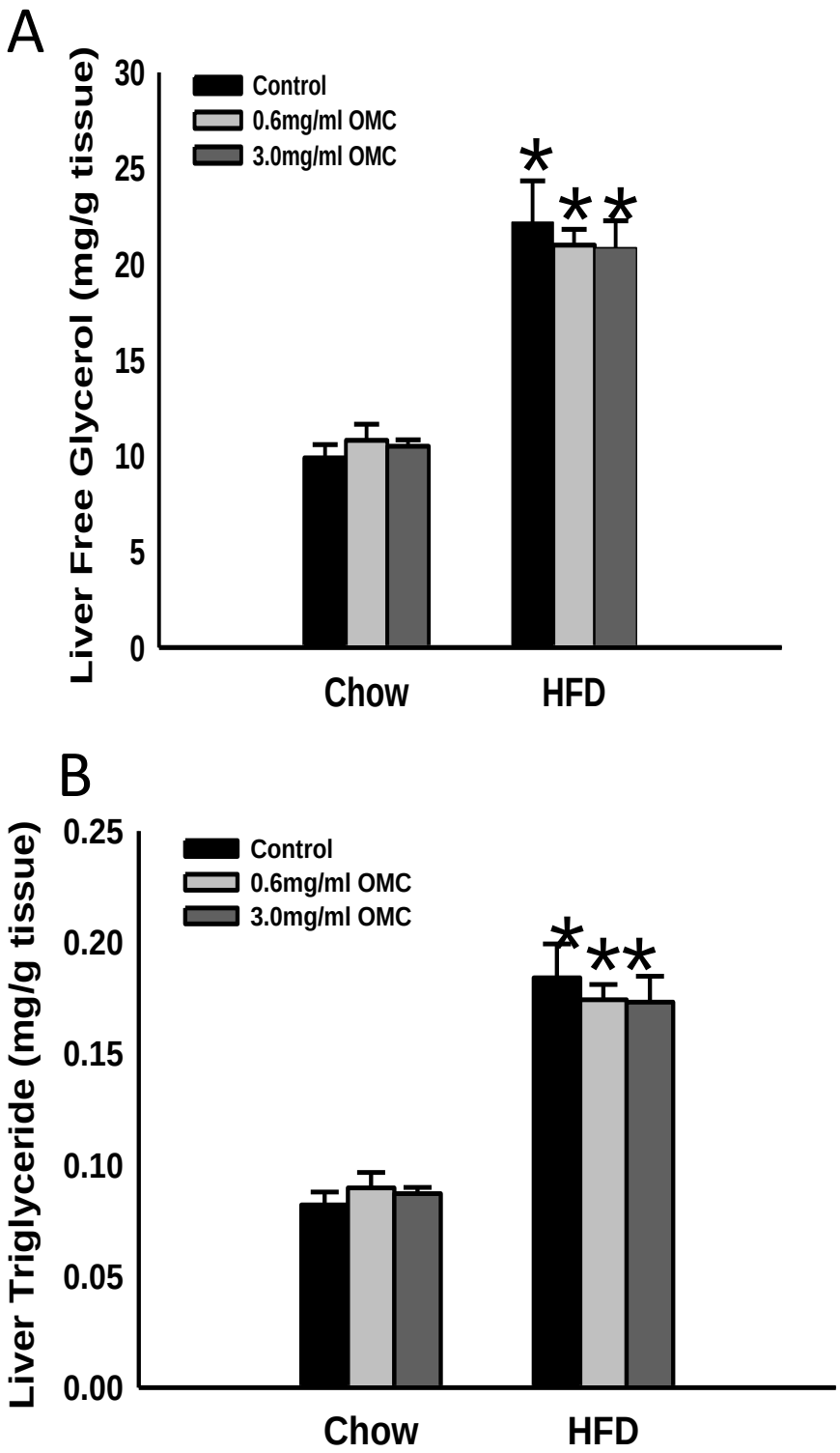


Figure 4: Western blot analyses of IL-1B in the small intestine

Protein expression of inflammatory cytokines in small intestine samples collected from male Sprague-Dawley rats from all treatment groups analyzed by Western blot. Densitometry values were normalized to Coomassie for all samples. Data are expressed as mean ±SEM and analyzed by Two-Way ANOVA. Representative blot of IL-1B in small intestine homogenate from rats in all treatment groups (n=6/group), p=0.603. Effects of OMC supplementation on IL-1B expression, *p=0.574*. HFD vs OMC interaction, *p=0.931*.


A
Liver Free Glycerol (mg/g tissue)
30
25
20
15
10
5
0
Control
0.6mg/ml OMC
3.0mg/ml OMC
*
**
Chow
HFD
B
Liver Triglyceride (mg/g tissue)
0.25
0.20
0.15
0.10
0.05
0.00
Control
0.6mg/ml OMC
3.0mg/ml OMC
*
**
Chow
HFD

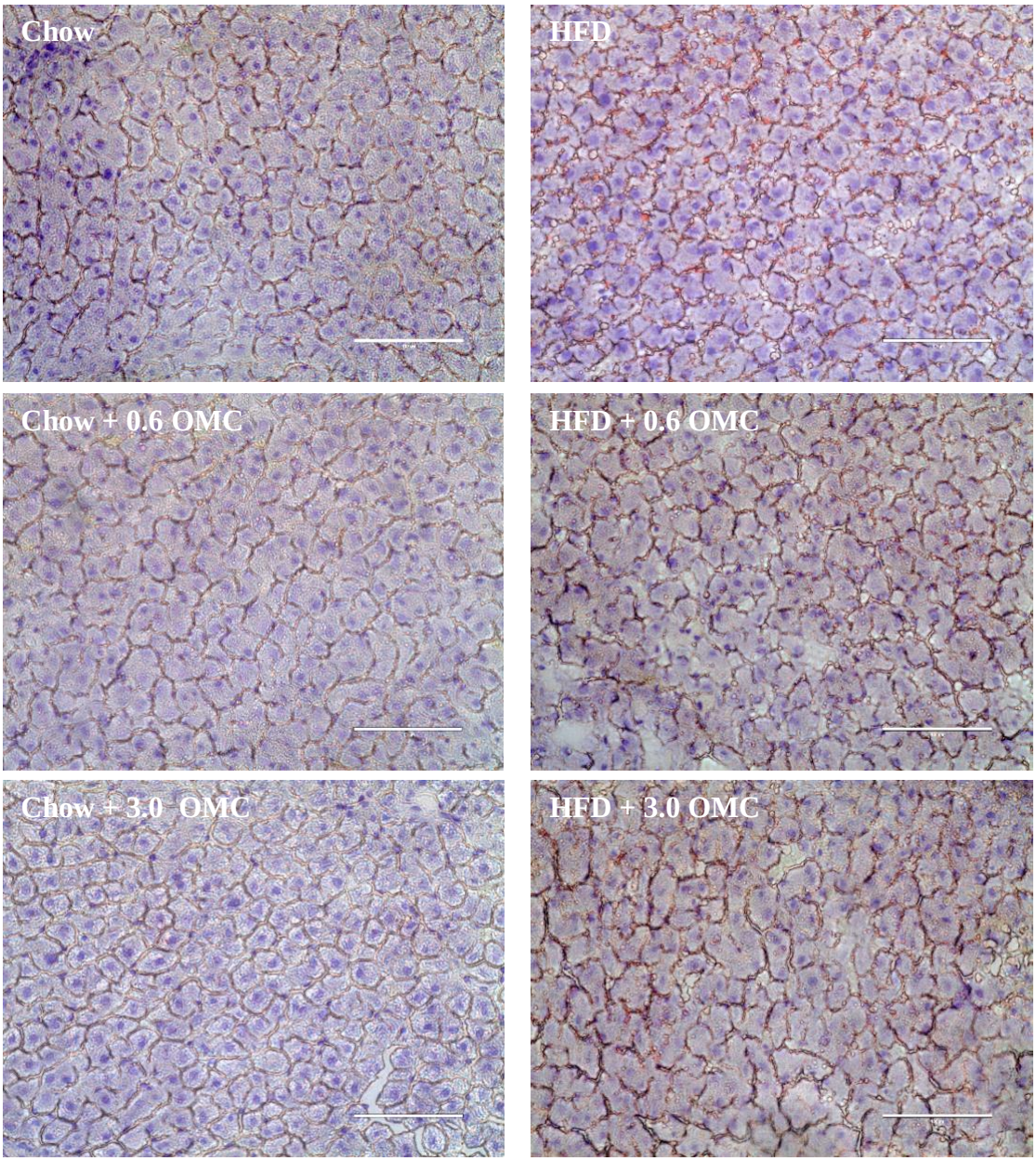


Figure 5: Hepatic Lipid Accumulation

(A) Liver free glycerol and (B) triglyceride from chow and HFD. Data are expressed as mean ±SEM and analyzed by Two-Way ANOVA, *$p<0.05$ vs. respective chow control.

Oil-Red O indicated the development of simple steatosis in rats fed a 10-week HFD. Representative images of Oil-Red O stained hepatic tissues collected from male Sprague-Dawley rats from chow, HFD and OMC-treated rats. Frozen tissue sections were stained with Oil-Red O to show lipid content and counterstained with hematoxylin to show nuclei (blue). Livers from HFD rats showed evidence of simple steatosis after 10 weeks.

CHAPTER SIX

CONCLUSIONS

High fat diet increases metabolic syndrome risk factors

My dissertation work demonstrates that the consumption of a westernized diet can increase risk factors related to metabolic syndrome (MetS) and gastrointestinal diseases. High fat diet (HFD) intake for 6 and 10 weeks (Chapter 2, Chapter 3 and Chapter 5), increased body mass and abdominal waist circumference. These findings were also accompanied by an increase in glucose intolerance and insulin resistance (Chapter 3). Additionally, the consumption of HFD promoted hepatocyte injury indicated by a rise in liver triglycerides and ALT activity (Chapter 2 and Chapter 5). Interestingly, a 6-week HFD promoted cecal inflammation but these findings conflict with our 10-week diet-induced model of metabolic syndrome. A 10-week HFD did not produce any differences in the expression of pro-inflammatory markers in the cecum and small intestine (Chapter 5). During the 6-week HFD, study animals were housed together and are known to be coprophagic [Hakkak et al., 2017]. Previous studies have shown that coprophagic activity could have a significant effect on gut microbial communities [Hakkak et al., 2017] and thereby could be a study limitation yet a casual factor in the development of intestinal inflammation in our animal models. During the 10-week treatment, study animals were singly housed. Earlier studies have reported that single housing increases stress in Sprague-Dawley rats [Turner et al., 2014; Pinelli et al., 2017]. This may also serve as a factor in the variations of outcomes between the 6 and 10-week studies.

Similar research has shown that murine models on a HFD for 2-16 weeks develop intestinal inflammation, typically found in the ileum and colon [Ding et al., 2010]. Still, there are few studies that examine inflammation specifically in the cecum of rodent models. Although, our studies show that poor dietary consumption can lead to an increase in metabolic disease risk, these data also indicate a need for continued research on the effects of metabolic syndrome on the development of inflammation in the gastrointestinal tract.

High fat diet can alter the gut microbiome and promote plasma endotoxemia

Observations of the gut microbiome have raised questions about human health and the pathology of disease. Several studies have investigated the intestinal microbiota of human and animal models to better understand the relationship between intestinal bacteria and the development of metabolic disorders [Liang et al., 2018]. I demonstrated that the gut microbiome can be significantly altered following the consumption of a 6 and 10-week HFD (Chapter 2 and Chapter 4). Similar to human studies that examine modifications of the gut microbiota, the cecum of Sprague-Dawley rats showed a significant increase in gram-positive bacteria (*Firmicutes*, *Actinobacteria, Clostridiales, Proteobacteria*, *Streptococcaceae, Erysipelotrich and Dorea*) associated with non-alcoholic fatty liver disease and irritable bowel syndrome [Turnbaugh et al., 2009; Jiang et al., 2015] (Chapter 2).

Additionally, I reported that study animals on a 6-week HFD showed an increase in the abundance of commensal gram-negative bacteria (*Bacteroidetes* and *Actinobacteria*; Chapter 4). This increase in gram-negative bacteria could explain the rise

in plasma lipopolysaccharides (Chapter 2 and Chapter 3). In the 6-week study, the expression of transcription factor NF-kB and pro-inflammatory cytokine IL-1B were significantly increased in the cecum (Chapter 4). The production of pro-inflammatory cytokines can initiate an immune response to destroy gram-negative bacteria subsequently releasing LPS and also degrading intestinal epithelial tight junction proteins [Lee et al., 2015]. This increase in intestinal permeability allows for favorable conditions of pathogenic bacteria and systemic inflammation induced by LPS and pro-inflammatory cytokines [Kim et al., 2012]. I also reported that a 10-week HFD can decrease fecal bacterial diversity and significantly alter the composition of fecal bacteria by increasing the abundance of microbes classified as "other", *Rickettsiales* and mitochondrial DNA which may serve as a reactive oxygen species mediator (Chapter 4).

Findings from these studies demonstrate the variability of microbial abundance along the gastrointestinal tract. The differences in microbial communities during a pathogenic state induced by a HFD have been linked to metabolic syndrome and the development of obesity and gastrointestinal diseases [Hillman et al., 2017]. These studies may continue to encourage the development of novel treatment methods focusing on promoting the growth of commensal bacteria lost during HFD-intake. Additionally, these studies can provide further understanding of the role of the gut microbiota in metabolic disease states.

OMC supplementation alleviates symptoms of metabolic syndrome

It has been reported that natural health products may alleviate symptoms of metabolic syndrome (Chapter 3 and Chapter 5). It has been hypothesized that a state of

chronic low-grade inflammation promotes the development of obesity, cardiovascular disease and type 2 diabetes [Emanuela et al., 2012]. Clinical and basic studies have reported poor dietary habits and low physical activity are essential in the development of metabolic disease [Pisavos et al., 2006]. There have been many efforts to treat MetS through dietary supplementation of probiotics and other natural health products [Mallappa et al., 2012; Winkler et al., 2018]. My dissertation work demonstrates that supplementation with either a low or high dose of an organometallic complex (OMC) for 10 weeks prevented increases in glucose concentrations, plasma LPS, and ALT activity in HFD-fed rats. The high dose of OMC supplementation reversed the impairment of endothelium-dependent vasodilation but did not exhibit any significant effects on the development of simple steatosis or the production of pro-inflammatory cytokines in the cecum or small intestine of HFD-fed rats. The composition of OMC is primarily fulvic acid (similar to Shilijat) and may act through antioxidant mechanisms to alleviate metabolic complications. However, the exact physiological mechanisms of fulvic acid are not fully understood. Therefore, more research is needed to determine the therapeutic effects of OMC supplementation during chronic inflammation induced by HFD-intake.

REFERENCES:

Saklayen, M. G. (2018). The Global Epidemic of the Metabolic Syndrome. *Current Hypertension Reports*, *20*(2), 12. https://doi.org/10.1007/s11906-018-0812-z

Oda, E. (2012). Metabolic syndrome: Its history, mechanisms, and limitations. *Acta Diabetologica*, *49*(2), 89–95. https://doi.org/10.1007/s00592-011-0309-6

Vague, J. (1956). The Degree of Masculine Differentiation of Obesities: A factor determining predisposition to diabetes, atherosclerosis, gout, and uric calculous disease. *The American Journal of Clinical Nutrition*, *4*(1), 20–34. https://doi.org/10.1093/ajcn/4.1.20

Kissebah, A.H. Ecans, D. J., Vydelingum, N., Adams, P. W., Murray, R., & Kalkhoff, R. K. (1982). Relation of Body Fat Distribution to Metabolic Complications of Obesity*. *The Journal of Clinical Endocrinology & Metabolism*, *54*(2), 254–260. https://doi.org/10.1210/jcem-54-2-254

Fujioka, S., Matsuzawa, Y., Tokunaga, K., & Tarui, S. (1987). Contribution of intra-abdominal fat accumulation to the impairment of glucose and lipid metabolism in human obesity. *Metabolism - Clinical and Experimental*, *36*(1), 54–59. https://doi.org/10.1016/0026-0495(87)90063-1

Reaven, G. M. (1988). Role of Insulin Resistance in Human Disease. *Diabetes*, *37*(12), 1595 LP-1607. https://doi.org/10.2337/diab.37.12.1595

Panchal, S. K., & Brown, L. (2011). Rodent models for metabolic syndrome research. *Journal of Biomedicine & Biotechnology*, *2011*, 351982. https://doi.org/10.1155/2011/351982

Parikh, R. M., & Mohan, V. (2012). Changing definitions of metabolic syndrome. *Indian Journal of Endocrinology and Metabolism*, *16*(1), 7–12. https://doi.org/10.4103/2230-8210.91175

Moore, J. X., Chaudhary, N., & Akinyemiju, T. (2017). Metabolic Syndrome Prevalence by Race/Ethnicity and Sex in the United States, National Health and Nutrition Examination Survey, 1988-2012. *Preventing Chronic Disease*, *14*, E24. https://doi.org/10.5888/pcd14.160287

Harley, I. T. W., & Karp, C. L. (2012). Obesity and the gut microbiome: Striving for causality. *Molecular Metabolism*, *1*(1–2), 21–31. https://doi.org/10.1016/j.molmet.2012.07.002

Cho, I., & Blaser, M. J. (2012). The human microbiome: at the interface of health and disease. *Nature Reviews Genetics*, *13*(4), 260–270.

https://doi.org/10.1038/nrg3182

Cani, P. D., & Everard, A. (2016). Talking microbes: When gut bacteria interact with diet and host organs. *Molecular Nutrition and Food Research*, *60*(1), 58–66. https://doi.org/10.1002/mnfr.201500406

Wang, H., Tang, X., Cheserek, M. J., Shi, Y., & Le, G. (2015). Obesity prevention of synthetic polysaccharides in high-fat diet fed C57BL/6 mice. *Journal of Functional Foods*, *17*, 563–574. https://doi.org/10.1016/j.jff.2015.06.012

Ganapathy, V., Thangaraju, M., Prasad, P. D., Martin, P. M., & Singh, N. (2013). Transporters and receptors for short-chain fatty acids as the molecular link between colonic bacteria and the host. *Current Opinion in Pharmacology*, *13*(6), 869–874. https://doi.org/10.1016/j.coph.2013.08.006

Bischoff, S. C., Barbara, G., Buurman, W., Ockhuizen, T., Schulzke, J.-D., Serino, M., … Wells, J. M. (2014). Intestinal permeability – a new target for disease prevention and therapy. *BMC Gastroenterology*, *14*(1), 189. https://doi.org/10.1186/s12876-014-0189-7

Raetz, C. R. H., & Whitfield, C. (2002). Lipopolysaccharide Endotoxins. *Annual Review of Biochemistry*, *71*(1), 635–700. https://doi.org/10.1146/annurev.biochem.71.110601.135414

Bäckhed, F., Manchester, J. K., Semenkovich, C. F., & Gordon, J. I. (2007). Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. *Proceedings of the National Academy of Sciences*, *104*(3), 979–984. https://doi.org/10.1073/pnas.0605374104

Yan, H., Diao, H., Xiao, Y., Li, W., Yu, B., He, J., … Livak, K. J. (2016). Gut microbiota can transfer fiber characteristics and lipid metabolic profiles of skeletal muscle from pigs to germ-free mice. *Scientific Reports*, *6*(August), 31786. https://doi.org/10.1038/srep31786

Wheeler, R., Turner, R. D., Bailey, R. G., Salamaga, B., Mesnage, S., Mohamad, S. A. S., … Foster, S. J. (2015). Bacterial cell enlargement requires control of cell wall stiffness mediated by peptidoglycan hydrolases. *mBio*, *6*(4), 1–10. https://doi.org/10.1128/mBio.00660-15

Wang, P., Li, N., Li, J. S., & Li, W. Q. (2002). The role of endotoxin, TNF-α, and IL-6 in inducing the state of growth hormone insensitivity. *World Journal of Gastroenterology*, *8*(3), 531–536.

Baker, R. G., Hayden, M. S., & Ghosh, S. (2011). NF-κB, inflammation, and metabolic disease. *Cell Metabolism*, *13*(1), 11–22.

https://doi.org/10.1016/j.cmet.2010.12.008

Patel, H., & Pate, V. H. (2015). Inflammation and Metabolic Syndrome: An Overview. *Current Research in Nutrition and Food Science*, *3*(3), 262–268. https://doi.org/10.1002/9781444347319.ch13

Brower, M., Grace, M., Kotz, C. M., & Koya, V. (2015). Comparative analysis of growth characteristics of Sprague Dawley rats obtained from different sources. *Laboratory Animal Research*, *31*(4), 166–173. https://doi.org/10.5625/lar.2015.31.4.166

Sweazea, K. L., Lekic, M., & Walker, B. R. (2010). Comparison of mechanisms involved in impaired vascular reactivity between high sucrose and high fat diets in rats. *Nutrition & Metabolism*, *7*, 48. https://doi.org/10.1186/1743-7075-7-48

Dobrian Anca, D., Davies Michael, J., Prewitt Russell, L., & Lauterio Thomas, J. (2000). Development of Hypertension in a Rat Model of Diet-Induced Obesity. *Hypertension: Journal of the American Heart Association*, *35*(4), 1009–1015.

Ghibaudi, L., Cook, J., Farley, C., van Heek, M., & Hwa, J. J. (2002). Fat Intake Affects Adiposity, Comorbidity Factors, and Energy Metabolism of Sprague-Dawley Rats. *Obesity Research*, *10*(9), 956–963. https://doi.org/10.1038/oby.2002.130

He, M., & Shi, B. (2017). Gut microbiota as a potential target of metabolic syndrome: the role of probiotics and prebiotics. *Cell & Bioscience*, *7*, 54. https://doi.org/10.1186/s13578-017-0183-1

Wos-Oxley, M., Bleich, A., Oxley, A. P. A., Kahl, S., Janus, L. M., Smoczek, A., … Pieper, D. H. (2012). Comparative evaluation of establishing a human gut microbial community within rodent models. *Gut Microbes*, *3*(3), 234–249. https://doi.org/10.4161/gmic.19934

Ding, S., Chi, M. M., Scull, B. P., Rigby, R., Schwerbrock, N. M. J., Magness, S., … Lund, P. K. (2010). High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse. *PloS One*, *5*(8), e12191–e12191. https://doi.org/10.1371/journal.pone.0012191

Hakkak, R., Korourian, S., Foley, S. L., & Erickson, B. D. (2017). Assessment of gut microbiota populations in lean and obese Zucker rats. *PloS One*, *12*(7), e0181451–e0181451. https://doi.org/10.1371/journal.pone.0181451

Turner, P. V, Sunohara-Neilson, J., Ovari, J., Healy, A., & Leri, F. (2014). Effects of single compared with pair housing on hypothalamic-pituitary-adrenal axis activity and low-dose heroin place conditioning in adult male Sprague-Dawley rats.

Journal of the American Association for Laboratory Animal Science : JAALAS, *53*(2), 161–167. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/24602542

Pinelli, C. J., Leri, F., & Turner, P. V. (2017). Long term physiologic and behavioural effects of housing density and environmental resource provision for adult male and female sprague dawley rats. *Animals*, *7*(6). https://doi.org/10.3390/ani7060044

Liang, D., Leung, R. K.-K., Guan, W., & Au, W. W. (2018). Involvement of gut microbiome in human health and disease: brief overview, knowledge gaps and research opportunities. *Gut Pathogens*, *10*, 3. https://doi.org/10.1186/s13099-018-0230-4

Kim, K.-A., Gu, W., Lee, I.-A., Joh, E.-H., & Kim, D.-H. (2012). High Fat Diet-Induced Gut Microbiota Exacerbates Inflammation and Obesity in Mice via the TLR4 Signaling Pathway. *PLOS ONE*, *7*(10), e47713. Retrieved from https://doi.org/10.1371/journal.pone.0047713

Lee, S. H. (2015). Intestinal permeability regulation by tight junction: implication on inflammatory bowel diseases. *Intestinal Research*, *13*(1), 11–18. https://doi.org/10.5217/ir.2015.13.1.11

Hillman, E. T., Lu, H., Yao, T., & Nakatsu, C. H. (2017). Microbial Ecology along the Gastrointestinal Tract. *Microbes and Environments*, *32*(4), 300–313. https://doi.org/10.1264/jsme2.ME17017

Emanuela, F., Grazia, M., Marco, D. R., Maria Paola, L., Giorgio, F., & Marco, B. (2012). Inflammation as a link between obesity and metabolic syndrome. *Journal of Nutrition and Metabolism*, *2012*. https://doi.org/10.1155/2012/476380

Mallappa, R. H., Rokana, N., Duary, R. K., Panwar, H., Batish, V. K., & Grover, S. (2012). Management of metabolic syndrome through probiotic and prebiotic interventions. *Indian Journal of Endocrinology and Metabolism*, *16*(1), 20–27. https://doi.org/10.4103/2230-8210.91178

Winkler, J., & Ghosh, S. (2018). Therapeutic Potential of Fulvic Acid in Chronic Inflammatory Diseases and Diabetes. *Journal of Diabetes Research*, *2018*, 1–7. https://doi.org/10.1155/2018/5391014

Pitsavos, C., Panagiotakos, D., Weinem, M., & Stefanadis, C. (2006). Diet, exercise and the metabolic syndrome. *The Review of Diabetic Studies : RDS*, *3*(3), 118–126. https://doi.org/10.1900/RDS.2006.3.118

COMPREHENSIVE REFERENCES

Ainslie DA, Proietto J, Fam BC, Thorburn AW. (2000) Short-term, high-fat diets lower circulating leptin concentrations in rats. *American Journal of Clinical Nutrition* **7**:438–442 DOI 10.1093/ajcn/71.2.438.

Angelakis E, Raoult D. (2018) Gut microbiota modifications and weight gain in early life. *Human Microbiome Journal* 7-8:10-14. DOI 10.1016/j.humic.2018.01.002.

Auberval N, Dal S, Bietiger W, Pinget M, Jeandidier N, Maillard-Pedracini E. (2014) Metabolic and oxidative stress markers in Wistar rats after 2 months on a high-fat diet. *Diabetology and Metabolic Syndrome* **6**:130 DOI 10.1186/1758-5996-6-130.

Bischoff SC, Barbara G, Buurman W, Ockhuizen T, Schulzke JD, Serino M, Wells JM. (2014) Intestinal permeability – a new target for disease prevention and therapy. *BMC Gastroenterology* **14**:189 DOI 10.1186/s12876-014-0189-7.

Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH. (2004) Prevalence of hepatic steatosis in an urban population in the United States: Impact of ethnicity. *Hepatology* **40**:1387–1395 DOI 10.1002/hep.20466.

Cani PD, Bibiloni R, Knauf C, Neyrinck AM, Delzenne NM. (2008) Changes in gut microbiota control metabolic diet–induced obesity and diabetes in mice. *Diabetes* **57**: 1470–1481 DOI 10.2337/db07-1403.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Knight R. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*. **7**:335–336 DOI 10.1038/nmeth.f.303.

Clarke SF, Murphy EF, Nilaweera K, Ross PR, Shanahan F, O'Toole PW, Cotter PD. (2012) The gut microbiota and its relationship to diet and obesity: new insights. *Gut Microbes* **3**:186–202 DOI 10.4161/gmic.20168.

Cox AJ, West NP, Cripps AW. 2015. Obesity, inflammation, and the gut microbiota. *The Lancet Diabetes and Endocrinology* **3**:207–215 DOI 10.1016/S2213-8587(14)70134-2.

Hakkak R, Korourian S, Foley SL, Erickson BD. 2017. Assessment of gut microbiota populations in lean and obese Zucker rats. *PLoS ONE* **12**: e0181451 DOI 10.1371/journal.pone.0181451.

Hansen TH, Gøbel RJ, Hansen T, Pedersen O. 2015. The gut microbiome in cardio-metabolic health. *Genome Medicine* **7**:33 DOI 10.1186/s13073-015-0157-z.

Harley IT, Karp CL. 2012. Obesity and the gut microbiome: Striving for causality. *Molecular Metabolism* **1**:21–31 DOI 10.1016/j.molmet.2012.07.002.

Hawkesworth S, Moore SE, Fulford AJC, Barclay GR, Darboe AA, Mark H, Prentice AM. 2013. Evidence for metabolic endotoxemia in obese and diabetic Gambian women. *Nutrition and Diabetes* **3**:e83 DOI 10.1038/nutd.2013.24.

Huang W, Dedousis N, O'Doherty RM. 2007. Hepatic steatosis and plasma dyslipidemia induced by a high-sucrose diet are corrected by an acute leptin infusion. *Journal of Applied Physiology* **102**:2260–2265 DOI 10.1152/japplphysiol.01449.2006.

Jackman MR, MacLean PS, Bessesen DH. (2010) Energy expenditure in obesity-prone and obesity-resistant rats before and after the introduction of a high-fat diet. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **299**: R1097–R1105 DOI 10.1152/ajpregu.00549.2009.

Jiang W, Wu N, Wang X, Chi Y, Zhang Y, Qiu X, Hu Y, Li J, Liu Y. (2015) Dysbiosis gut microbiota associated with inflammation and impaired mucosal immune function in intestine of humans with non-alcoholic fatty liver disease. *Scientific Reports* **5**:1–7 DOI 10.1038/srep08096.

Jouihan H. (2012) Measurement of liver triglyceride content. *Bio-protocol* **2**: e223 DOI 10.21769/BioProtoc.223.

Kaakoush NO. (2015) Insights into the role of *Erysipelotrichaceae* in the human host. *Frontiers in Cellular and Infection Microbiology* **5**:84 DOI 10.3389/fcimb.2015.00084.

Kim KA, Gu W, Lee IA, Joh EH, Kim DH, Faulds M, Anderson N. 2012. High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. *PLoS ONE* **7**:e47713 DOI 10.1371/journal.pone.0047713.

Lecomte V, Kaakoush NO, Maloney CA, Raipuria M, Huinao KD, Mitchell HM, Morris MJ. (2015) Changes in gut microbiota in rats fed a high fat diet correlate with obesity-associated metabolic parameters. *PLoS ONE* **10**:1–22 DOI 10.1371/journal.pone.0126931.

Li D, Chen H, Mao B, Yang Q, Zhao J, Gu Z, Zhang H, Chen W. (2017) Microbial biogeography and core microbiota of the rat digestive tract. *Scientific Reports* **8**: 45840 DOI 10.1038/srep45840.

Liu X, Henkel AS, LeCuyer BE, Schipma MJ, Anderson KA, Green RM. (2015) Hepatocyte X-box binding protein 1 deficiency increases liver injury in mice fed a

high-fat/sugar diet. *American Journal of Physiology – Gastrointestinal and Liver Physiology* **309**: G965–974 DOI 10.1152/ajpgi.00132.2015.

Mokhtari Z, Gibson DL, Hekmatdoost A. (2017) Nonalcoholic fatty liver disease, the gut microbiome, and diet. *Advances in Nutrition* **8**:240–252 DOI 10.3945/an.116.013151.

Moore XJ, Chaudhary N, Akinyemiju T. (2017) Metabolic syndrome prevalence by race/ethnicity and sex in the United States, National Health and Nutrition Examination Survey, 1988-2012. *Preventing Chronic Disease* **14**:1–16 DOI 10.5888/pcd14.160287.

Nagao-Kitamoto H, Kamada N. (2017) Host-microbial cross-talk in inflammatory bowel disease. *Immune Network* **17**:1–12 DOI 10.4110/in.2017.17.1.1.

Nagy-Szakal D, Hollister EB, Luna RA, Szigeti R, Tatevian N, Smith CW, Versalovic J, Kellermayer R. (2013) Cellulose supplementation early in life ameliorates colitis in adult mice. *PLoS ONE* **8**: e56685 DOI 10.1371/journal.pone.0056685.

Netto Candido TL, Bressan J, Alfenas RCG. (2018) Dysbiosis and metabolic endotoxemia induced by high-fat diet. *Nutricion Hospitalaria* **35**: 1432-1440. DOI 10.20960/nh.1792.

Oliveira RB, Canuto LP, Collares-Buzato CB. [Epub ahead of print] Intestinal lumina; content from high-fat-fed prediabetic mice changes epithelial barrier function in vitro. *Life Science*. DOI 10.1016/j.lfs.2018.11.012.

Rajilić-Stojanović M, Biagi E, Heilig HGHJ, Kajander K, Kekkonen RA, Tims S, De Vos WM. (2011) Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. *Gastroenterology* **141**:1792–1801 DOI 10.1053/j.gastro.2011.07.043.

Roth CL, Elfers CT, Figlewicz DP, Melhorn SJ, Morton GJ, Hoofnagle A, Yeh MM, Nelson JE, Kowdley KV. (2012) Vitamin D deficiency in obese rats exacerbates nonalcoholic fatty liver disease and increases hepatic resistin and Toll-like receptor activation. *Hepatology* **55**:1103–1111 DOI 10.1002/hep.24737.

Schmidt SL, Harmon KA, Sharp TA, Kealey EH, Bessesen DH. (2012) The effects of overfeeding on spontaneous physical activity in obesity prone and obesity resistant humans. *Obesity* **20**:2186–2193 DOI 10.1038/oby.2012.103.

Schneider CA, Rasband WS, Eliceiri KW. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9**:671–675 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5554542/.

Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. (2011) Metagenomic biomarker discovery and explanation. *Genome Biology* **12**: R60. DOI 10.1186/gb-2011-12-6-r60.

Tan Y, Lao W, Xiao L, Wang Z, Xiao W, Kamal M, Paul Seale J, Qu X. (2013) Managing the combination of nonalcoholic fatty liver disease and metabolic syndrome with Chinese herbal extracts in high-fat-diet fed rats. *Evidence-Based Complementary and Alternative Medicine* **2013**:306738 DOI 10.1155/2013/306738.

Vaughn AC, Cooper EM, Dilorenzo PM, O'Loughlin LJ, Konkel ME, Peters JH, Hajnal A, Sen T, Lee SH, de la Serre CB, Czaja K. (2017) Energy-dense diet triggers changes in gut microbiota, reorganization of gut-brain vagal communication and increases body fat accumulation. *Acta Neurobiologiae Experimentalis* **77**:18–30 https://www.ncbi.nlm.nih.gov/pubmed/28379213.

Yin J, Li Y, Han H, Chen S, Gao J, Liu G, Wu X, Deng J, Yu O, Huang X, Fang R, Li T, Reiter RJ, Zhang D, Zhu C, Zhu G, Ren W, Yin Y. (2018) Melatonin reprogramming of gut microbiota improves lipid dysmetabolism in high-fat-diet-fed mice. *Journal of Pineal Research*. **65**:e12524.

Younossi Z, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, George J, Bugianesi E. (2018) Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol.* 15:11-20 DOI 10.1038/nrgastro.2017.109

Zhu L, Baker RD, Baker SS. (2015) Gut microbiome and nonalcoholic fatty liver diseases. *Pediatric Research* **77**:245–251 DOI 10.1038/pr.2014.157.

Beltran-Sanchez H, Harhay MO, Harhay, MM, McElligott, S. Prevalence and trends of Metabolic Syndrome in the adult US population, 1999–2010. J Am Coll Cardiol (2013); 62(8): 697-703. doi:10.1016/j.jacc.2013.05.064.

Rochlani, Y, Pothineni, NV, Kovelamudi, S, Mehta, JL. Metabolic syndrome: Pathophysiology, management, and modulation by natural compounds. Ther. Adv. Cardiovasc. Dis (2017); 11(8):215–225. doi: 10.1177/1753944717711379.

Bastard JP, Jardel C, Bruckert E, Blondy P, Capeau J, Laville M, et al. Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. J Clin Endocrinol Metab (2000); 85(9): 3338-42. doi:10.1210/jcem.85.9.6839.

Tsigos C, Kyrou I, Chala E, Tsapogas P, Stavridis JC, Raptis SA, et al. Circulating tumor necrosis factor alpha concentrations are higher in abdominal versus peripheral

obesity. Metabolism (1999); 48(10):1332-1335. doi: 10.1016/S0026-0495(99(90277-9.

Almeda-Valdes P, Herrera-Mercadillo RJ, Aguilar-Salinas CA, Uribe M, Méndez-Sánchez N. The Role of Diet in Patients with Metabolic Syndrome. Curr Med Chem (2017). doi: 10.2174/0929867324666170518095316.

Ostman C, Smart NA, Morcos D, Duller A, Ridley W, Jewiss D. The effect of exercise training on clinical outcomes in patients with the metabolic syndrome: a systematic review and meta-analysis. Cardiovasc Diabetol (2017); 16(1):110, 2017. doi: 10.1186/s12933-017-0590-y.

Azadbakht L, Mirmiran P, Esmaillzadeh A, Azizi T, Azizi F. Beneficial effects of a Dietary Approaches to Stop Hypertension eating plan on features of the metabolic syndrome. Diabetes Care (2005); 28(12): 2823-31. https://doi.org/10.2337/diacare.28.12.2833.

Gusdon AM, Song K, Qu S. Nonalcoholic Fatty Liver Disease: Pathogenesis and Therapeutics from a Mitochondria-Centric Perspective. Oxidative Medicine and Cellular Longevity (2014); 1–20. https://doi.org/10.1155/2014/637027.

Moss JWE, Williams, JO, Ramji DP. Nutraceuticals as therapeutic agents for atherosclerosis. Biochim Biophys Acta (2018); 1864:1562-72. doi: 10.1016/j.bbadis.2018.02.006.

Ríos-Hoyo A, Cortés MJ, Ríos-Ontiveros H, Meaney E, Ceballos G, Gutiérrez-Salmeán G. Obesity, Metabolic Syndrome, and Dietary Therapeutical Approaches with a Special Focus on Nutraceuticals (Polyphenols): A Mini-Review. Int J Vitam Nutr Res (2014); 84(3-4):113-23. doi: 10.1024/0300-9831/a000198.

Deneau J, Ahmed T, Blotsky R, Bojanowski K. Anti-diabetic activity of a mineraloid isolate, in vitro and in genetically diabetic mice. Int J Vitam Nutr Res (2011); 81(1): 34-42. doi: 10.1024/0300-9831/a000048.

Gerbaix M, Metz L, Ringot E, Courteix D. Visceral fat mass determination in rodent: Validation of dual-energy x-ray absorptiometry and anthropometric techniques in fat and lean rats. Lipids Health Dis (2010); 9:140. doi:10.1186/1476-511X-9-140.

Bernardis LL. Prediction of carcass fat, water and lean body mass from Lee's "nutritive ratio" in rats with hypothalamic obesity. Experientia (1970); 26(7): 789-790. doi: 10.1007/BF02232553.

Novelli EL, Diniz YS, Galhardi CM, Ebaid GM, Rodrigues HG, Mani F, et al. Anthropometrical parameters and markers of obesity in rats. Lab Anim (2007); 41(1): 111-119. doi: 10.1258/002367707779399518.

Cacho J, Sevillano J, de Castro J, Herrera E, Ramos MP. Validation of simple indexes to assess insulin sensitivity during pregnancy in Wistar and Sprague-Dawley rats. Am J Physiol Endocrinol Metab (2008). 295(5):E1269-E1276.

Antunes LC, Elkfury JL, Jordana MN, Foletto KC, Bertoluci MC. Validation of HOMA-IR in a model of insulin resistance induced by a high-fat diet in Wistar rats. Arch Endocrinol Metab (2016) 60(2): 138-142.

Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman, JI, Donato, KA, et al. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation (2009); 120(16): 1640-5. doi:10.1161/CIRCULATIONAHA.109.192644.

Panchal SK, Poudyal H, Iyer A, Nazer R, Alam MA, Diwan V, et al. High-carbohydrate, high-fat diet-induced metabolic syndrome and cardiovascular remodeling in rats. Journal of Cardiovascular Pharmacology (2011); 57(1): 611–624. https://doi.org/10.1097/FJC.0b013e3181feb90a

Robberecht, H, De Bruyne T, Hermans N. Biomarkers of the metabolic syndrome: Influence of minerals, oligo- and trace elements. J Trace Elem Med Biol (2017); 43:23-28. doi: 10.1016/j.jtemb.2016.10.005.

Chang Q, Lu Z, He M, Gao, R, Bai, H, Shi, B, et al. Effects of dietary supplementation of fulvic acid on lipid metabolism of finishing pigs. J Anim Sci (2014); 92(11): 4921-6. doi:10.2527/jas/2014-8137.

Hsu H-Y, Wen M-H. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. J Biol Chem (2002); 277:22131–9. https://doi.org/10.1074/jbc.M111883200.

McMurray F, Patten DA, Harper ME. Reactive Oxygen Species and Oxidative Stress in Obesity—Recent Findings and Empirical Approaches. Obesity (2016); 24(11): 2301-2310. doi: 10.1002/oby.21654

Taniyama Y, Griendling KK. Reactive oxygen species in the vasculature: Molecular and cellular mechanisms. Hypertens (2003); 42: 1075-1081. doi: 10.1161/01.HYP.0000100443.09293.4F.

Van Guilder GP, Hoetzer GL, Dengel DR, Stauffer BL, DeSouza CA. Impaired endothelium-dependent vasodilation in normotensive and normoglycemic obese adult humans. J Cardiovasc Pharmacol (2006); 47(2): 310–313. https://doi.org/10.1097/01.fjc.0000205097.29946.d3.

Lopez-Moreno J, Garcia-Carpintero S, Jimenez-Lucena R, Haro C, Rangel-Zuniga OA, Blanco-Rojo R, et al. Effect of dietary lipids on endotoxemia influences postprandial inflammatory response. Journal of Agriculture and Food Chemistry (2017); 65(35):7756–7763. doi: 10.1021/acs.jafc.7b01909.

Wang, X., & Quinn, P. Endotoxins: Lipopolysaccharides of Gram-Negative Bacteria. Sub-cellular biochemistry (2010); 53:3-25. https://doi.org/10.1007/978-90-481-9078-2_1.

Chien SJ, Chen TC, Kuo HC, Chen, CN, Chang, SF. Fulvic acid attenuates homocysteine-induced cyclooxygenase-2 expression in human monocytes. BMC Complement Altern Med (2015); 15: 61. doi: 10.1186/s12905-015-0583-x.

Van Rensburg CE, van Straten A, Dekker J. An *in vitro* investigation of the antimicrobial activity of oxifulvic acid. J Antiomicrob Chemother (2000); 46(5):853. https://doi.org/10/1093/jac/46.5.853.

Ghosal S, Singh SK, Kumar Y, Srivastava R, Goel RK, Dey R, Bhattacharya SK. Anti-ulcerogenic activity of fulvic acids and 4'-methoxy-6-carbomethoxybiphenyl isolated from Shilajit. Phytother Res (1988); 2(4): 187-191. doi:10.1002/ptr.2650020408.

Aslam MN, Paruchuri T, Bhagavathula N, Varani J. A mineral-rich red algae extract inhibits polyp formation and inflammation in the gastrointestinal tract of mice on a high-fat diet. Integr Cancer Ther (2010); 9(1): 93-9. doi:10.1177/1534735409360360.

Aslam MN, Bergin I, Naik M, Paruchuri T, Hampton A, Rehman M, et al. A multi-mineral natural product inhibits liver tumor formation in C57BL/6 mice. Biol Trace Elem Res (2012);64(7):1020-8. doi:10.1080/01635581.2012.713160.

O'Gorman DM, O'Carroll C, Carmody RJ. Evidence that marine-derived, multi-mineral, Aquamin inhibits the NF-κB signaling pathway in vitro. Phytother Res (2012); 26(4):630-2. doi: 10.1002/ptr.3601.

Crinigan C, Calhoun M, Sweazea KL. Short-term high fat intake does not significantly alter markers of renal function or inflammation in young male Sprague-Dawley rats. J Nutr Metab (2015). 157520. doi: 10.1155/2015/157520.

Rodriguez NC, Urrutia EC, Gertrudis BH, Chaverri JP, Mejia GB. Antioxidant activity of fulvic acid: A living matter-derived bioactive compound. Journal of Food, Agriculture & Environment (2011); 9(3-4), 123-127. https://doi.org/10.1155/2018/5391014.

Li S, Tan HY, Wang N, Zhang, ZJ, Lao, L, Wong, CW, et al. The Role of Oxidative Stress and Antioxidants in Liver Diseases. International Journal of Molecular Sciences (2015); 16(11): 26087–26124. https://doi.org/10.3390/ijms161125942.

Kai M, Miyoshi M, Fujiwara M, Nishiyama Y, Inoue T, Maeshige N, et al. A lard-rich high-fat diet increases hepatic peroxisome proliferator-activated receptors in endotoxemic rats. J Surg Res (2017); 212:22-32. doi: 10.1016/j.jss.2016.11.048.

Han CY. Roles of Reactive Oxygen Species on Insulin Resistance in Adipose Tissue. Diabetes Metab J (2016); 40(4): 272–279. https://doi.org/10.4093/dmj.2016.40.4.272.

Kanety H, Feinstein R, Papa MZ, Hemi R, Karasik A. Tumor necrosis Factor alpha-induced phosphorylation of insulin receptor substrate-1 (IRS-1). Possible mechanism for suppression of insulin-stimulated tyrosine phosphorylation of IRS-1. J Biol Chem (1995); 270(40): 23780-23784. doi: 10.1074/jbc.270.40.23780.

Lamar RT, OLK DC, Mayhew L, Bloom PR. A new standardized method for quantification of humic and fulvic acids in humic ores and commercial product. Journal of AOAC International (2014); 97(3): 721–730. doi: https://doi.org/10.5740/jaoacint.13-393.

Liang D, Leung RK, Guan W, Au WW. Involvement of gut microbiome in human health and disease: brief overview, knowledge gaps and research opportunities. *Gut Pathog*. 2018; 10:3. Published 2018 Jan 25. doi:10.1186/s13099-018-0230-4

Parséus A, Sommer N, Sommer F, et al. Microbiota-induced obesity requires farnesoid X receptor. *Gut*. 2016;66(3):429-437.

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V, Koh, G. Y., Nagy, A., … Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(44), 15718 LP-15723. https://doi.org/10.1073/pnas.0407076101

Nagpal, R., Newman, T. M., Wang, S., Jain, S., Lovato, J. F., & Yadav, H. (2018). Obesity-Linked Gut Microbiome Dysbiosis Associated with Derangements in Gut Permeability and Intestinal Cellular Homeostasis Independent of Diet, *2018*.

He, C., Cheng, D., Peng, C., Li, Y., Zhu, Y., & Lu, N. (2018). High-Fat Diet Induces Dysbiosis of Gastric Microbiota Prior to Gut Microbiota in Association With Metabolic Disorders in Mice. *Frontiers in microbiology*, *9*, 639. doi:10.3389/fmicb.2018.00639

Sonnenburg, J. L., & Academy, S. (2018). HHS Public Access, *535*(7610), 56–64. https://doi.org/10.1038/nature18846.Diet

Conlon, M. A., & Bird, A. R. (2015). The impact of diet and lifestyle on gut microbiota and human health. *Nutrients*, *7*(1), 17–44. https://doi.org/10.3390/nu7010017

Jung, C., & Hugot, J. (2010). Peyer ' s Patches : The Immune Sensors of the Intestine, *2010*. https://doi.org/10.4061/2010/823710

Boutagy, N. E., McMillan, R. P., Frisard, M. I., & Hulver, M. W. (2015). Metabolic endotoxemia with obesity: Is it real and is it relevant?. *Biochimie*, *124*, 11-20.

Martinez, K. B., Leone, V., & Chang, E. B. (2017). Western diets, gut dysbiosis, and metabolic diseases: Are they linked? *Gut Microbes*, *8*(2), 130–142. https://doi.org/10.1080/19490976.2016.1270811

Crawford, M., Whisner, C., Al-Nakkash, L., Sweazea KL. (2019). Six-week high fat diet alter the gut microbiome and promotes cecal inflammation, endoxtin production and simple steatosis without obesity in male rats. *Lipids.*

Gill, S. R., Pop, M., Deboy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., Gordon, J. I., Relman, D. A., Fraser-Liggett, C. M., Nelson, K. E. (2006). Metagenomic analysis of the human distal gut microbiome. *Science (New York, N.Y.)*, *312*(5778), 1355-9.

Dethlefsen, L., McFall-Ngai, M., & Relman, D. A. (2007). An ecological and evolutionary perspective on human–microbe mutualism and disease. *Nature*, *449*, 811. Retrieved from https://doi.org/10.1038/nature06245

Sanz, Y., Santacruz, A., & Gauffin, P. (2019). 3rd International Immunonutrition Workshop Session 8 : Probiotics in the defence and metabolic balance of the organism Gut microbiota in obesity and metabolic disorders Proceedings of the Nutrition Society Proceedings of the Nutrition Society, (October 2009), 434–441. https://doi.org/10.1017/S0029665110001813

Sharma, S., & Tripathi, P. (2019). ScienceDirect. *The Journal of Nutritional Biochemistry*, *63*, 101–108. https://doi.org/10.1016/j.jnutbio.2018.10.003

Franco-Obregón A, Gilbert JA. The Microbiome-Mitochondrion Connection: Common Ancestries, Common Mechanisms, Common Goals. *mSystems*. 2017;2(3):e00018-17. Published 2017 May 9. doi:10.1128/mSystems.00018-17

Vaughn AC, Cooper EM, DiLorenzo PM, et al. Energy-dense diet triggers changes in gut microbiota, reorganization of gut-brain vagal communication and increases body fat accumulation. *Acta Neurobiol Exp (Wars)*. 2017;77(1):18-30.

Flemer B, Gaci N, Borrel G, et al. Fecal microbiota variation across the lifespan of the healthy laboratory rat. *Gut Microbes*. 2017;8(5):428-439

Hu, Q., Ren, H., Ren, J., Liu, Q., Wu, J., Wu, X., … Li, J. (2018). Released Mitochondrial DNA Following Intestinal Ischemia Reperfusion Induces the Inflammatory Response and Gut Barrier Dysfunction. *Scientific Reports*, *8*(1), 7350. https://doi.org/10.1038/s41598-018-25387-8

Chunchai T, Thunapong W, Yasom S, et al. Decreased microglial activation through gut-brain axis by prebiotics, probiotics, or synbiotics effectively restored cognitive function in obese-insulin resistant rats. *J Neuroinflammation*. 2018;15(1):11. Published 2018 Jan 9. doi:10.1186/s12974-018-1055-2

Kang YJ, Diao XN, Zhao GY, et al. Extensive diversity of Rickettsiales bacteria in two species of ticks from China and the evolution of the Rickettsiales. *BMC Evol Biol*. 2014;14:167. Published 2014 Jul 30. doi:10.1186/s12862-014-0167-2

Ley, R. E., Turnbaugh, P. J., Klein, S., & Gordon, J. I. (2006). Human gut microbes associated with obesity. *Nature*, *444*, 1022. Retrieved from https://doi.org/10.1038/4441022a

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., & Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*, *444*, 1027. Retrieved from https://doi.org/10.1038/nature0541

Watanabe, M., Yokotsuka, M., Yamaoka, K., Adachi, M., Nemoto, A., & Tango, T. (2017). Effects of a lifestyle modification programme to reduce the number of risk factors for metabolic syndrome: a randomised controlled trial. *Public Health Nutrition*, *20*(1), 142–153. https://doi.org/10.1017/S1368980016001920

Camilleri, M., Malhi, H., & Acosta, A. (2017). Gastrointestinal Complications of Obesity. *Gastroenterology*, *152*(7), 1656–1670. https://doi.org/10.1053/j.gastro.2016.12.052

National Institutes of Health, U.S. Department of Health and Human Services. *Opportunities and Challenges in Digestive Diseases Research: Recommendations of the National Commission on Digestive Diseases.* Bethesda, MD: National Institutes of Health; 2009. NIH Publication 08–6514.

Jensen, V. S., Hvid, H., Damgaard, J., Nygaard, H., Ingvorsen, C., Wulff, E. M., Lykkesfeldt, J., … Fledelius, C. (2018). Dietary fat stimulates development of NAFLD more potently than dietary fructose in Sprague-Dawley rats. *Diabetology & metabolic syndrome*, *10*, 4. doi:10.1186/s13098-018-0307-8

Pappachan, J. M., Babu, S., Krishnan, B., & Ravindran, N. C. (2017). Non-alcoholic Fatty Liver Disease: A Clinical Update. *Journal of Clinical and Translational Hepatology*, *5*(4), 384–393. https://doi.org/10.14218/JCTH.2017.00013

Fu, X.-Y., Li, Z., Zhang, N., Yu, H.-T., Wang, S.-R., & Liu, J.-R. (2014). Effects of gastrointestinal motility on obesity. *Nutrition & Metabolism*, *11*(1), 3. https://doi.org/10.1186/1743-7075-11-3

Ng, S. C., Bernstein, C. N., Vatn, M. H., Lakatos, P. L., Loftus, E. V, … Tysk, C. (2013). Geographical variability and environmental risk factors in inflammatory bowel disease. *Gut*, *62*(4), 630 LP-649. https://doi.org/10.1136/gutjnl-2012-303661

Kobyliak, N., Conte, C., Cammarota, G., Haley, A. P., Styriak, I., Gaspar, L., … Kruzliak, P. (2016). Probiotics in prevention and treatment of obesity: a critical view. *Nutrition & Metabolism*, *13*(1), 14. https://doi.org/10.1186/s12986-016-0067-0

Thounaojam, M. C., Nammi, S., & Jadeja, R. (2016). Natural Products for the Treatment of Obesity, Metabolic Syndrome, and Type 2 Diabetes 2016. *Evidence-Based Complementary and Alternative Medicine : ECAM*, *2016*, 9072345. https://doi.org/10.1155/2016/9072345

Deneau, J. (2011). Anti-Diabetic Activity of a Mineraloid Isolate, in vitro and in Genetically Diabetic Mice. *International Journal for Vitamin and Nutrition Research*, *81*(1), 34–43.

Bashiardes, S., Shapiro, H., Rozin, S., Shibolet, O., & Elinav, E. (2016). Non-alcoholic fatty liver and the gut microbiota. *Molecular Metabolism*, *5*(9), 782–794. https://doi.org/https://doi.org/10.1016/j.molmet.2016.06.003

Guo, S., Nighot, M., Al-Sadi, R., Alhmoud, T., Nighot, P., & Ma, T. Y. (2015). Lipopolysaccharide Regulation of Intestinal Tight Junction Permeability Is Mediated by TLR4 Signal Transduction Pathway Activation of FAK and MyD88. *Journal of Immunology (Baltimore, Md. : 1950)*, *195*(10), 4999–5010. https://doi.org/10.4049/jimmunol.1402598

Crawford, M., Gumpricht, E., Sweazea, KL. (2019). A Novel Organometallic Complex Attenuates Risk Factors in a Rodent Model of Metabolic Syndrome. In Review.

Sakai, J., Cammarota, E., Wright, J. A., Cicuta, P., Gottschalk, R. A., Li, N., … Bryant, C. E. (2017). Lipopolysaccharide-induced NF-κB nuclear translocation is primarily dependent on MyD88, but TNFα expression requires TRIF and MyD88. *Scientific Reports*, *7*(1), 1428. https://doi.org/10.1038/s41598-017-01600-y

Liu, T., Zhang, L., Joo, D., & Sun, S.-C. (2017). NF-κB signaling in inflammation. *Signal Transduction And Targeted Therapy*, *2*, 17023. Retrieved from https://doi.org/10.1038/sigtrans.2017.23

Guma, M., Stepniak, D., Shaked, H., Spehlmann, M. E., Shenouda, S., Cheroutre, H., … Karin, M. (2011). Constitutive intestinal NF-κB does not trigger destructive inflammation unless accompanied by MAPK activation. *The Journal of Experimental Medicine*, *208*(9), 1889–1900. https://doi.org/10.1084/jem.20110242

Ding, S., Chi, M. M., Scull, B. P., Rigby, R., Schwerbrock, N. M. J., Magness, S., … Lund, P. K. (2010). High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse. *PloS One*, *5*(8), e12191–e12191. https://doi.org/10.1371/journal.pone.0012191

Hayden, M. S., & Ghosh, S. (2014). Regulation of NF-κB by TNF family cytokines. *Seminars in Immunology*, *26*(3), 253–266. https://doi.org/10.1016/j.smim.2014.05.004

De Plaen, I. G., Tan, X. D., Chang, H., Wang, L., Remick, D. G., & Hsueh, W. (2000). Lipopolysaccharide activates nuclear factor kappaB in rat intestine: role of endogenous platelet-activating factor and tumour necrosis factor. *British Journal of Pharmacology*, *129*(2), 307–314. https://doi.org/10.1038/sj.bjp.0703055

Nishimura, S., Manabe, I., & Nagai, R. (2009). Adipose tissue inflammation in obesity and metabolic syndrome. *Discovery Medicine*, *8*(41), 55-60.

Hersoug, L.-G., Møller, P., & Loft, S. (2018). Role of microbiota-derived lipopolysaccharide in adipose tissue inflammation, adipocyte size and pyroptosis during obesity. *Nutrition Research Reviews*, *31*(2), 153–163. https://doi.org/DOI: 10.1017/S0954422417000269

Yaligar, J., Gopalan, V., Kiat, O. W., Sugii, S., Shui, G., Lam, B. D., … Velan, S. S. (2014). Evaluation of dietary effects on hepatic lipids in high fat and placebo diet fed rats by in vivo MRS and LC-MS techniques. *PloS One*, *9*(3), e91436–e91436. https://doi.org/10.1371/journal.pone.0091436

Winkler, J., & Ghosh, S. (2018). Therapeutic Potential of Fulvic Acid in Chronic Inflammatory Diseases and Diabetes. *Journal of Diabetes Research*, *2018*, 1–7. https://doi.org/10.1155/2018/5391014

Junek, R., Morrow, R., Schoenherr, J. I., Schubert, R., Kallmeyer, R., Phull, S., & Klöcking, R. (2009). Bimodal effect of humic acids on the LPS-induced TNF-α release from differentiated U937 cells. *Phytomedicine*. https://doi.org/10.1016/j.phymed.2008.10.003

Petrovic, M., & Kastelan-Macan, M. (1996). The uptake of inorganic phosphorus by insoluble metal-humic complexes. *Water Science and Technology*, *34*(7–8), 253–258. https://doi.org/10.2166/wst.1996.0629

Mirza, M. A., Ahmad, N., Agarwal, S. P., Mahmood, D., Khalid Anwer, M., & Iqbal, Z. (2011). Comparative evaluation of humic substances in oral drug delivery. *Results in Pharma Sciences*, *1*(1), 16–26. https://doi.org/https://doi.org/10.1016/j.rinphs.2011.06.001

Visser, S. A. (1987). Effect of humic substances on mitochondrial respiration and oxidative phosphorylation. *Science of The Total Environment*, *62*, 347–354. https://doi.org/https://doi.org/10.1016/0048-9697(87)90521-3

Li, S., Tan, H.-Y., Wang, N., Zhang, Z.-J., Lao, L., Wong, C.-W., & Feng, Y. (2015). The Role of Oxidative Stress and Antioxidants in Liver Diseases. *International Journal of Molecular Sciences*, *16*(11), 26087–26124. https://doi.org/10.3390/ijms161125942

Casas-Grajales, S., & Muriel, P. (2015). Antioxidants in liver health. *World Journal of Gastrointestinal Pharmacology and Therapeutics*, *6*(3), 59–72. https://doi.org/10.4292/wjgpt.v6.i3.59

APPENDIX A

CHAPTER 3 STATISTICAL RESULTS IN DETAIL

10-week Body Mass: Two-way ANOVA

Source of Variation	DF	F	p
Diet	1	17.53	<0.001
Dose	2	0.545	0.584
Diet x Dose	2	0.157	0.855
Residual	36		
Total	41		

10-week Abdominal Circumference: Two-way ANOVA

Source of Variation	DF	F	p
Diet	1	19.07	<0.001
Dose	2	3.70	0.035
Diet x Dose	2	0.129	0.879
Residual	36		
Total	41		

Epidydimal Fat Pad Mass: Two-way ANOVA

Source of Variation	DF	F	p
Diet	1	51.43	<0.001
Dose	2	1.76	0.187
Diet x Dose	2	0.372	0.692
Residual	36		
Total	41		

Serum Glucose Two-way RM ANOVA table

Source of Variation	DF	F	p
Group	5	7.599	<0.001
Animal	36		
Time	1	4.178	0.048
Group x Time	5	1.424	0.239
Residual	36		
Total	83		

Serum Insulin Two-way RM ANOVA table

Source of Variation	DF	F	p
Group	5	5.460	<0.001
Animal	36		
Time	1	0.0952	0.759
Group x Time	5	0.638	0.672
Residual	36		
Total	83		

Urine H_2O_2 Two-way ANOVA

Source of Variation	DF	F	p
Diet	1	8.053	0.009
Dose	2	1.304	0.289
Diet x Dose	2	0.211	0.811
Residual	25		
Total	30		

Acetylcholine Dilation: Two-way RM ANOVA

Source of Variation	DF	F	p
Diet	1	7.058	0.006
OMC	1	107.8	<0.001
Diet x OMC	1	3.008	<0.001

Acetylcholine Dilation: Chow Curves, Two-way RM ANOVA

Source of Variation	DF	F	p
Diet	1	1.546	0.240
OMC	1	152.8	<0.001
Diet x OMC	1	1.242	0.234

Acetylcholine Dilation, Chow control vs HFD control: Two-way RM ANOVA

Source of Variation	DF	F	p
Diet	1	0.582	0.030
Dose	1	115.6	<0.001
Diet x Dose	1	1.657	0.106

Plasma SOD Activity at 10 weeks Two-way ANOVA

Source of Variation	DF	F	p
Diet	1	4.346	0.044
Dose	2	1.026	0.369
Diet x Dose	2	0.681	0.513
Residual	35		
Total	40		

Plasma ALT Activity at 10 weeks Two-way ANOVA

Source of Variation	DF	F	p
Diet	1	45.133	<0.001
Dose	2	3.437	0.043
Diet x Dose	2	3.396	0.045
Residual	35		
Total	40		

Plasma AST Activity at 10 weeks Two-way ANOVA

Source of Variation	DF	F	p
Diet	1	3.569	0.067
Dose	2	3.691	0.035
Diet x Dose	2	0.238	0.790
Residual	35		
Total	40		

Plasma Endotoxin Concentrations at 10 weeks Two-way ANOVA

Source of Variation	DF	F	P
Diet	1	23.893	<0.001
Dose	2	0.237	0.790
Diet x Dose	2	0.0890	0.915
Residual	34		
Total	39		

APPENDIX B

CHAPTER 5 STATISTICAL RESULTS IN DETAIL

Cecum NF-kB Two-way ANOVA

Source of Variation	DF	F	p
Diet	2	1.050	0.362
Dose	1	0.723	0.402
Diet x Dose	2	0.157	0.855
Residual	30		
Total	35		

Cecum IL-1B Two-way ANOVA

Source of Variation	DF	F	p
Diet	2	0.358	0.702
Dose	1	3.744	0.062
Diet x Dose	2	0.246	0.783
Residual	31		
Total	36		

Small Intestine NF-kB Two-way ANOVA

Source of Variation	DF	F	p
Diet	2	0.296	0.746
Dose	1	1.096	0.304
Diet x Dose	2	0.545	0.586
Residual	29		
Total	34		

Small Intestine IL-1B Two-way ANOVA

Source of Variation	DF	F	p
Diet	2	0.565	0.574
Dose	1	0.277	0.603
Diet x Dose	2	0.0713	0.931
Residual	29		
Total	34		

Liver Triglyceride/Glycerol Concentrations at 10 weeks

Source of Variation	**DF**	**F**	**p**
Diet	1	134.695	<0.001
Dose	2	0.0955	0.909
Diet x Dose	2	0.689	0.510
Residual	32		
Total	37		