Do Probiotics Protect Against the Deleterious Effects of a High-Fat Diet?

Dissertation

Submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy
In

Human Nutrition, Foods and Exercise

by

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May 15, 2014
Blacksburg, VA

Keywords: Probiotics, high-fat diet, metabolic endotoxemia, skeletal muscle

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ABSTRACT

High-fat diets and obesity have been linked to unfavorable changes in gut bacteria and increased leakage of bacterially-derived lipopolysaccharide (endotoxin) from the intestinal tract into circulation, which is associated with low-grade inflammation, metabolic dysregulation and degradation of tight-junction proteins between intestinal cells. Probiotic supplementation is the practice of ingesting live strains of bacteria that are proposed to have a beneficial effect on the host by enriching the intestine with healthy bacteria. The purpose of this project was to determine if probiotic supplementation would prevent increased inflammatory tone, decreased oxidative capacity, and decreased tight-junction protein expression associated with high-fat feeding and elevated endogenous endotoxin. Male C57BL/6J mice were fed either a control (CD, 10% fat) or high-fat (HFD, 60% fat) diet for 4 weeks while receiving a daily oral gavage of water (C-VSL#3, HF-VSL#3) or probiotics (C+VSL#3, HF+VSL#3) equivalent to 1.2 billion live cultures. Changes in body weight, body composition, respiratory exchange ratio, energy expenditure, and glucose and insulin tolerance were measured in live mice. Markers of metabolic function were measured in whole muscle homgenates and mitochondria isolated from red and white skeletal muscle. Plasma endotoxin was measured in blood collected from fasted mice at the time of euthanization. The large and small intestines were collected and mRNA levels of tight-junction proteins and markers of nutrient sensing were measured. To determine a possible protective effect against endogenous LPS, a second cohort of mice were given an intraperitoneal injection of 0.1μg/kg LPS or

saline to induce endotoxemia after four weeks of the aforementioned feeding protocol. Markers of metabolic function and inflammation were measured in mitochondria, skeletal muscle and liver. VSL#3 supplementation improved glucose homeostasis and markers of inflammation while enhancing nutrient sensing in the gut.

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DEDICATION

I dedicate this dissertation to my grandfather, Richard Hagerty, who taught me the value of lifelong learning and discovery. Instead of simply telling me the answer to one of my many questions, he would guide me through the process of learning the answer myself. He embodied the spirit of research and imbued in me an appreciation for the search of new knowledge. That passion has motivated me throughout my doctoral career and will motivate my future students in the same way.

ACKNOWLEDGEMENTS

I would like to thank Dr. Matthew Hulver for his ceaseless confidence in my abilities to perform research and teach. He has always supported my teaching efforts, and without his thoughtful nomination I would not be one of the inaugural members of the Graduate Teaching Scholars. At times, Dr. Hulver had more confidence in my abilities than I did, and his calm encouragement was a great source of motivation. I would also like to thank Dr. Donna Westfall-Rudd who has advised me as a Graduate Teaching Scholar for the latter half of my doctorate. Dr. Westfall-Rudd has been a well of advice and guidance, and from her I have learned what it really takes to be a dynamic teacher. To the rest of my committee members—Dr. Madlyn Frisard, Dr. Kevin Davy, Dr. Eva Schmelz, and Dr. Andrew Neilson—I give my gratitude for your thoughtful advice and insight.

I would like to thank my parents, Micheal and Silke Fundaro, for all their love, support and advice on this journey. I am incredibly thankful to my wonderful fiance', Jeffery Willis, who has been so understanding, patient, and loving, especially when stress has gotten the best of me. Additionally, I wish to thank my amazing and hilarious friends and colleagues, whose humor and commiseration has helped me take myself less seriously. Finally, I thank my grandmothers, Penny Hagerty and Evelyn Fundaro, for their love and support.

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LIST OF ABBREVIATIONS

Lipopolysaccharide (LPS)

Toll-Like Receptor 4 (TLR-4)

Fatty Acid Oxidation (FAO)

Fatty Acid Synthase (FAS)

Carbohydrate Responsive Element Binding Protein (ChREBP)

Sterol Responsive Element Binding Protein (SREBP-1)

5' Adenosine Monophosphate-Activated Protein Kinase (AMPK)

Short-Chain Fatty Acids (SCFA)

G-Protein Coupled Receptors (GPCR)

Acetyl CoA Carboxylase (ACC)

Carnitine:Palmitoyl Transferase-1 (CPT1)

Fasting-Induced Adipose Factor (FIAF)

Lipoprotein Lipase (LPL)

Cannabinoid Receptor 1 and 2 (CB1 and CB2)

Mitogen-Activated Protein Kinase (MAPK)

Extracellular Signal-Related Kinase (ERK)

Nuclear Factor kappa B (NFκB)

Fatty Acid Amide Hydrolase (FAAH)

Monoacylglycerol Hydrase (MGL)

Tumor Necrosis Factor α (TNFα)

Monocyte Chemoattractant Protein-1 (MCP-1)

Crohn's Disease (CD)

Ulcerative Colitis (UC)

Irritable Bowel Disease (IBD)

Colony Forming Units (CFU)

Zonula Occludins 1, 2 and 3 (ZO-1, ZO-2, ZO-3)

Glucagon-Like Peptide 2 (GLP-2)

Interleukin 10 (IL-10)

Monoacylglycerols (MAG)

Diacylglycerols (DAG)

Very Low Density Lipoproteins (VLDL)

Low Density Lipoproteins (LDL)

CHAPTER I: INTRODUCTION

Rates of both child and adult obesity in the United States have increased drastically in the past 30 years [1]. Obesity is closely associated with metabolic dysregulation including dyslipidemia, insulin resistance, and type II diabetes [2]. High-fat diets, obesity, and type II diabetes have been linked to unfavorable changes in gut bacteria and increased leakage of bacterially-derived lipopolysaccharide (endotoxin) from the intestinal tract into circulation[3]. Lipopolysaccharide, or LPS, may migrate from the intestine into circulation via compromised tight junction proteins, resulting in a condition known as 'metabolic endotoxemia'[4]. When LPS binds its ligand, Toll-like receptor 4 (TLR-4), an inflammatory cascade is initiated and inflammatory cytokines are produced [5]. Persistent elevation of plasma endotoxin can induce chronic low-grade inflammation which has been implicated in the development of metabolic dysregulation and gut permeability [6].

Recent research has shown that the profiles of intestinal bacteria differ between lean and obese individuals, and obese individuals are likely to present with 'dysbiosis' or an over-abundance of non-beneficial gut bacteria [7]. Certain bacterial profiles favor increased energy harvesting from the host diet, increased intestinal permeability, and higher levels of gram-negative bacteria which contain LPS. In contrast, some strains of bacteria are commonly found in lean hosts and are associated with greater insulin sensitivity and integrity of intestinal tight junctions as compared to obese hosts [8]. Additionally, new links have been discovered between LPS and the endocannabinoid system which can regulate feeding behaviors, lipid uptake, and adipose cell differentiation [9-11].

Probiotic supplementation is the practice of ingesting live, non-pathogenic bacteria that can enrich the bacterial colony of the host and promote gut health [12]. Beneficial bacteria provided

through probiotic supplementation have been shown to decrease gut permeability, decrease inflammatory markers, improve diet-induced insulin resistance, and increase the health of individual enterocytes [13]. However, most of these findings have occurred in murine models, and human studies have not consistently replicated these results [14, 15]. Probiotic supplementation has been utilized primarily as a therapy for inflammatory bowel diseases, including colitis and irritable bowel syndrome, to correct the overgrowth of harmful bacteria and improve mucous production to protect the intestinal lining [16].

Although there are clear links between the profile of gut bacteria and the phenotype of the host, few studies have examined the effect that enrichment with probiotics may have on preventing or treating obesity [3]. In most cases, studies illustrate correlations between host phenotype and pre-existing colonies, and the role of bacteria in actually inducing obesity or endotoxemia remains to be seen. While several studies have examined the protective effects of single-strain probiotics against diet-induced obesity and metabolic regulation, no other study has utilized a multi-strain probiotic as an intervention to determine effects on skeletal muscle metabolism. Therefore, it is the purpose of this study to determine the role of multi-strain probiotic supplementation as a non-invasive dietary method for protection against high-fat diet-induced metabolic dysregulation.

CHAPTER II: REVIEW OF LITERATURE

2. Introduction

In the past 30 years, obesity rates have doubled in adults and tripled in children. Today, a staggering 17% of children and over 30% of adults are classified as obese [1]. It is well documented that obesity is accompanied by insulin resistance, dyslipidemia, and type II diabetes, the latter of which affects 220 million people globally [2]. Obesity is now considered a low-grade inflammatory condition, causing immune responses that dysregulate metabolism and lead to the aforementioned co-morbidities [17]. Both obesity and type II diabetes have been linked to metabolic endotoxemia, a modest increase in circulating levels of lipopolysaccharide (LPS) which binds to Toll-like receptor 4 (TLR-4) and activates the innate immune system, resulting in low-grade inflammation [5, 18]. In response to pro-inflammatory signaling, skeletal muscle becomes insulin-resistant and loses oxidative capacity, thereby resulting in the storage of fats rather than oxidation, a state known as metabolic dysregulation or metabolic inflexibility. [19].

In recent years, researchers have also discovered links between obesity and the gut microbiome, which refers to the bacteria that colonize the length of the intestinal tract. Obese and diabetic individuals' bacterial profiles and gut permeability differ significantly from those of lean individuals [2, 9, 20]. These differences correlate with changes in insulin sensitivity, propensity for diet-induced obesity, endotoxemia, circulating cytokine concentrations, and gut hormones [2, 21, 22]. Colonizing germ-free mice (lacking gut bacteria) with gut bacteria from normal, diet-induced obese mice causes dramatic weight gain independent of food intake [7, 23]. When *ob/ob* mice, which lack the appetite-regulating hormone leptin, are used for colonization, this weight gain effect is exacerbated. Cani, et al. has coined the terms 'dysbiosis' and 'microbesity' to describe a gut microbiome which favors obesity and leads to increased gut permeability and

plasma LPS due to its overgrowth of pathogenic bacteria [7, 13, 24]. While the mechanisms are not clear, it is becoming more obvious that the diet readily influences the gut microbiome, and in turn the bacteria influence the metabolic health of their host.

The human intestinal tract is colonized by roughly 10 trillion bacteria comprised of hundreds of species [7]. Gut bacteria were initially perceived as benign organisms with no influence on human health unless they entered the blood stream and induced sepsis, but in the past 20 years they have been recognized for their potential to influence intestinal cell health and whole-body metabolism [13]. Now, the gut microbiome is being considered as a potential therapeutic target to combat obesity and its related complications, including low-grade inflammation and metabolic dysregulation. Recent research has begun to elucidate a "brain-gut-microbiota axis," wherein the gut bacteria influence energy balance and obesity via regulation of gut hormones and activation of the endocannabinoid receptors [11, 17]. Research has thus far focused on the use of supplements, including prebiotics and probiotics, to influence the activity and bacterial variety of the gut microbiome.

The conceptualization and commercialization of probiotics—bacteria that positively affect a host's health—have led to new research pursuits to better understand the role of the gut microbiome and methods by which to improve its beneficial properties [12]. It is hypothesized that probiotics can effectively enrich the gut to shift a potentially harmful microbiome phenotype to one that benefits and protects the host. In humans, probiotic supplementation has correlated with decreased plasma cholesterol levels, decreased bowel-disease-related intestinal inflammation, decreased diarrhea and decreased visceral fat mass [16, 25, 26]. In mice, they have been shown to increase intestinal cell and mucous proliferation, thereby decreasing gut permeability and possibly indirectly reducing the harmful effects of a high-fat diet in a

microbiome-dependent manner [12, 27]. Prebiotics—fibers that are indigestible by humans but fermentable by bacteria—have been used as successful therapies to improve the health of intestinal cells and reduce high-fat diet-induced metabolic dysregulation by increasing certain strains of gut bacteria [28]. The bacteria ferment these fibers to short-chain fatty acids, which can then be utilized for energy by the bacteria and intestinal cells, promoting proliferation and the production of proteins that increase gut integrity [29]. The possibility to reduce and even prevent diet-induced obesity and metabolic dysregulation with non-invasive dietary supplements shows promise in combating the current epidemic of obesity and its co-morbidities [21]. This review discusses recent research illustrating the mechanisms by which gut bacteria and probiotics can influence skeletal muscle metabolism, intestinal function and integrity, and obesity-related endotoxemia. In addition, it proposes possible avenues for further research into proposed processes by which probiotic supplementation may ameliorate the deleterious inflammatory effects of chronic endotoxemia.

3. The Gut Microbiome and Gut Colonization

3.1 The Microbiome

The gut microbiome is essentially a microscopic ecosystem composed of trillions of bacteria living off the intestinal contents of the host and producing both waste products and energy [7]. The propensity of the bacteria to convert fibrous material to energy usable by the host has been shown to greatly influence the metabolic health of the host [21]. Similarly, by-products of the death of certain bacteria have been shown to have deleterious effects on the metabolic health of the host. ‘Good’ bacteria are non-pathogenic and bind to the epithelial wall of the lumen, promoting cell and mucous proliferation as well as the formation of the antibody IgA to fight infectious bacteria [30]. The ‘bad’ bacteria release toxins in the form of lipopolysaccharide,

which can induce inflammation and metabolic dysregulation at the level of the intestine as well as skeletal muscle [6, 31]. Certain bacteria have been linked to obesity and overweight in mice and humans, while others appear to prevent diet-induced obesity and insulin resistance [3, 13]. Bacterial strains compete for the available nutrients, using bacteriocins and controlling luminal pH to kill their competitors and prevent their proliferation [12]. Controlling the dominating species of bacteria through the use of probiotics has been shown to benefit the health of the host, and as researchers classify the expansive number of bacterial species, correlations can be found between the genotype of the gut microbiome and the phenotype of its host [2, 3, 7, 21, 22, 32].

3.2 Colonization

In utero, infants' intestinal tracts are completely free of bacteria. Even before birth, the mother's gut microbiome can influence the infant's birth weight, and from the beginning of life, the profile of the inherited gut microbiota correlates with weight gain through adolescence [33]. Colonization of the gut begins at birth as the infant is exposed to the vaginal canal, and continues throughout infancy while the infant nurses and is exposed to a bacteria-rich environment up to age two, at which point the gut microbiome stabilizes [32, 34]. Tsukumo, et al. reported that cesarean sections result in a delay of infant gut colonization by up to a month compared to babies birthed vaginally [27]. In addition, breastfeeding was shown to colonize the infant gut primarily with the beneficial *Bifidobacterium*, which produced high amounts of acetate and lactate. These acidic compounds reduced the pH of the intestine making it difficult for pathogenic bacteria to flourish. Many studies have illustrated links between the type of bacteria colonizing the gut and the degree of obesity and insulin resistance in the host. In humans, the most prevalent phyla of bacteria are the *Firmicutes, Actinobacteria* and *Bacteroidetes*, which make up roughly 80% of the total bacterial population [21]. The dominant phyla in an individual's gut can determine its

"enterotype", or co-occurring genre and species related to a specific phyla, and in turn, the enterotype influences the phenotype of the host [3]. High levels of *Firmicutes* have been linked with obesity and high amounts of *Bacteriodetes* with a propensity toward leanness [20, 21]. *Proteobacteria*, a Gram-negative phylum, have been measured at unusually high levels in insulin-resistant humans [3]. Dietary factors can also influence the enterotype of the gut according to studies that have shown increased *Bacteroides* levels during a diet high in protein and saturated fat, and increased levels of *Prevotella* (*Firmicutes*) during a diet high in simple sugars and carbohydrates [3].

Obesity has clear links to the composition and metabolic activity of gut bacteria [2, 3, 6, 9]. The microbiome of obese individuals is less diverse and has a high ratio of *Firmicutes* to *Bacteroides* [3]. Obese individuals also contain greater numbers of methanogens, or archaea that produce methane, which makes fiber fermentation more efficient and allows for greater energy harvesting from the diet [3, 9]. It appears that obese individuals contain bacteria that are adept at efficiently extracting energy from the host diet, which, in cases of obesity, is often Westernized and high in fat and sugar [3]. LPS-producing, pathogenic bacteria are also higher in both obese individuals and those with Type II diabetes while some beneficial strains are reduced; this phenomenon is termed "gut dysbiosis [7, 13, 21]." Fortunately, these effects can be ameliorated when individuals lose weight as a result of a calorie-restricted diet [3]. While the mechanism has not been fully elucidated, there are several theories that attempt to explain this phenomenon.

4. Mechanisms of Metabolic Influence

4.1 Energy Harvesting

One possible cause of a propensity toward obesity with certain gut colonization points to products of fiber fermentation by gut bacteria. Gut bacteria are capable of fermenting fibers

undigestible by humans, but the products differ [21]. While *Bacteriodetes* produce hydrogen, *Firmicutes* are capable of producing short-chain fatty acids (SCFA) which can be used for energy by the intestinal cells and as precursors to fatty acids and cholesterol [13]. Although the ingested fiber isn't bioavailable to a human gut, the SCFA's produced from it can account for 6-10% of basal energy requirements for people in developed countries [3]. This is known as 'energy harvesting', and it is one possible explanation for increased obesity in individuals colonized with high levels of this bacteria.

4.2 Short Chain Fatty Acids & G-protein Coupled Receptor Signaling

Recent research has shown these SCFA's can act as ligands to enteroendocrine cell-bound G-protein coupled receptors (GPR) in the gut which regulate energy homeostasis [3, 9, 13, 21]. GPR43 expression is increased in subcutaneous fat of mice fed a high-fat diet. Binding of GPR 43 by the SCFA's acetate and propioniate has been linked to increased adipogenesis, inhibited lipolysis, and decreased whole-body energy expenditure [3, 9, 13, 21]. When GPR41 was bound by the SCFA butyrate, SCFA uptake increased. Meanwhile, GPR 41 -/- mice were resistant to diet-induced obesity even after conventionalization, indicating that this receptor may be required for the gut microbiome to promote diet-induced obesity [3, 9, 13, 21].

However, it should be noted that in some cases supplementation of certain fibers known as preobiotics led to increased resting energy expenditure and insulin sensitivity and a decrease in *de novo* fatty acid synthesis in mice which contradicts the idea that fiber is a singular cause of increase adipogenesis [22, 29]. Gao, et. al showed that butyrate, a SCFA product of fiber fermentation, increased insulin sensitivity and energy expenditure in mice by increasing PGC-1a activity in skeletal muscle [29]. In addition, the enterocytes of the intestine can utilize the SCFA's for energy, which generally results in greater proliferation and tight-junction integrity,

thereby reducing the negative effects of high-fat feeding on the gut. Due to these conflicting results, the 'energy harvesting' theory has lost some support in recent years.

4.3 Fatty Acid Oxidation & de novo Fatty Acid Synthesis

Another possible explanation for decreased fatty acid oxidation in animals colonized with gut bacteria is the suppression of AMP-activated protein kinase (AMPK), which acts as an energy gauge and deactivates acetyl-CoA carboxylase (ACC) to increase mitochondrial fatty-acid oxidation . AMPK becomes active when levels of ATP decrease due to its dephosphorylation during times of high energy demand [35]. Active AMPK phosphorylates and suppresses the activity of ACC. ACC inactivation prevents the conversion of acetyl CoA to malonyl CoA. When malonyl CoA levels are low, fatty acids can enter the mitochondria via carnitine:palmitoyl transferase-1 (CPT1) where they can be oxidized to produce energy. When AMPK is suppressed, malonyl CoA levels remain elevated, preventing fatty acids from entering the mitochondria where they would be oxidized. Multiple studies and reviews reported germ-free mice experienced increased AMPK activity resulting in elevated PGC-1a levels, which increases mitochondrial biogenesis and oxidative capacity [7, 9, 36]. In a murine model of high-fat diet-induced obesity, mice lacking gut bacteria experienced obesity resistance, 40% more phosphorylated AMPK and ACC but a 15% decrease in CPT1, indicating lower levels of both fatty acid synthesis and oxidation, respectively [3]. Introduction of gut bacteria into germ-free mice resulted in suppression of AMPK and a subsequent decrease in fat oxidation. ACC and fatty acid synthase (FAS) can also be controlled by Carbohydrate Responsive Element Binding Protein (ChREBP) and Sterol Responsive Element Binding Protein (SREBP-1), both of which increase in hepatic cells after gut colonization [3, 7]. This leads to increased *de novo* fatty acid

synthesis and higher monosaccharide uptake from the intestine in colonized mice as compared to germ-free mice.

4.4 Fasting-induced Adipose Factor & Fatty Acid Uptake

Gut colonization, or the addition of gut bacteria to a previously bacteria-free mouse, has also been shown to suppress fasting-induced adipose factor (FIAF), a lipoprotein lipase (LPL) inhibitor [3, 13]. The suppression of FIAF led to greater LPL activity and therefore greater triglyceride uptake and possible storage in adipocytes. One study showed a decrease in ileal FIAF expression and a 122% increase in LPL activity in epididymal adipose tissue along with increased adiposity after conventionalization of germ-free mice [3]. Conventionalized mice—those who have undergone manual colonization—experience greater uptake and clearance of serum lipids compared to germ-free mice as well. In contrast, during high fat feeding, treatment with antibiotics intended to kill bacteria ameliorated diet-induced weight gain [37]. Germ-free mice have been shown to have reduced intestinal vascularity and digestive capabilities, so it is possible that some protection against diet-induced obesity is due to inefficient nutrient digestion and absorption [32].

4.5 Endocannabinoid System

Known as the 'brain-gut' axis, the endocannabinoid system connects the brain, gut, and liver through the vagus nerve, hypothalamus, and gastric hormone production to regulate certain digestive actions and signal energy requirements [3, 7, 11, 17]. Endocannabinoids are endogenous ligands, including anandamide and AG-2, which bind to cannabinoid receptors that are expressed in skeletal muscle, adipose tissue, liver, pancreas, bone, immune cells, and the nervous system [11, 17]. Cannabinoid receptors (CB1 and CB2) are G-coupled protein receptors that inhibit adenylate cyclase and cAMP production when bound and modify gene transcription

through a mitogen-activated protein kinase (MAPK), extracellular signal-related kinase (ERK) and nuclear factor kappa B (NFkB) pathway [17]. Endocannabinoids are produced only when needed and once synthesized, they are rapidly degraded by fatty acid amide hydrolase (FAAH) and monoacylglycerol hydrase (MGL). It has been shown that high-fat feeding, obesity, gut microbiota, and LPS play influential roles in endocannabinoid system activity [11, 17]. Obese individuals experience greater endocannabinoid eCB) levels in plasma and adipose tissue as well as increased CB1 expression compared to lean individuals [11, 17]. Muccioli, et. al showed that acute high-fat feeding increased intestinal eCB production and suppressed FAAH and MGL, but Cluny, et. al reported that chronic high-fat feeding resulted in normal eCB system function, which may indicate that obesity rather than high-fat feeding may cause chronic dysregulation of the eCB system [11, 17]. LPS alone has been shown to increase the synthesis of endocannabinoids and CB1 receptor expression in cell culture and mice, mimicking the effect of diet-induced obesity [7]. In addition, CB1 receptor activation has been linked to higher LPS levels in obese mice, indicating a possible self-promoting cycle [11, 17]. The eCB system has been shown to control gut permeability by changing the distribution of tight junction proteins, which exist between enterocytes to regulate influx and efflux of various substances in the intestine, including LPS [17]. Muccioli, et. al blocked the CB1 receptor using a receptor antagonist which reduced plasma LPS levels and decreased gut permeability [11]. In contrast, the application of LPS and a CB1 agonist to intestinal cells resulted in decreased tight junction protein expression. Evidence supports the theory that increases in LPS and CB1 receptor activation due to obesity correlates with increased gut permeability, allowing LPS to enter circulation and cause an inflammatory response. Fortunately, gut bacteria were shown to have exerted a great deal of influence over the eCB system after treatment with prebiotics (fibers)

[11]. Presumably, this was due to an increase in non-pathogenic bacteria and/or an increase in fermentation of fiber to SCFA's that the bacteria could use for energy as well as a pH buffer to decrease the growth of pathogenic strains. Obese mice fed a prebiotic fiber for 5 weeks experienced decreased intestinal CB1 expression, plasma LPS, and anandamide production. Similarly, obese mice treated with a CB1 receptor antagonist for 12 days showed reduced plasma LPS and increased gut barrier proteins, indicating decreased gut permeability [11]. In another study, obese mice fed prebiotic fiber for two weeks experienced a normalization of eCB tone in the gut, decreased gut permeability and plasma LPS, and a stunted fat mass growth [28]. Interestingly, these reductions were related to increases in markers of adipocyte differentiation and lipogenesis, including ACC, FAS, and SREBP-1 [11]. This could be indicative of the production of new adipocytes to prevent hypertrophy of other adipose cells, which leads to inflammation. As adipocytes undergo hypertrophy, they can become hypoxic and starved of nutrients due to the distance between the cell wall and the nucleus. In response, they secrete low levels of cytokines such as tumor necrosis factor α (TNFα) and monocyte chemoattractant protein-1 (MCP-1) [17]. LPS was shown to inhibit this adipogenic effect [11]. A logical explanation for these phenomena would be as follows: obesity-induced CB1 expression led to increased gut permeability and LPS levels; the LPS was free to circulate and increase inflammation while simultaneously preventing adipogenesis, thereby increasing adipose cell size and furthering low-grade inflammation.

There are many possibilities and conflicting views surrounding the type and mode of bacterial influence on the host's metabolic health. Currently it is only clear that these findings are reliant on the specific species of bacteria being introduced into the gut and the pre-existing gut microbiome of the host. These findings suggest a high-fat diet and/or obesity in conjunction with

a *Firmicute*- and Gram-negative-rich bacterial colony resulted in greater energy harvesting, increased circulating LPS, increased fatty acid synthesis, dysregulated hormone response to feeding, and increased eCB tone leading to gut leakiness [20, 28].

4.6 Metabolic Endotoxemia

When considering obesity as an inflammatory state, another possibility arises linking gut microbiota to inflammation via lipopolysaccharide (LPS), or endotoxin. The cell walls of Gram-negative bacteria in the intestine contain the LPS which acts as a ligand to Toll-like receptor 4 (TLR-4) of the immune system [22]. Plasma LPS levels in normal, healthy individuals generally remain under 0.2 ng/mL, but a high-fat diet can increase this to 2 ng/mL and individuals with increased gut permeability can show levels up to 10 ng/ml [6]. Studies have illustrated that LPS can bind TLR-4 and cause metabolic endotoxemia, a chronic, low-grade inflammation due to consistently, moderately elevated levels of cytokines [5].

When TLR-4 is activated, it causes the production of inflammatory cytokines, which have been shown to play a role in insulin resistance. TNFa, for instance, phosphorylates serine residues on insulin receptors, rendering them inactive [36]. In mice, low-dose LPS injections were shown to increase expression of TNFa and multiple inflammatory interleukins [38]. In addition, insulin-stimulated muscle glucose uptake was inhibited and insulin clearance was reduced, indicating some level of insulin-resistance at the level of muscle tissue. High-fat feeding has been linked to a greater level of Gram-negative bacteria in the gut and obesity has been linked to greater TLR-4 expression, and both have been linked to increased plasma LPS levels [3, 21, 24].

The tight junctions that separate enterocytes become compromised due to down-regulation of certain hormones during high-fat feeding, obesity and diabetes, and have also been associated with a loss of *Bifidobacterium* (*Actinobacteria*) [3]. Impaired tight junctions result in increased

gut permeability [39]. Both in vitro and in vivo models have illustrated that physiologically-relevant levels of LPS ranging from .1-10 ng/mL can cause increased gut permability and TLR-4 expression in gut epithelial cells [6]. LPS has been shown to associate with chylomicrons, which transport fatty acids to peripheral tissues, and plasma LPS levels are elevated after high-fat feeding and in obese individuals [40-42]. Additionally, obesity has been associated with increased bacterial translocation of Gram-negative bacteria from the lumen and mucosa to the blood and mesenteric adipose tissue in mice after one week of high-fat feeding compared with chow-fed mice [3]. While the mechanisms are still unclear, these findings illustrate that LPS may translocate through the enterocyte, be carried out on a chylomicron, or leak through the paracellular junction. Once in circulation, free LPS can bind TLR-4 which may lead to the condition referred to as endotoxemia [5].

5. Toll-Like Receptor 4 Activation and Metabolic Dysregulation

5.1 TLR-4 Signaling in Skeletal Muscle

Skeletal muscle is a metabolically-active organ that makes up a great deal of total body mass and is active in the endocrine system, making it a main site of substrate disposal as well as cytokine—or myokine—production [5, 43]. Healthy skeletal muscle responds to an increase in glucose or fatty acid availability by increasing oxidation of the dominant substrate [44]. This cycle, named for its discoverer Sir Philip Randle, has been considered as an originating site of metabolic dysregulation since it regulates both fatty acid and glucose oxidation [45]. Chronic high-fat feeding has been shown to induce insulin resistance via the Randle cycle by inhibiting the activity of pyruvate dehydrogenase and phosphofructokinase, thereby preventing glucose oxidation through glycolysis. The inability of muscle to adapt to increased substrate availability has been termed ‘metabolic inflexibility’. It is associated with inflammatory diseases such as

obesity and diabetes as well as high-fat feeding and excess caloric intake resulting in adipocyte hypertrophy [44, 46].

Skeletal muscle cells express TLR-4 at the cell surface where it can bind LPS that is carried through the plasma by LPS binding protein. Once bound, TLR-4 induces an inflammatory cascade resulting in the release of cytokines linked to skeletal muscle insulin resistance and metabolic inflexibility [47, 48]. In diabetes, obesity, or during high-fat feeding when circulating LPS levels are high, skeletal muscle metabolism becomes abnormal, resulting in 'metabolic inflexibility' [44, 46, 49]. This results in a propensity toward glucose utilization and increased adipose and intramuscular fat deposition [50]. The cycle of inflammation and dysregulation is furthered as fat cell size increases due to greater fat deposition, causing inflammatory cytokines to be released from adipose tissue as well [23]. TLR-4 knockout mice are resistant to the obesogenic, metabolic, and inflammatory effects of either a high-fat diet or LPS infusion, indicating that this is a key regulator in the development of obesity and metabolic inflexibility [3].

5.2 TL-4 Signaling in the Intestine

In a healthy gut, TLR-4 is expressed at very low levels and there is little inflammatory response to the constant presence of LPS [51, 52]. However, obesity and high doses of LPS increase the expression of TLR-4 and CD-14, respectively. CD14 associates LPS with the TLR-4 receptor. These have been found in the Golgi apparatus of mucosal cells in the intestinal crypts. There is little expression of TLR-4 on the apical membrane enterocytes, most likely due to the constant presence of LPS in the lumen which would induce high levels of inflammation [53]. Most luminal LPS is bound to micelles and packaged in chylomicrons before eventual deactivation in the liver. However, free LPS can bind to CD14 and can then be internalized to facilitate the

binding of LPS to TLR-4 which is co-localized with the Golgi apparatus. At that point the immune response begins, leading to inflammation in the gut. TLR's have been shown to regulate the gut immune response via influence on tight junction proteins and the production of inflammatory cytokines which are essential to fending off pathogens, maintaining homeostasis [54]. Mice lacking either TLR-4, MyD88 (a TLR pathway intermediate), or gut bacteria experience increased disease states and higher morbidity in models of intestinal inflammation, illustrating the essentiality of both the microbiome and TLR signaling in maintaining gut health. Pathologies arise when this signaling pathway is dysregulated, resulting in chronic inflammation. In the intestine, this manifests as inflammatory bowel disease; in skeletal muscle, this manifests as metabolic inflexibility.

5.3 Emerging Links between Skeletal Muscle and the Microbiome

Few studies have examined the link between skeletal muscle metabolism and the gut microbiome, but they appear to be linked through circulating LPS, which is increased in obesity, type II diabetes, and high-fat feeding [6, 31, 49]. Obese and diabetic individuals have higher circulating LPS levels, greater numbers of Gram-negative bacteria, and greater TLR-4 expression than lean individuals [3, 5]. Visceral adiposity—high levels of adipose tissue around the organs—has also been linked to increased gut permeability [55]. A high-fat diet increases both intestinal permeability and numbers of Gram-negative bacteria which release LPS [2]. LPS can bind to TLR-4 on skeletal muscle and enterocytes resulting in an inflammatory cascade. TLR-4 is abundant in skeletal muscle and has been shown to play a role in FA-induced insulin resistance and obesity-related LPS-induced inflammation [5]. TLR-4 binding results in the release of inflammatory cytokines such as tumor necrosis factor a (TNFa), which has been shown to induce insulin resistance in skeletal muscle, and the transcription factor NF-kB which

may lead to increased gut permeability by decreasing tight-junction proteins [6, 31, 38]. This heightened expression of TLR-4 in concert with elevated intestinal permeability and Gram-negative bacteria may result in a self-promoting cycle of endotoxemia and metabolic dysregulation in obese or diabetic individuals and those eating a chronic high-fat diet. The end result is metabolic inflexibility in skeletal muscle, one of the largest metabolically active tissues in the body.

6. Probiotics

6.1 Benefits of Probiotic Supplementation

Probiotic supplementation is the practice of ingesting live, non-pathogenic bacteria that can enrich the bacterial colony of the host and promote gut health [12]. Enrichment refers to the addition of healthy bacteria and should not be confused with colonization, which refers to adding an entirely new population of bacteria to a previously uncolonized gut. The host and bacteria share a symbiotic or mutualistic relationship; the bacteria are able to produce energy from non-digestible fibers and other nutrients provided by the host, and the health of the host is greatly influenced by the bacteria [56]. Beneficial bacteria provided through probiotic supplementation have been shown to decrease gut permeability, decrease inflammatory markers, improve diet-induced insulin resistance, and increase the health of individual enterocytes [13]. Probiotic supplementation has been utilized primarily as a therapy for inflammatory bowel diseases, including colitis and irritable bowel syndrome, to correct the overgrowth of harmful bacteria and improve mucous production to protect the intestinal lining [16]. Recently probiotics have become more widely available for regular use by individuals without bowel illness.

Probiotics are generally available as over-the-counter nutritional supplements containing millions to billions of bacteria in a capsule that protects them from the highly acidic contents of

the stomach and duodenum, but breaks down during digestion so the bacteria can enrich primarily the large intestine. Because probiotics are considered a dietary supplement and not a drug, the Food and Drug Administration only regulates their safety, efficacy, and accuracy of labeling [57]. Currently little is known about appropriate dosing and effectiveness. Generally the host must ingest enough capsules to release multiple billions of bacteria into their intestines each day for effective enrichment, and some companies recommend doses into the hundreds of billions for individuals with inflammatory bowel diseases. In addition, the probiotic bacteria must be capable of surviving transit through the highly acidic stomach and then adhere to the mucous layer of the enterocytes upon entering the intestines [58]. While the mechanisms of probiotics in improving host health are not entirely clear, it is theorized that they function to block the growth and adhesion of pathogenic bacteria to the intestinal mucous, enhance the innate immune response via Toll-like receptors located in the enterocytes, and provide substrates for enterocyte signaling and energy production [3, 12, 13]. Probiotics have also been shown to protect gut health by changing the pH of the gut, outcompeting pathogenic bacteria, and increasing mucous production [12, 58]. In addition, some probiotic bacteria produced bacteriocins, which kill competing bacteria. Certain probiotic bacteria also increase the expression of MUC genes which enhances the production of mucous covering the enterocytes [3, 12]. This protects them from the highly acidic environment and also prevents pathogenic bacteria from coming into contact with the enterocytes. More research is needed to further elucidate the extent to which these proposed mechanisms occur and how effectively the probiotic bacteria enrich the pre-existing colonies.

6.2 Probiotic Therapy for Inflammatory Bowel Diseases

Probiotic supplementation in humans has been shown to reduce the severity of diarrhea and gut permeability in Crohn's disease (CD), ulcerative colitis (UC), and irritable bowel disease (IBD) [16, 27, 58, 59]. In doses of 10-20 billion colony-forming units (CFUs), probiotics reduced infectious and antibiotic-associated diarrhea duration and occurrence in children and adults. Remission in CD and UC was increased significantly in patients who supplemented probiotics for 6 months and 6 weeks, respectively [59] . Adults and children with IBD experienced significantly less bloating and fewer relapses while taking probiotics compared to placebo [16]. A murine model of UC showed complete prevention of colitis-related gut permeability and enterocyte apoptosis during probiotic supplementation, suggesting that probiotics may increase proteins associated with maintaining cellular tight junctions [27].

6.3 Probiotics and Gut Permeability

Gut permeability refers to the ability of substances to leak between enterocytes. High gut permeability is linked to increased plasma LPS, most likely because LPS from Gram-negative bacteria is a small molecule that can diffuse between the intestinal cells [22]. Possible causes of increased gut permeability include high-fat feeding, visceral adiposity, type II diabetes, and obesity [2, 55, 60]. However, even in these states, probiotics have been shown to decrease gut permeability in mice through a variety of mechanisms, including the promotion of hormone production and the fermentation of fibers into SCFA's the enterocytes can utilize for energy [58]. Due to the variability in pre-existing gut colonies, many of the mechanisms are unclear and the findings are not always consistent. However, some studies do illustrate the beneficial effects and provide compelling explanations [12, 27, 58].

Tight junctions occur at the apical membrane between two epithelial cells [30]. At the site of the junction (the 'kissing point') are the transmembrane receptors occludin and claudin, which

associate in the intermembrane space. They are held in place by zonula-occludins-1,2 and 3 (ZO-1,2,3) that associate with actin which can manipulate the size of the tight junction through relaxation or contraction. Multiple studies have shown that endotoxemia, high-fat feeding, and obesity are associated with intestinal permeability, or leakiness [20, 21, 41]. Impaired tight-junctions can lead to bacterial translocation of intestinal bacteria into the circulatory and lymph system, causing inflammation.

In contrast, probiotic supplementation correlated with the increased tight junction proteins including zonula-occludins 1 (ZO-1) and occludin [12, 27, 58]. The expression of these proteins may, in part, be regulated by glucagon-like peptide 2 (GLP-2), an intestinal hormone secreted to facilitate nutrient absorption in response to nutrient intake. It is also involved in intestinal cell growth, maintenance of tight junctions, inflammation reduction, and the formation of chylomicrons [39]. Cani, et. al showed that prebiotic (fiber) supplementation caused a selective increase in *Bifidobacterium*, a bacterial strain commonly found in probiotic supplements [37]. The increase of *Bifidobacterium* correlated with increased GLP-2 production, which is associated with increased tight junction proteins. While it is not yet clear how GLP-2 promotes cell growth and junction integrity, some studies have shown that it acts on insulin-like growth factor 1 (IGF-1) which is a peptide that promotes cell proliferation, survival, and differentiation [39, 61]. It may also prevent apoptosis in a PI3Kase-dependent manner. GLP-2 has also been shown to increase the anti-inflammatory cytokine interleukin-10 (IL-10) and decrease levels of TNFa and other inflammatory cytokines. In reducing levels of these cytokines, it reduces potential down-regulation of the tight junction proteins. While studies have yet to link probiotic supplementation with an increase in GLP-2 production, it is often referred to as a mechanism by which changing the gut microbiome through probiotic supplementation can positively influence host health [3, 7,

13, 58]. Metabolic endotoxemia is generally related to a decrease in *Bifidobacterium* and a subsequent increase in gut permeability; enriching the gut with this bacteria through probiotic supplementation could repair gut permeability in a GLP-2 dependent manner [3].

Probiotic supplementation has also been shown to prevent the inflammatory-bowel-disease-induced enterocyte apoptosis, as well as losses of the tight-junction proteins ZO-1, occludin, and the claudins at the apical side of enterocytes [27]. The induction of colitis in murine models caused increased gut permeability by reducing both protein levels and membrane localization of these proteins, compromising the tight-junction complexes. In addition, enterocyte apoptosis increased and the diversity of the gut bacteria decreased. Probiotic supplementation completely ameliorated these effects, maintaining normal tight-junction function and the profile of the gut bacteria.

6.4 Probiotics and Metabolic Syndrome

Recently, researchers have begun to examine the effects of probiotic supplementation on obesity as an inflammatory disease and its related metabolic dysfunctions, such as insulin resistance, which are commonly grouped under the title of metabolic syndrome [14, 26]. Although there is conflict regarding the specific strains related to an obese or lean phenotype, there is consistent evidence that both the microbiome and gut integrity is less robust in obese humans and mice versus their lean counterparts [3]. This has led researchers to postulate that enriching the gut with beneficial bacteria may lead to improvements in anthropometric measurements. However, only a limited number of studies have shown minor improvement in biomarkers of metabolic health in obese humans, including weight loss, improved waist-hip ratio, decreased body mass index, and decreased blood pressure [26, 62]. Human studies utilizing probiotics as an intervention during metabolic syndrome or obesity showed no effect on gut permeability or inflammatory markers,

though insulin sensitivity improved in some cases [14, 15, 63, 64]. Effects in murine models of obesity are striking in comparison, as mice fed probiotics consistently exhibit increased insulin sensitivity and fatty acid oxidation in addition to decreased adiposity, endotoxemia, and markers of inflammation [65-68].

6.5 Possible Role of Probiotics in Lipopolysaccharide Clearance

LPS in the gut can be rendered temporarily inactive during fat absorption before its eventual deacetylation and inactivation in Kupffer cells, a process which is dependent upon the concentration of lipopolysaccharide binding protein and the number of chylomicrons produced [40, 69]. LPS associates with the micelle, a globule of digested fatty acids which is taken up by the enterocyte and disassembled. The contents of the micelle are repackaged as a nascent chylomicron, which binds the lipid A portion of the LPS molecule, rendering the LPS incapable of binding TLR-4. The chylomicron then enters circulation via the lymph system and can scavenge excess LPS circulating in the plasma [70]. Once it reaches the liver the LPS disassociates before being deacetylated and filtered from the body. This process prevents uncontrolled LPS binding and excess inflammation in response to the LPS, which is constantly being released from the intestine as Gram-negative bacteria die.

CD36, a fatty acid uptake receptor, binds monoacylglycerols (MAGs) and diacylglycerols (DAGs) as they approach the brush border of the enterocytes, and then transports them to the endoplasmic reticulum where they are packaged into chylomicrons. LPS bound to micelles which carry the MAGs and DAGs is also bound in the chylomicron because LPS associates with ApoB48 , the apolipoprotein attached to chylomicrons to identify them to pathogen-sensing cells. Increased LPS association with chylomicrons reduced levels of free LPS available to bind TLR-4 and cause inflammation [40, 71]. GLP-2 has been shown to increase chylomicron formation in a

CD-36 dependent manner [71]. A selective increase in *Bifidobacteirum*, a strain used in nearly all multi-strain probiotic supplementation, has been found to increase GLP-2 expression [37]. This is one proposed mechanism by which GLP-2 increases LPS clearance, and how probiotics might indirectly promote clearance through increasing GLP-2 production.

As chylomicrons distribute triglycerides to the periphery, their density changes as cholesterol levels remain generally stable. Once devoid of triglycerides, chylomicron remnants return to the liver where they can be re-used in the synthesis of very-low density lipoproteins (VLDL). VLDL cholesterol is distributed to the periphery and eventually the lipoproteins become low-density lipoproteins (LDL). This LDL cholesterol can be oxidized, which has been regarded as deleterious to health. Normally, oxidized cholesterol contributes to atherosclerotic plaque. However, it has been shown that oxidized LDL cholesterol can inhibit formation of the complex of LPS, CD-14, and TLR-4 required for TLR-4 activation [72]. Schlieffen, et. al showed that oxidized phospholipids competed with LPS for TLR-4 binding, and when bound to TLR-4 did not produce levels of inflammation equal to those induced by LPS [73]. This mechanism is novel, however, and requires more research to determine whether the benefits outweigh the increased disease risk. This pathway may serve as a mechanism to control metabolic dysregulation induced by a high-fat diet by utilizing otherwise harmful oxidized LDL cholesterol in a protective manner.

7. Limitations and Unanswered Questions

Currently there is little conclusive evidence supporting the hypothesis that probiotic supplementation is effective in improving human health. While murine models have successfully and repeatedly illustrated benefits, there are intrinsic limitations to testing these hypotheses in humans.

Compared to laboratory mice, there is great variability between individual human lifestyles, diets, and genetic profiles. As a result, there is variability between individual microbiomes which are influenced by all of these factors [74]. Mice engage in coprophagia, or the ingestion of their own fecal matter, which has been shown to influence markers in serum and could affect the microbiome [75]. It is also important to note that probiotic strains are native to the human gut, but not all of them are commonly found in the murine gut. Rawls, et al. showed that the profile of transplanted gut bacteria will change to match that of the native microbiome, illustrating that the microbiomes of different species are adaptable but certainly variable [76]. It is possible that coprophagia and enrichment with foreign strains of bacteria could confound the results seen in murine models of probiotic supplementation.

While a trend exists in the predominating bacterial phyla between lean and obese humans, variability and conflicting information confound correlational evidence that certain strains of bacteria are specifically beneficial or pathogenic [3]. While laboratory mice can be reared without gut bacteria and are so genetically similar that there is little difference in their gut microbiome at baseline, humans are extremely variable and there is no feasible or ethical way to induce total annihilation of their gut bacteria. Therefore, it is nearly impossible to control this variable in human subject testing. Additionally, while it is possible to test the expression of tight-junction protein-related genes directly from mouse intestinal tissue, it is not feasible to remove sections of human intestine for this purpose. Though it is possible to test gut permeability through the ingestion of sugars that cross the intestinal barrier through paracellular junctions, thus far this method has provided little evidence that probiotics improve obesity-related gut permeability in human models although probiotic supplementation has been effective in murine models of gut permeability [14, 27].

Most studies examining the effects of probiotics or gut bacteria in humans rely on anthropomorphic measurements, inflammatory markers in plasma, gut permeability markers in urine, and bacterial DNA analysis from fecal samples [26]. However, this provides little evidence that probiotic supplementation changes the profiles of the living microbiome within a human intestine. While it is possible that fecal samples are representative of the actual profile of the microbiome, there is also a chance that it is only representative of the bacteria that are failing to compete for nutrients and adhesion to intestinal mucuous [77]. In this case, the fecal sample would not be an effective measurement of active bacteria capable of influencing host metabolism.

In addition to the limitations inherent to human research, this area of study is also full of opportunities for future research. While links are emerging between gut permeability, plasma LPS, metabolic disease, and the gut microbiome, there are very few clear connections due to a lack of mechanistic studies and many questions remain. What are the mechanisms by which gut bacteria regulate gut permeability? What are the roles of gut peptides and the endocrine system in the cross-talk between gut bacteria and peripheral metabolism? What are the deciding factors in forming an individual gut microbiome, and could researchers one day synthesize gut microbiomes for implantation to ‘cure’ obesity?

8. Conclusion

The gut microbiome is capable of influencing the metabolic function of its host through a variety of mechanisms that have yet to be fully elucidated. Studies have shown that the production of SCFA’s, increases in tight junction proteins and GLP-2, and improved LPS clearance are all ways the gut bacteria promote health. In contrast, LPS release by some bacteria can be deleterious to the metabolic regulation of the host. High-fat feeding, obesity, and diabetes have

all been linked to increased gut permeability which allows LPS to leak between the enterocytes of the intestine and easily reach the blood stream. Probiotic supplementation has correlated with positive changes in gut permeability, visceral adiposity, and markers of endotoxemia and inflammation. The gut appears to be connected to skeletal muscle metabolism through the release of LPS. It is hypothesized that LPS may travel through paracellular junctions between intestinal cells whose permeability has increased as a result of a high-fat diet and/or obesity. Once the LPS enters circulation, it can bind TLR-4 on skeletal muscle and cause an inflammatory response which, over time, causes the skeletal muscle to become insulin resistant and metabolically inflexible. The impairment of skeletal muscle metabolism can lead to type II diabetes and obesity. It may be possible to prevent or treat certain aspects of these metabolic diseases through the use of probiotics to enrich the gut with beneficial bacteria. However, intrinsic limitations in human research must be overcome in order to fully explore and elucidate possible mechanisms by which probiotics can impact the native gut microbiome and cause changes in host health.

CHAPTER III: SPECIFIC AIMS AND HYPOTHESES

Despite a plethora of research and attention aimed at improving the obesity epidemic, obesity is still a prevalent disease in the United States and is becoming a serious concern all over the world. Diet-induced obesity has been linked to insulin resistance, type II diabetes, dyslipidemia, and overall metabolic dysregulation characterized by metabolic inflexiblity in skeletal muscle. Additionally, obesity is associated with dysbiosis of the intestinal tract leading to the loss of intestinal tight junction proteins and possibly metabolic endotoxemia.

Metabolic endotoxemia can induce low-grade inflammation which appears to play a major role in the development of reduced oxidative capacity related to metabolic inflexibility. The LPS, or endotoxin, must leave the intestinal tract and enter circulation in order to induce metabolic endotoxemia. The gut bacteria of obese individuals is characterized by increased potential for energy harvesting, and high-fat feeding and obesity correlate with increased plasma endotoxin levels. Our lab and others have shown increased plasma endotoxin levels following a high-fat meal in both humans and murine models. Additionally, both high-fat feeding and LPS stimulation reduced insulin sensitivity and glucose uptake, both of which are indicative of metabolic inflexibility. Research has shown that probiotic supplementation can increase the expression of intestinal tight junction proteins in models of inflammatory bowel diseases. Additionally, specific strains of probiotic bacteria improved insulin sensitivity in mouse models.

Our working hypothesis is that probiotic supplementation will prevent the deleterious of a high-fat diet, which include adiposity, metabolic endotoxemia, low-grade inflammation, disrupted glucose homeostasis and reduced oxidative capacity by reducing fatty acid uptake, maintaining the expression of tight junction proteins, maintaining insulin and glucose sensitivity, and maintaining normal regulation of the immune response. The specific aims of this project include:

Specific Aim 1: To determine the effects of 4 weeks of a high fat diet with or without VSL#3 probiotic supplementation on weight gain and body composition, respiratory exchange ratio and energy expenditure, glucose and insulin tolerance, mitochondrial biogenesis, and skeletal muscle substrate handling in C57BL/6 mice.

Hypothesis: A high fat diet will induce weight gain and increased fat mass, reduced oxidative capacity, reduced energy expenditure, reduced glucose and insulin tolerance, and reduced mitochondrial function. VSL#3 will prevent this effect.

Objective 1: Feed C57BL6 mice a high fat or control diet with or without VSL#3 probiotic supplementation. Measure changes in bodyweight and body composition during 4 weeks of feeding.

Objective 2: Feed C57BL6 mice a high fat or control diet with or without VSL#3 probiotic supplementation. Measure respiratory exchange ratio and energy expenditure in vivo. Determine insulin and glucose tolerance in vivo.

Objective 3: Feed C57BL6 mice a high fat or control diet with or without VSL#3 probiotic supplementation. Measure skeletal muscle substrate handling and mitochondrial function ex vivo after feeding a high-fat diet with or without probiotic supplementation.

Specific Aim 2: To study the effects of 4 weeks of a high fat diet +/- VSL#3 on changes in plasma endotoxin, tight junction protein expression, and markers of lipid handling in the small intestine.

Hypothesis: A high fat diet will reduce expression of tight-junction proteins and increase plasma endotoxin levels. VSL#3 supplementation will maintain normal expression of tight-junction proteins and prevent metabolic endotoxemia. VSL#3 supplementation will decrease genes associated with fatty acid uptake in small intestine epithelial cells.

Objective 1: Feed mice a high fat diet with or without VSL#3 supplementation for 4 weeks. Measure plasma lipopolysaccharide, tight junction protein mRNA expression, and expression of genes associated with fatty acid handling in intestinal tissue.

Specific Aim 3: To study the effects of 4 weeks of a high fat diet +/- VSL#3 and LPS injection on LPS-mediated alterations in markers of inflammation in skeletal muscle and liver as well as mitochondrial function in skeletal muscle.

Hypothesis: A high fat diet will induce inflammation in skeletal muscle and liver while decreasing mitochondrial function in skeletal muscle. This will be exacerbated by LPS injection but reduced by VSL#3 supplementation.

Objective 1: Feed mice a high fat diet with or without VSL#3 supplementation. Inject mice with a low dose of LPS or saline after 4 weeks of feeding. Measure skeletal muscle inflammation, substrate handling and mitochondrial function in skeletal muscle tissue.

Objective 2: Feed mice a high fat diet with or without VSL#3 supplementation. Inject mice with a low dose of LPS or saline after 4 weeks of feeding. Measure markers of inflammation in liver tissue.

CHAPTER IV: THE EFFECTS OF VSL#3 ON GLUCOSE HOMEOSTASIS AND INTESTINAL MARKERS OF NUTRIENT SENSING DURING HIGH-FAT FEEDING

4.1 Abstract

High-fat diets and obesity have been linked to unfavorable changes in gut bacteria and tight junction proteins leading to increased leakage of bacterially-derived lipopolysaccharide (endotoxin) from the intestinal tract into circulation. Metabolic endotoxemia, or chronically elevated circulating endotoxin, is associated with low-grade inflammation and metabolic dysregulation. Probiotic supplementation is the practice of ingesting live strains of bacteria that are proposed to have a beneficial effect on the host by enriching the intestine with healthy bacteria. The purpose of this project was to determine if probiotic supplementation would prevent the dysregulation of metabolism and tight-junction proteins associated with high fat feeding in mice. Male C57BL/6J mice were fed either a control (CD) (10% fat) or high-fat (HFD) (60% fat) diet for 4 weeks while receiving a daily oral gavage of water (C-VSL#3, HF-VSL#3) or probiotics (CD+VSL#3, HF+VSL#3) equivalent to 1.2 billion live cultures. Changes in body weight, body composition, respiratory exchange ratio,energy expenditure, and glucose and insulin tolerance were measured in live mice. Markers of metabolic function were measured in mitochondria and red and white skeletal muscle. Plasma endotoxin was measured in blood collected from fasted mice. The large and small intestines were collected and mRNA levels of tight-junction proteins and markers of nutrient sensing were measured. Fasting glucose and glucose AUC were significantly reduced during glucose and insulin tolerance tests with VSL#3 supplementation. VSL#3 supplementation significantly reduced expression levels of fasting-induced adipose factor while increasing levels of GLP-2 receptor expression in the small intestine. Body composition, tight junction proteins and fasting plasma endotoxin were unaltered

by VSL#3 supplementation. Skeletal muscle metabolism, energy expenditure, and mitochondrial function were unaltered by HFD or VSL#3 supplementation. Results indicate that probiotic supplementation may have provided protection against HFD-induced weight gain, insulin resistance, and hyperglycemia while increasing nutrient sensing in the gut.

Key words: probiotics, hyperglycemia, insulin sensitivity, high-fat diet, skeletal muscle metabolism

4.2 Introduction

Chronic high fat feeding elicits deleterious metabolic effects such as increased adiposity, hyperlipidemia, insulin resistance, and metabolic inflexibility [2, 46, 78]. Recent studies have linked the gut microbiome to metabolic and anthropometric changes that occur with a high-fat diet (HFD) [7, 79, 80].

The dominant phyla of bacteria within the microbiome differ between lean and obese individuals [7]. Colonizing a germ-free mouse with bacteria from an obese mouse causes rapid weight gain in the germ-free mouse [7, 23]. Additionally, the removal of gut bacteria through antibiotic administration prevents diet-induced obesity and co-morbidities [37, 81]. Research has shown that colonization of germ-free mice may induce adiposity by decreasing fatty acid oxidation (FAO) while increasing fatty acid uptake by suppressing fasting-induced adipose factor (FIAF) [3, 36]. Other research has shown that certain species of bacteria increase energy harvesting from the diet by fermenting undigestible fiber to nutritive short-chain fatty acids, thereby increasing the host's caloric intake [3, 13].

Dietary changes, such as shifting from high-carbohydrate to high-fat intake, can also cause changes in the profile of the microbiome [79]. A HFD has been linked to increased gut permeability through down-regulation of tight junction proteins between intestinal epithelial

cells, leading to an increase in plasma lipopolysaccharide (LPS), also known as endotoxin [2, 24, 42]. Chronically-elevated LPS (metabolic endotoxemia) has been implicated as a possible cause of metabolic inflexibility and insulin resistance due to its pro-inflammatory properties [24, 49, 82].

Probiotics are live, non-pathogenic bacteria that can be added to the diet through supplements or fermented products in order to enrich the gut microbiome [12]. Recently, probiotic supplementation has become a proposed therapy to prevent the deleterious effects of chronic high-fat feeding, such as excess adiposity, insulin resistance, gut permeability and metabolic endotoxemia [7]. Human trials have illustrated that certain probiotic strains can prevent visceral adiposity and dyslipidemia, but findings concerning insulin resistance are inconsistent and there is no evidence of protection against HFD-induced gut permeability [3, 14, 15, 25, 62, 83, 84]. Animal studies have illustrated improved insulin sensitivity, reduced weight gain, reduced plasma endotoxin, and increased markers of FAO [8, 65-67, 85-87]. In addition, animal studies of inflammatory bowel diseases showed that probiotics maintained tight junction proteins including ZO-1 and occludin, and a prebiotic-associated increase in specific bacteria led to an increase in glucagon-like peptide 2 (GLP-2), which may prevent the HFD-induced loss of tight junction proteins [27, 39, 60, 88].

The majority of the aforementioned studies examining the effect of probiotic supplementation during HFD utilized single-strain probiotics, primarily within the *Lactobacillus* and *Bifidobacterium* genera. VSL#3 is a probiotic medical food containing eight strains of bacteria from the genera *Lactobacillus, Bifidobacterium,* and *Streptococcus*. It has classically been studied as an effective therapy for individuals with inflammatory bowel disease, especially ulcerative colitis [89, 90]. Inflammatory bowel diseases often lead to increased gut permeability

through the loss of tight junction proteins, and VSL#3 has been shown to maintain normal levels of these proteins [27]. A limited number of studies have illustrated its anti-inflammatory and anti-steatotic effects in the liver during HFD [68, 91-93]. Additionally, Ma, et al. showed that VSL#3 supplementation protected against HFD-induced glucose intolerance following a glucose tolerance test in mice [68]. Thus far, evidence supports VSL#3 as a promising intervention to protect against the deleterious metabolic effects of HFD. However, to date the researchers are unaware of any study that has examined this multi-strain probiotic as it pertains to the prevention of HFD-induced adiposity, whole-body insulin resistance, metabolic endotoxemia or changes in fatty acid handling. The purpose of this study is to determine the potential protective effect of VSL#3 against the aforementioned deleterious effects of chronic high-fat feeding.

4.3 Methods

Animal studies. Male C57Bl/6J mice were ordered from Jackson Laboratories (Bar Harbor, Maine, USA) at 5 weeks of age and single-housed. They were acclimated to standard show for one week and scruffed daily for acclimation to being handled. The mice were divided into 4 groups (n=20): Control (10% fat) –VSL#3, Control +VSL#3, High Fat (60% fat) –VSL#3, and High Fat +VSL#3. Probiotic supplementation was administered by oral gavage with 150uL Omnipur water with or without 45mg of VSL#3 powder (1.2 billion cells) (Sigma-Tau Laboratories, Gaithersburg, MD, USA). The feeding lasted 4 weeks, at which point mice were euthanized by CO2 asphyxiation followed by cervical dislocation. All studies were conducted under an approved protocol by the Institutional Animal Care and Use committee at Virginia Tech, Blacksburg, VA.

Anthropometric changes. Body mass was recorded weekly. Body composition was measured at week 0 and week 4 using the Bruker MiniSpec magnetic nMR machine (Bruker Biospin Corp.,

Billerica, MA, USA).

Whole-body glucose and insulin sensitivity. Whole-body glucose tolerance and insulin tolerance were measured using an intraperitoneal glucose tolerance test or insulin tolerance test following 12 or 4 hours of fasting, respectively, as previously described [94].

Mouse energy metabolism. A Labmaster system (TSE Systems, Chesterfield, MO, USA) was used to measure indirect calorimetry and locomotor activity in individual mice. Metabolic chambers were used to measure VO_2 consumption and VCO_2 production in individual mice. Air with a concentration of 20.9% oxygen and 0.05% CO2 (Airgas Company, Radnor Twp., Pennsylvania, USA) flowed into the TSE system at a rate of 0.4 L/min. Mouse ambulatory movements were recorded via a photobeam-based activity monitoring system. Data on the aforementioned parameters was collected every 15 minutes. The results were used to calculate the respiratory exchange ratio (RER) and total energy expenditure/gram lean mass (EE). The formula $VO_2 \times [3.815 + (1.232 \times RER)] \times 4.1868$ was used to calculate energy expenditure (kJ/h), which was then normalized to the lean mass determined by NMR [95]. Measurements were made continuously and simultaneously for 48 hours after approximately 20 hours of adaptation for singly housed mice. The average values for the last 24 hours were used for analysis.

Assay of fatty acid oxidation. Quadriceps and gastrocnemius muscles were fractioned into red and white portions and collected for analysis. Fatty acid oxidation was assessed by measuring and summing $^{14}CO_2$ production and ^{14}C-labeled acid-soluble metabolites from the oxidation of [1-^{14}C]-palmitic acid (Perkin-Elmer, Waltham, MA), as previously described [5]. Citrate synthase, phosphofructokinase, and malate dehydrogenase maximal activities were measured in skeletal muscle as previously described [5].

Mitochondrial isolation and respiration analysis. Mitochondria were isolated from red gastrocnemius muscle as previously described with modifications [96]. Tissue samples were collected in buffer containing 67mM sucrose, 50mM Tris/HCl, 50mM KCl, 10mM EDTA/ Tris, and 10% bovine serum albumin (all from Sigma-Aldrich, St. Louis, MO). Samples were minced and digested in 0.05% trypsin (Invitrogen, Carlsbad, CA) for 30 minutes. Samples were homogenized and mitochondria were isolated by differential centrifugation. The experiments consisted of 3-min mixing, 2-min wait, and 3-min measurement cycle. Oxygen consumption was measured under basal conditions in the presence of the mitochondrial inhibitors 0.5 μmol/L oligomycin (Calbiochem) or 0.25 μmol/L rotenone (Sigma), or in the presence of the mitochondrial uncoupler, 0.3 μmol/L carbonylcyanide-p-trifluoromethoxyphenylhydrazone FCCP (Sigma) to assess maximal oxidative capacity. All experiments were performed at 37 °C. Oxygen consumption rate (OCR) was calculated by the oligomycin or FCCP-induced rates expressed as pmols/minute. Respiratory control ratio (RCRo) was calculated as the ratio between ADP-stimulated respiration and respiration in the presence of oligomycin, an ATP synthase inhibitor. Data are presented as the mean of three independent experiments performed in replicates of five and corrected for protein.

Plasma endotoxin. Fasting plasma endotoxin was measured in blood collected from cardiac punch after mice were sacrificed at the end of 4 weeks. Plasma endotoxin concentrations were determined using PyroGene Recombinant Factor C Endotoxin Detection fluorescence assay kits (Lonza Walkersville, Inc.,Walkersville, MD, USA). Limulus Amebocyte Lysate assay plates, Limulus Amebocyte Lysate reagent water, and pyrogen-free tubes (Lonza) were used to minimize exogenous endotoxin contamination. Assays were performed in duplicate on 25μL diluted plasma diluted in 75μL LAL water. Fluorescence was quantified using a BioTek

Synergy2 plate reader (Winooski, VT, USA), and data were analyzed using Gen 5 software (v1.08; BioTek).

RNA extraction and qRT-PCR. Intestine was collected from mice after sacrifice. The small intestine was separated into three equal sections (proximal, medial and distal) and the large intestine was separated from the distal section of small intestine. Intestinal contents were extruded and sections were minced before being placed in TRIzol (Life Technologies, Grand Island, NY, USA) and flash-frozen in liquid nitrogen. RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) and DNase I treatment (Qiagen, Valencia, CA), according to the manufacturer's instructions. qRT-PCR was performed using a Life Technologies ViiA 7 rtPCR (Life Technologies, Grand Island, NY) instrument and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) was used according to the manufacturer's specifications. Target gene expression in rodent intestine was normalized to β-actin RNA levels. Primers for ZO-1, ZO-2, Occludin, FIAF and GLP-2 were purchased as pre-validated assays from Life Technologies (Grand Island, NY). Relative quantification of target genes were calculated using the $2^{-\Delta C}{}_{T}$ method which has been described in Applied Biosystems User Bulletin no. 2 (P/N 4303859).

Statistical analysis. All data are represented as mean ± SEM. A 2-way ANOVA with multiple comparisons was performed to determine an interaction between diet x probiotic supplementation as well as main effects. If a significant interaction or main effect was found, an unprotected Fisher's LSD was performed *post-hoc* to determine differences between groups. Differences were only reported within the statistically significant main effect. Analysis was completed using GraphPad Prism 6 (La Jolla, CA, USA). Data was considered significant at $p<.05$.

4.4 Results

Body mass, weight gain and body composition. Mouse body weights were not stratified until week four, indicating a lack of HFD-induced obesity for the first three weeks of feeding (Figure 1A). After 4 weeks of feeding, HFD mice had gained significantly more weight (Figure 1B) and exhibited a significantly greater increase in %BF (Figure 1C) than control fed mice regardless of VSL#3 supplementation. HF-VSL#3 mice exhibited significantly greater fat mass than C-VSL#3 mice (Figure 2A). However, the body weights (Figure 2B) and total fat mass of C+VSL#3 and HFD+VSL#3 (Figure 2A) were not significantly different at week four. There were no differences in lean mass (data not shown).

Figure 1

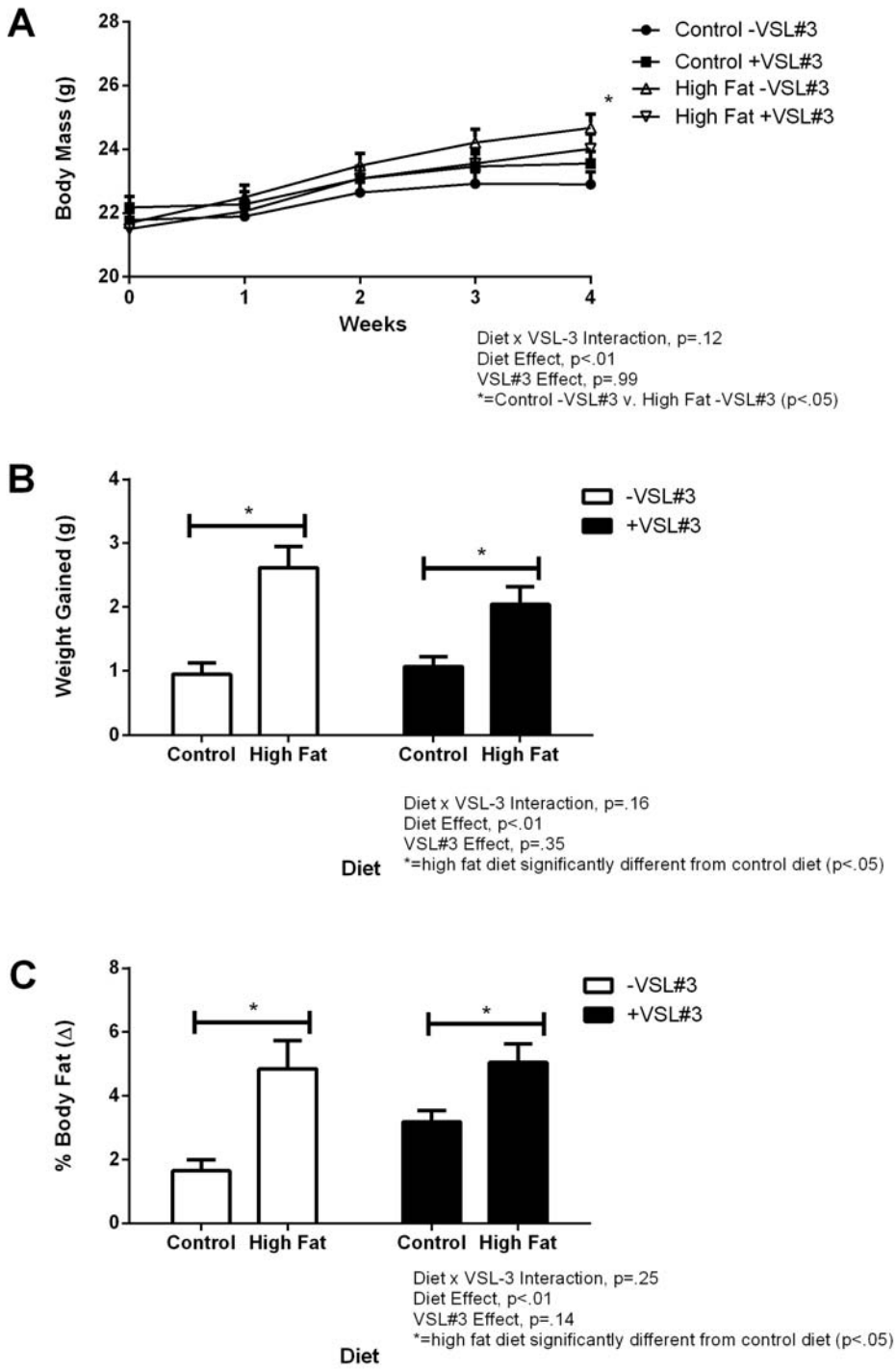


Figure 1. Changes in body mass (A), total weight gain (B) and changes in % body fat (C) in mice fed a control or high-fat diet with or without supplementation of VSL#3 probiotics at 1.2 billion cultures per day (n=10-15). Data are presented as mean ±SEM.

Figure 2

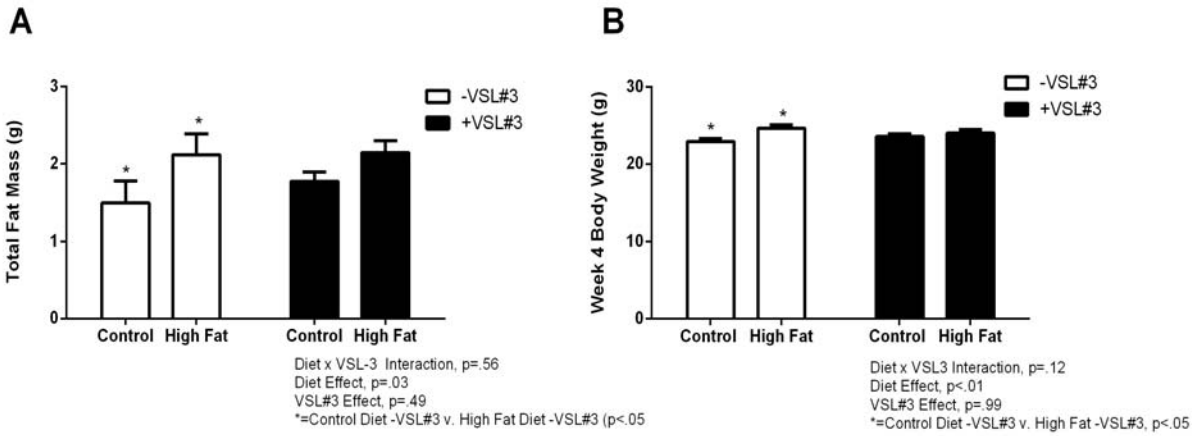


Figure 2. Total fat mass in grams (A) and total body weight (B) in the fourth week of feeding (n=10-15). Data are presented as mean ±SEM.

Respiratory exchange ratio and energy expenditure. RER and EE were measured to determine whether VSL#3 supplementation would influence substrate preference or metabolic rate. Regardless of VSL#3 probiotic supplementation, HFD resulted in a significantly lower respiratory exchange ratio (RER) in mice compared to control diet (Table 1). These results are indicative of the switch from primarily glucose oxidation to fatty acid oxidation during high fat feeding. There were no changes in energy expenditure (Table 1).

Markers of Skeletal Muscle Metabolism				
	-VSL#3		+VSL#3	
	Control	**High Fat**	**Control**	**High Fat**
Energy Expenditure (KJ/kg FFM/hr)	104.77±2.63	107.38±1.76	106.98±3.03	104.24±2.28
Respiratory Exchange Ratio (vCO_2/vO_2)	0.85±.03	0.75±.01	0.86±.03	0.75±.01
White Skeletal Muscle				
Total Fatty Acid Oxidation	16.59±2.64	18.40±2.54	15.64±2.22	19.94±2.56
Citrate Synthase	373.10±38.21	451.47±53.31	473.42±71.69	505.24±81.37
Phosphofructokinase	34.23±11.57	62.50±9.10	50.65±9.52	46.67±4.15
Malate Dehydrogenase	590.33±185.27	408.66±39.43	412.72±42.24	447.58±33.96
Red Skeletal Muscle				
Total Fatty Acid Oxidation	76.93±9.13	78.93±9.57	68.50±10.15	78.81±11.00
Mitochondrial Fatty Acid Oxidation	111.07±6.19	109.46±10.37	98.44±10.36	98.71±13.93
Citrate Synthase	746.86±54.4	814.54±90.53	720.63±62.40	794.02±62.07
Phosphofructokinase	22.66±7.78	37.50±4.84	40.5±7.72	36.44±4.30
Malate Dehydrogenase	883.70±99.86*	517.97±79.69*	625.77±49.19	485.70±178.78

Table 1. Markers of skeletal muscle metabolism, including whole-body energy expenditure and respiratory exchange ratio as measured via indirect calorimetry (n=4), total fatty acid oxidation in red and white skeletal muscle (n=7-10), enzyme activities in red and white skeletal muscle (n=2-5), and total fatty acid oxidation in mitochondria isolated from red skeletal muscle (n=3-5). Fatty acid oxidation is expressed as μmol/mg protein/hr. Enzyme activity is expressed as nmol/mg protein/min. *MDH enzyme activity in red muscle was significantly different in C-VSL#3 versus HF-VSL#3 mice ($p<.05$). All data are presented as mean±SEM.

Fasting glucose, insulin tolerance, and glucose tolerance. Following a 12-hour fast, blood glucose was measured to determine the presence of HFD-induced hyperglycemia. Fasting blood glucose was significantly lower in HF+VSL#3 mice compared to HF-VSL#3 mice (Figure 3A). Interestingly, glucose levels in C+VSL#3 and HF+VSL#3 mice were not significantly different. Fasting blood glucose of C-VSL#3 mice was not significantly different from any treatment group. An i.p. glucose tolerance test (GTT) was performed to determine whole-body glucose sensitivity. There were no significant differences in blood glucose at any time point during the

GTT (Figure 3B). However, VSL#3 supplementation resulted in a significantly reduced glucose AUC regardless of diet, and C+VSL#3 mice trended toward a decrease in glucose AUC compared to C-VSL#3 mice (p=.08) (Figure 3C). An i.p. insulin tolerance test (ITT) was performed to determine whole-body insulin sensitivity as well. Following a 4-hour fast, blood glucose levels in HFD mice were significantly higher than those of CD mice regardless of VSL#3 supplementation, but there were no significant differences at any other time point during the ITT (Figure 3D). HF-VSL#3 mice exhibited significantly higher glucose AUC values than C-VSL#3 mice (Figure 3E). However, similar to the fasting glucose results, C+VSL#3 and HF+VSL#3 values were not significantly different. These results demonstrate that VSL#3 supplementation leads to reduced blood glucose whether in a metabolically fed or fasted state.

Figure 3

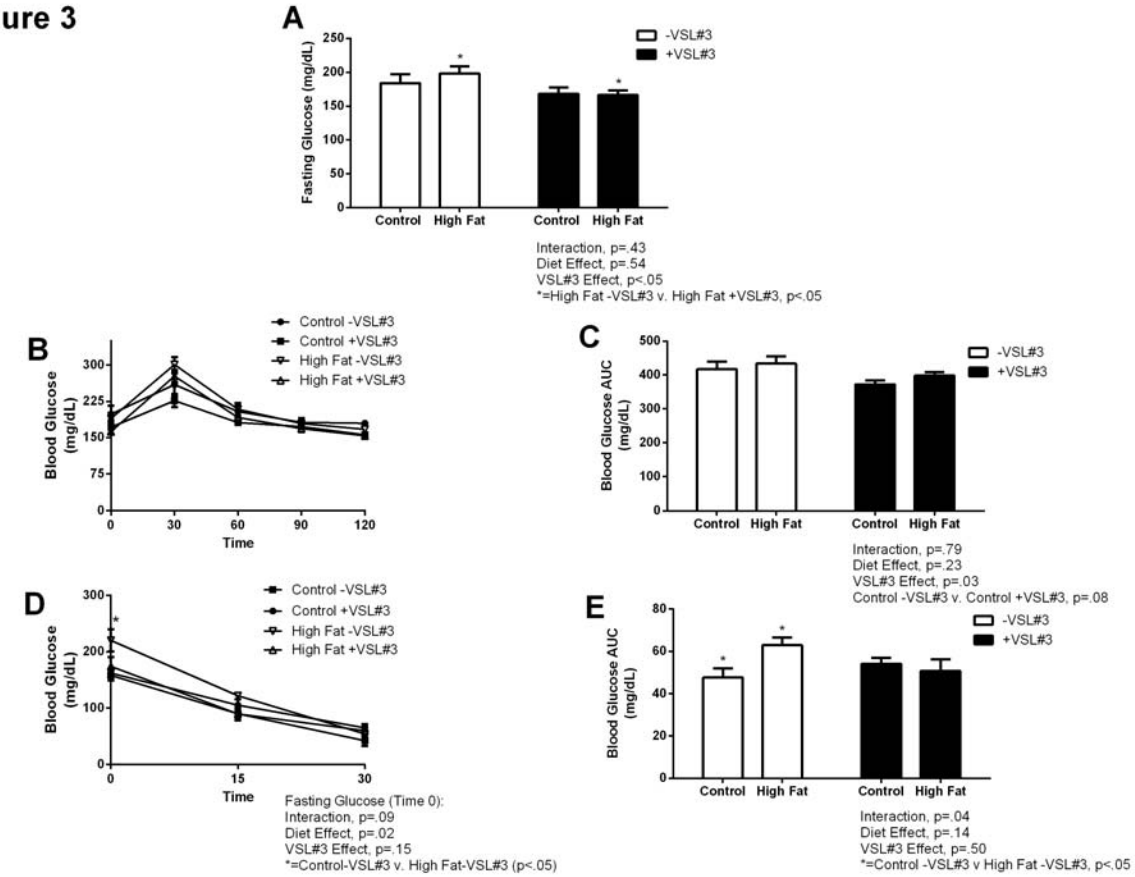


Figure 3. Levels of 12-hour fasted glucose in all mice (n=14-15)(A). Blood glucose (B) and glucose area under the curve (C) in mice receiving i.p. GTT (n=9-10). Blood glucose (D) and glucose area under the curve (E) in mice receiving i.p. ITT (n=4-5). Data are presented as mean ±SEM.

Skeletal muscle fatty acid oxidation and enzyme activity. Total fatty acid oxidation, CO_2 production, and acid-soluble metabolite (ASM) production was measured in red and white skeletal muscle homogenate to determine whether VSL#3 supplementation would protect against high-fat feeding-induced loss of fatty acid oxidative capacity indicated by reduced CO_2 production and increased ASM's. Neither VSL#3 supplementation nor the HFD elicited changes in total fatty acid oxidation in white or red skeletal muscle (Table 1). Similarly, CO_2 production and acid-soluble metabolite production were unchanged (data not shown). Malate dehydrogenase activity was significantly decreased in the red muscle of HF-VSL#3 mice compared to C-VSL#3 mice, but there was no significant difference between C+VSL#3 and HF+VSL#3 mice (Table 1). These results indicate that fatty acid oxidative capacity was not reduced during high fat feeding.

Mitochondrial fatty acid oxidation and biogenesis. Mitochondrial measures were taken to determine whether VSL#3 would affect the oxidative capacity and functionality of mitochondria. There were no differences in mitochondrial biogenesis (data not shown) or fatty acid oxidation between any groups (Table 1).

Fasting plasma LPS. Plasma LPS was measured in blood collected from cardiac punches following animal sacrifice to determine whether VSL#3 supplementation could reduce HFD-induced metabolic endotoxemia. Regardless of VSL#3 supplementation, plasma LPS was significantly and unexpectedly reduced in HFD mice compared to control-fed animals (Figure 4A). These are, perhaps, indicative of a significant difference in the clearance or bioactivity of LPS in HFD versus control-fed animals during the fasted state.

Figure 4

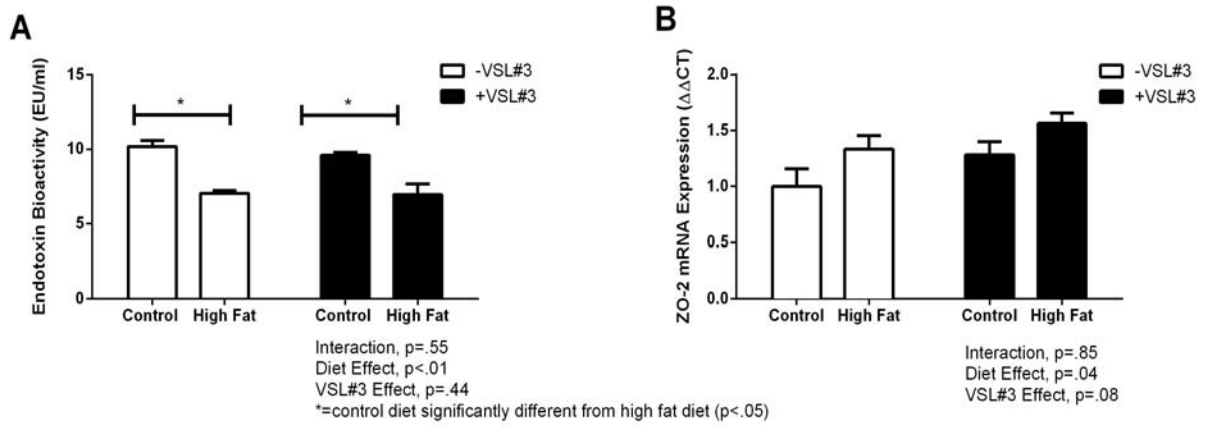


Figure 4. LPS bioactivity in plasma following a 12-hour fast (n=5-8) (A). Blood was collected from cardiac punch and spun down to separate plasma. ZO-2 mRNA expression in medial small intestine, corrected to control using ΔΔCT method (n=4-6) (B). Data are presented as mean ±SEM.

Gene expression of tight junction proteins. mRNA expression of the tight junction proteins ZO-1, ZO-2, and occludin were measured to determine whether VSL#3 supplementation would protect against the high-fat feeding-induced reduction in expression levels. Interestingly, ZO-2 expression in the medial section of small intestine was significantly increased in HFD mice compared to control fed mice (Figure 4B). VSL#3 supplementation trended toward an increase in ZO-2 expression (p=.08). There were no changes in any other measures (data not shown).

Expression of genes associated with fatty acid digestion and absorption. Expression levels of GLP-2 receptor and FIAF were measured in the small intestine to determine whether VSL#3 supplementation would influence HFD-induced changes in these markers of fatty acid handling. In the proximal section, expression of GLP-2 receptor was significantly lower in HF+VSL#3 mice compared to C+VSL#3 mice, while the levels in mice that did not receive VSL#3 supplementation were not significantly different (Figure 5A). There were no changes in FIAF (Figure 5B). Interestingly, in the medial section of the small intestine, there were no changes in GLP-2 receptor (Figure 5C) but changes occurred in FIAF. Expression of FIAF was significantly

higher in HF+VSL#3 mice compared to C+VSL#3 mice, but the differences between HF-VSL#3 and C-VSL#3 mice were not significant (Figure 5D). There were no differences in any measures in the distal section of the small intestine (data not shown). These results indicate that VSL#3 supplementation exerts effects in the proximal and medial sections of small intestine which influences genes associated with fatty acid digestion and absorption.

Figure 5

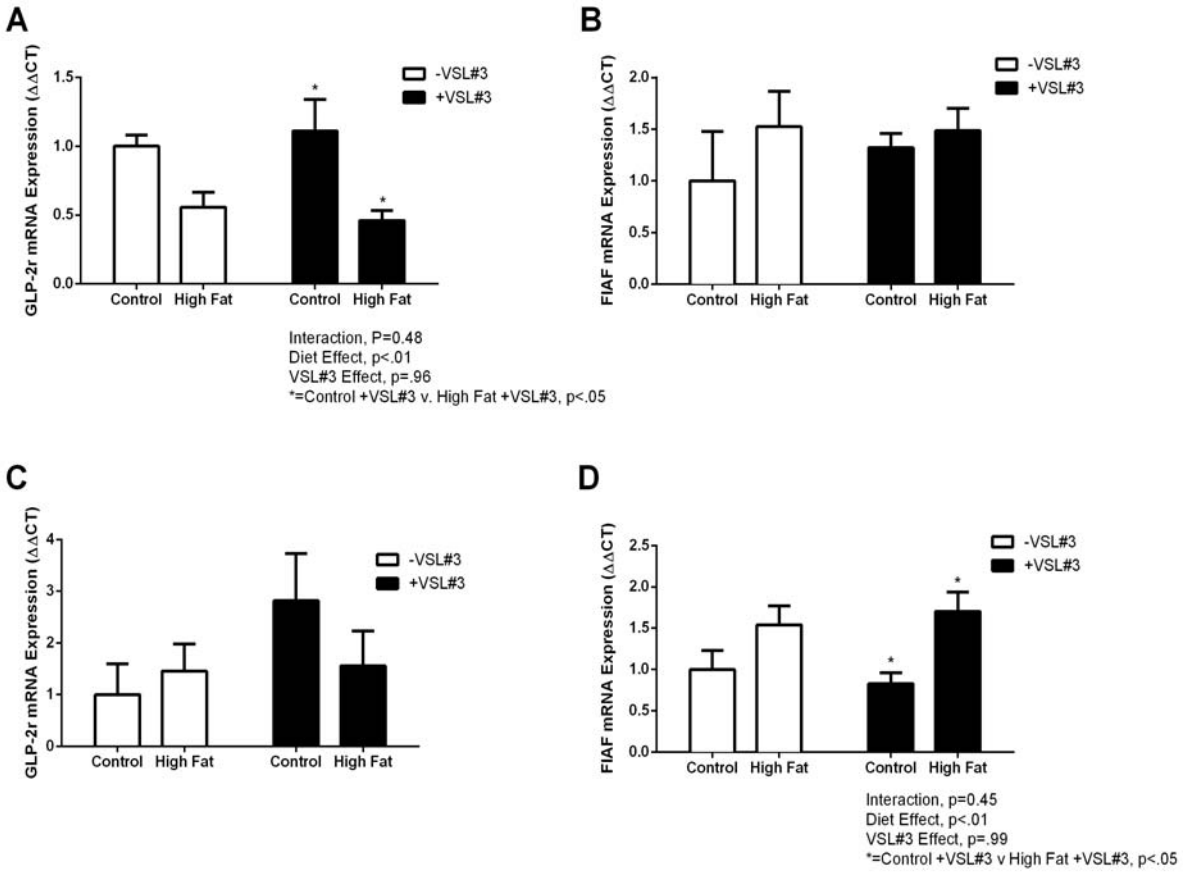


Figure 5. mRNA expression levels of glucagon-like peptide 2 receptor (n=5-10) (A) and fasting-induced adipose factor in proximal sections of small intestine (B) (n=4-7). mRNA expression levels of glucagon-like peptide 2 receptor (n=6-10) and fasting-induced adipose factor (n=6-9) in medial sections of small intestine. All values are corrected to control using ΔΔCT method. Data are presented as mean±SEM

4.5 Discussion

Our objective was to determine whether supplementation with the multi-strain probiotic VSL#3 would protect against HFD-induced weight gain, metabolic dysregulation, endotoxemia, and loss of tight-junction proteins. Our results show that VSL#3 probiotic supplementation provides

modest protection against diet-induced weight gain and insulin resistance during HFD. Additionally, VSL#3 supplementation provided significant protection against HFD-induced fasting hyperglycemia. Interestingly, compared to mice that did not receive probiotics, mice given VSL#3 supplementation experienced greater changes in FIAF and GLP-2 receptor expression when comparing control diet to HFD. While this is the first study that has utilized VSL#3 in such a way, it is in agreement with previous studies that have used single-strain probiotics as interventions during HFD.

Multiple studies have illustrated that single-strain probiotics in the *Lactobacillus* and *Bifidobacterium* genera are protective against hyperglycemia and insulin resistance during HFD in rodent models [65, 67, 86, 97]. The current study is in agreement with these studies as well as a previous study which illustrated that VSL#3 supplementation protected against glucose intolerance after 8 weeks of HFD in mice [68]. Our study showed that fasting glucose was significantly lower in HF+VSL#3 mice compared to HF-VSL#3 mice. Kim, et al. found that supplementation with a single-strain probiotic during HFD reduced the expression of the gluconeogenic genes PEPCK and glucose-6-phosphotase [65]. It is possible that VSL#3 supplementation elicited the same effect, resulting in lower levels of fasting blood glucose. Interestingly, this effect was not seen in control-fed mice; this is similar to Kim, et al. who found a significant improvement in glucose tolerance in HFD but not control-fed mice [65]. The results of the insulin tolerance test indicate that VSL#3 supplementation may have elicited intermediate effects in preventing insulin resistance based on similarities of glucose AUC in control and HFD mice fed VSL#3 probiotics. Similar results were seen during the glucose tolerance test, during which +VSL#3 mice trended toward decreased blood glucose levels compared to –VSL#3 mice. Although a number of studies have shown similar results, few have examined a possible

mechanism. Kim, et al. found increased expression levels of GLUT4 in the skeletal muscle of HFD mice fed a single-strain probiotic, which could indicate increased insulin-dependent glucose uptake [65]. In a study utilizing a combination of fiber and single-strain probiotic during HFD, hepatic insulin-receptor substrate-1 (IRS-1) serine phosphorylation was decreased by supplementation, indicating the protection of insulin signalling [86]. Possibly due to the short duration of the study, the results of our insulin and glucose tolerance tests indicate that the HFD mice were still sensitive to insulin and glucose tolerant. In this case, it could be extrapolated that VSL#3 supplementation may play a key role in preventing insulin resistance by maintaining euglycemia during the early stages of HFD-induced metabolic changes.

In –VSL#3 mice, the high fat diet significantly increased fat mass, percent body fat, total weight gain, and body weight at the end of 4 weeks of HFD compared to control-fed mice. While some of these findings also occurred in +VSL#3 mice, total fat mass and week 4 body weight was not significantly different between control+VSL#3 and HF+VSL#3 mice, indicating intermediate protective effects against diet-induced weight gain and adiposity. These results are in agreement with other studies that have shown protection against HFD-induced weight gain [23, 65, 87]. Other studies utilized longer feeding protocols of 10-12 weeks, which may explain the greater effects exhibited by their probiotic interventions. Similar to the previous glucose tolerance and insulin resistance data, because of the short length of our feeding protocol, it is likely that these data represent prevention of excess fat mass and weight gain considering the differences in body weight which emerged at four weeks of feeding. It could be extrapolated that a longer feeding protocol would result in a greater stratification of weight gain and body fat between groups, and indeed the time-courses of weight gain in previous studies illustrated that significant differences did not occur until after 3 to 4 weeks of HFD [8, 78].

Ours is the first study that has examined the effects of a multi-strain probiotic on the expression of fasting-induced adipose factor (FIAF or ANGPTL4), lipoprotein lipase inhibitor that likely acts on pancreatic lipase in the small intestine [98]. A previous study has shown that a *Lactobacillus* strain decreased fat storage through an increase in the expression FIAF [87]. In contrast, FIAF was suppressed in germ-free mice after colonization with bacteria from obese mice, leading to increased fatty acid uptake [99]. These conflicting results were likely due to the fact that the microbiome of obese mice exhibits increased capacity for energy harvesting from the diet while specific bacterial species within a single genus can have drastically different metabolic effects on the host [36, 99]. Additionally, the short-chain fatty acid butyrate, a product of fermentation by certain bacterial strains, has been shown to drastically increase FIAF expression [98]. Given that FIAF expression has been shown to increase with probiotic supplementation and high-fat feeding, we expected that its expression would increase in mice fed HFD as well as those given VSL#3. In the medial small intestine section of –VSL#3 mice, levels in control-fed and HFD mice were not significantly different. However, VSL#3 supplementation resulted in significantly different levels between control-fed and HFD mice, with greater expression in the HFD mice. While the current study is in agreement with Aronsson, et al. regarding an increase in FIAF with probiotic supplementation, their study did not include a HFD treatment, so these findings are novel. Increased expression of FIAF indicates reduced fatty acid (FA) uptake at the level of the intestine, which could explain the protection against weight gain and insulin resistance since elevated free fatty acids can cause inflammation-induced insulin resistance in peripheral tissues [100].

Studies have linked increases in *Bifidobacterium* to increases in circulating GLP-2, which is secreted from enteroendocrine cells following food intake and is involved in chylomicron

formation [39, 60, 88]. It is also suspected to play a role in maintaining tight junction proteins and regulating intestinal cell proliferation [71]. Due to the short half-life of this peptide and the overnight fast of our mice, it was prudent in our case to instead measure the expression of the GLP-2 receptor at the level of the intestine, where it is negatively regulated by circulating GLP-2 [61]. Based on the proposed regulation of GLP-2 receptor by its ligand, we expected to see a decrease in receptor expression during HFD compared to control-fed mice as GLP-2 levels would be highest due to increased nutrient intake and need for chylomicron formation. This was supported by our data which showed a significant decrease in GLP-2 receptor in HF+VSL#3 mice compared to C+VSL#3 mice in proximal small intestine. In contrast, the difference between C-VSL#3 and HF-VSL#3 mice was not significant. These results may indicate that VSL#3 supplementation improved nutrient sensing by enteroendocrine cells, thereby increasing GLP-2 to a greater extent during HFD. Despite the possible increase in the GLP-2 peptide based on these assumptions, there were no changes in tight junction proteins with the exception of ZO-2 in the medial intestine. Unexpectedly, ZO-2 expression increased modestly during high-fat feeding regardless of probiotic supplementation. Other studies have shown that a HFD decreases the expression of other tight junction proteins such as occludin and ZO-1, while probiotic supplementation can increase their expression [27, 80, 88]. One study has shown enhanced expression of ZO-2 expression in mice fed a single-strain probiotic, but none have shown an increase during HFD [101]. This could be indicative of a compensatory mechanism, but due to the lack of changes in any other tight junction proteins, this finding must be interpreted conservatively.

While several studies have shown that a high-fat meal acutely increases post-prandial plasma endotoxin levels in mice and humans, fewer studies have consistently replicated this finding in a

fasted state [24, 42, 94, 102]. The endotoxin concentrations of the mice in this study are similar to those seen in other studies which have shown post-prandial or fasting levels of LPS that reached roughly 5-8EU/mL, indicating that our mice may have exhibited metabolic endotoxemia [24, 83]. Cani, et al. illustrated that chronic HFD resulted in metabolic endotoxemia, or chronically elevated levels of plasma LPS that could induce low-grade inflammation [24]. However, our results show higher levels of plasma endotoxin in fasted mice fed the control diet. Interestingly, the time course of Cani, et al. illustrated a short period of time during which plasma LPS was higher in control than HFD mice, and it is within this window of time that our mice were sacrificed and blood was collected to measure plasma endotoxin, possibly indicating normal circadian fluctuations in endotoxin. Lipopolysaccharide binding protein (LBP) is released primarily from the liver in response to the presence of inflammatory cytokines such as interleukin-6, and binds the lipid A portion of LPS [103]. Once bound, the LPS/LBP complex can associate with immune receptors or circulating lipoproteins such as chylomicrons. While either interaction can result in an inflammatory response, the latter results in hepatic LPS deacetylation and clearance as well [69, 104]. Chylomicrons reduce LPS toxicity by binding it via LBP and transporting it to the liver where the inflammatory lipid A portion is removed [40, 70]. In a fasted state, the chylomicrons would have already been taken up by hepatocytes, resulting in low levels of plasma LPS. It is likely that the LPS had been cleared from the blood more rapidly under high-fat feeding conditions, perhaps as a result of post-prandial metabolic endotoxemia.

There were no changes in skeletal muscle fatty acid oxidation, and the significant differences in respiratory exchange ratio were appropriate given the high fat content of the HFD compared to the control diet. Malate dehydrogenase (MDH) was significantly reduced during high-fat feeding

without VSL#3 supplementation, but the mechanism and effect of this observation is unclear. Malate dehydrogenase is allosterically regulated by citrate, but no changes in citrate synthase were observed, making citric acid cycle flux unlikely [105]. A previous study showed that MDH expression was increased after high-fat feeding, which contradicts our findings [106]. These results indicate a limitation in the duration of the feeding protocol. The mice did not experience HFD-induced metabolic dysregulation, likely due to the short duration of the study resulting in the lack of excessive adiposity in HFD mice. This may have been due to the stress or discomfort of being handled and gavaged daily eliciting a stress response that would increase energy-producing pathways and blunt weight gain. The lack of HFD-induced metabolic dysregulation does limit the scope of our results because the mice did not serve as models of HFD-induced obesity or late-stage metabolic inflexibility. However, Hildebrandt, et al. showed that HFD-induced changes at the level of the gut microbiome occurred without the presence of obesity, and these changes resulted in modified fatty acid handling and weight gain [107]. Despite the fact that there was no metabolic inflexibility at the level of skeletal muscle, our results still illustrated HFD-induced changes in metabolism and therefore opportunities for VSL#3 supplementation to maintain normal metabolic function.

In addition to the short duration of the feeding protocol, a second limitation of this study is the lack of information about whether, or how, VSL#3 or HFD may have influenced the microbiome. Other studies have shown that diet rapidly changes the profile of gut microbiota, and there is evidence that HFD may lead to dysbiosis, or an overabundance of non-beneficial bacteria [20, 80, 107]. Studies have also shown that VSL#3 supplementation led to *Bifidobacterium* and *Lactobacillus* enrichment as well as an overall increase in species diversity [108, 109]. Based on previous data, it is reasonable to surmise that changes at the level of the

microbiome may have occurred, and that these changes influenced the current results. Additionally, this may explain why intestinal changes were localised in the proximal and medial sections of small intestine despite the fact that the large intestine is the most densely populated with bacteria [110]. The phyla *Actinobacteria,* which contains the genus *Bifidobacterium*, colonizes the length of the small intestine, while *Lactobacillus* species are hardy and can colonize the stomach and duodenum where acidity is too high for dense colonization. VSL#3 consists of multiple species of both the aforementioned genera, so it is possible that the greatest enrichment occurred in the proximal and medial sections of intestine.

This study was the first to examine the effects of VSL#3, a multi-strain probiotic, on HFD-induced metabolic derangements in skeletal muscle. Interestingly, our results occurred independently from metabolic changes at the level of skeletal muscle or mitochondria, which may point to mechanisms occurring at the level of intestine or liver. Our results show that VSL#3 significantly reduced fasting glucose and provided modest protection against the development of insulin resistance during high-fat feeding. While the mechanisms are unknown, based on other studies this could be due to a decrease in gluconeogenesis in the liver or increased insulin signaling at the level of skeletal muscle. In addition, our results include novel findings that VSL#3 supplementation increased FIAF expression and reduced GLP-2 receptor expression during HFD, which may indicate reduced fatty acid uptake and increased nutrient sensing by enteroendocrine cells. The increase in FIAF without a change in FAO indicates that the protection against weight gain by week four may have been due to decreased triglyceride digestion in the gut. The decrease in GLP-2 receptor may reflect an increase in the GLP-2 peptide which has been shown to protect against HFD induced gut permeability. In our case this may have been a mechanism by which the HFD-fed mice exhibited significantly reduced fasting

plasma LPS. Based on these results, VSL#3 is a novel potential therapy in preventing deleterious effects of HFD, and given its previous safe use in humans, should be utilized further in clinical trials.

CHAPTER V: VSL#3 SUPPLEMENTATION REDUCES TLR-2, TLR-4 AND TNFα EXPRESSION IN SKELETAL MUSCLE

6.1 Abstract

Both high-fat diets and obesity have been linked to changes in gut bacteria which may lead increased leakage of bacterially-derived lipopolysaccharide (endotoxin) from the intestinal tract into circulation where it can induce low-grade inflammation. Chronically-elevated levels of plasma endotoxin can induce persistent low-grade inflammation resulting in a condition known as 'metabolic endotoxemia' which has been implicated in the development of impaired fatty acid oxidation. Probiotic supplementation is the practice of ingesting live strains of bacteria that are proposed to have a beneficial effect on the host by enriching the intestine with healthy bacteria. The purpose of this project was to determine if probiotic supplementation would prevent the inflammatory and metabolic stress induced in skeletal muscle by a model of metabolic endotoxemia. Male C57BL/6J mice (n=5 per group) were fed either a control (CD) (10% fat) or high-fat (HFD) (60% fat) diet for 4 weeks while receiving a daily oral gavage of water or probiotics equivalent to 1.2 billion live cultures. Mice received an injection of saline or lipopolysaccharide (0.1µg/kg bodyweight) 4 hours before being sacrificed for tissue collection. Markers of metabolic function and inflammation were measured in mitochondria, skeletal muscle and liver. Due to a lack of high-fat feeding effect, groups were collapsed across diet treatments and the interaction between low-dose LPS injection and probiotic supplementation was measured. VSL#3 supplementation was associated with decreased mRNA expression levels of TLR-4, TLR-2 and TNFa in skeletal muscle following saline injection. While it did not protect against LPS-induced changes in TNFa or IL-10 in the liver, VSL#3 supplementation amplified both the inflammatory and anti-inflammatory responses to low-dose LPS injection.

Results indicate that probiotic supplementation modifies TLR-2, TLR-4 and TNFa mRNA expression in skeletal muscle and TNFa and IL-10 expression in the liver.

Key words: probiotics, inflammation, lipopolysaccharide

6.2 Introduction

High-fat feeding has been closely associated with increased leakage of bacterially-derived lipopolysaccharide (LPS), or endotoxin, from the intestinal tract into circulation [3, 42, 49, 80]. Chronically-elevated levels of plasma endotoxin, or 'metabolic endotoxemia, can induce persistent low-grade inflammation [4, 7]. This low-grade inflammation has been implicated in the development of metabolic dysregulation [24, 38, 44, 111, 112]. Elevated levels of LPS have been found in obese and type II diabetic individuals, indicating a link to metabolic dysregulation [113, 114].Toll-like receptor 4 (TLR-4), an integral part of the innate immune system, is a pathogen-associated molecular pattern (PAMP) receptor that is abundantly expressed in skeletal muscle, intestinal epithelial cells, and to a lesser extent in various liver cells [5, 6, 115]. LPS binds TLR-4, resulting in the release of inflammatory cytokines such as tumor necrosis factor α (TNFα), which may induce insulin resistance and the development of liver steatosis [31, 38, 92]. Metabolic endotoxemia, or chronically-elevated levels of LPS, could induce chronic inflammation and metabolic dysregulation through chronic activation of the TLR-4 pathway [24]. Similarly, TLR-2 has been shown to initiate the same inflammatory cascade in high-glucose environments and in conjunction with TLR-4 activation [100].

Probiotic supplementation is the practice of ingesting live, non-pathogenic bacteria that may enrich the bacterial colony of the host and promote gut health [12]. Although there are clear links between diet, gut bacteria, and metabolic function, few studies have examined the effect that probiotic supplementation may have on preventing or treating inflammation and metabolic

dysregulation induced by metabolic endotoxemia in skeletal muscle [3, 65]. Beneficial bacteria provided through probiotic supplementation have been shown to normalize inflammatory tone in plasma, intestine and liver in human and animal models [8, 90, 116, 117]. Common findings include a decrease in pro-inflammatory TNFα and an increase in the anti-inflammatory cytokine IL-10 [91, 93, 118].

Previous studies have primarily utilized single-strain probiotics, commonly from the *Lactobacillus* and *Bifidobacterium* genera. VSL#3 is a multi-strain probiotic, containing strains from the aforementioned genera as well as *Streptococcus* [119]. VSL#3 is a highly effective therapy in treating inflammatory bowel diseases (IBD), likely due to its high concentration and multiple strains of bacteria [89, 119]. Despite its effectiveness in treating IBD, it has not yet been studied as a therapy against inflammation in skeletal muscle. Indeed, the researchers are not aware of any studies that have examined the effects of a multi-strain probiotic on skeletal muscle metabolism or inflammation in a model of metabolic endotoxemia. Therefore, it was the purpose of this study to determine a possible role of probiotic supplementation as a non-invasive dietary method for protection against metabolic endotoxemia. Researchers hypothesized that probiotic supplementation would abrogate the inflammatory effects of a low-dose LPS injection modeling metabolic endotoxemia, thereby preventing subsequent metabolic dysregulation.

6.3 Methods

Animal Studies. Male C57Bl/6J mice were ordered from Jackson Laboratories (Bar Harbor, Maine, USA) at 5 weeks of age and single-housed. They were acclimated to standard show for one week and scruffed daily for acclimation to being handled. The mice were divided into 4 groups (n=5): Control (10% fat) –VSL#3 (C-VSL#3), Control +VSL#3 (C+VSL#3), High Fat (60% fat) –VSL#3 (HF-VSL#3), and High Fat+VSL#3 (HF+VSL#3). Probiotic supplementation

was administered by oral gavage with 150uL Omnipur water with or without 43mg of VSL#3 powder (1.2 billion cells) (Sigma-Tau Laboratories, Gaithersburg, MD, USA). The feeding lasted 4 weeks, at which point mice received an intraperitoneal injection of saline or lipopolysaccharide (LPS) (0.1µg/kg bodyweight) 4 hours before being sacrificed via inhalation of CO2. All studies were conducted under an approved protocol by the Institutional Animal Care and Use committee at Virginia Tech, Blacksburg, VA.

Assay of Fatty Acid Oxidation. Quadriceps and gastrocnemius muscles were fractioned into red and white portions and collected for analysis. Fatty acid oxidation was assessed by measuring and summing $^{14}CO_2$ production and ^{14}C-labeled acid-soluble metabolites from the oxidation of [1-^{14}C]-palmitic acid (Perkin-Elmer, Waltham, MA), as previously described [5]. Citrate synthase, phosphofructokinase, and malate dehydrogenase maximal activities were measured in skeletal muscle as previously described [5].

Mitochondrial isolation and respiration analysis. Mitochondria were isolated from red gastrocnemius muscle as previously described with modifications [96]. Tissue samples were collected in buffer containing 67mM sucrose, 50mM Tris/HCl, 50mM KCl, 10mM EDTA/ Tris, and 10% bovine serum albumin (all from Sigma-Aldrich, St. Louis, MO). Samples were minced and digested in 0.05% trypsin (Invitrogen, Carlsbad, CA) for 30 minutes. Samples were homogenized and mitochondria were isolated by differential centrifugation. The experiments consisted of 3-min mixing, 2-min wait, and 3-min measurement cycle. Oxygen consumption was measured under basal conditions in the presence of the mitochondrial inhibitors 0.5 µmol/L oligomycin (Calbiochem) or 0.25 µmol/L rotenone (Sigma), or in the presence of the mitochondrial uncoupler, 0.3 µmol/L carbonylcyanide-p-trifluoromethoxyphenylhydrazone FCCP (Sigma) to assess maximal oxidative capacity. All experiments were performed at 37 °C.

Oxygen consumption rate (OCR) was calculated by the oligomycin or FCCP-induced rates expressed as pmols/minute. Respiratory control ratio (RCRo) was calculated as the ratio between ADP-stimulated respiration and respiration in the presence of oligomycin, an ATP synthase inhibitor. Data are presented as the mean of three independent experiments performed in replicates of five and corrected for protein.

RNA extraction and qRT-PCR. RNA was extracted using an RNeasy Mini Kit (Qiagen) and DNase I treatment (Qiagen, Valencia, CA), according to the manufacturer's instructions. qRT-PCR was performed using a Life Technologies ViiA 7 rtPCR (Life Technologies, Grand Island, NY) instrument and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) was used according to the manufacturer's specifications. Target gene expression in rodent intestine was normalized to β-actin RNA levels. Primers for TLR-2, TLR-4, TNFα, IL-10 and β-actin were purchased as prevalidated assays from Life Technologies (Grand Island, NY). Relative quantification of target genes were calculated using the $2^{-\Delta C}{}_{T}$ method which has been described in Applied Biosystems User Bulletin no. 2 (P/N 4303859).

Statistical Analysis. Due to a lack of diet effect measured using 3-way ANOVA F-test values, the model was simplified by collapsing across diets. Data were then analyzed using 2-way ANOVA with an unprotected Fisher's LSD post-hoc when significant interaction or main effects were found. Analysis was completed using GraphPad Prism 6 (La Jolla, CA, USA). All data are represented as mean ± SEM and significance is set at $p<.05$.

6.4 Results

Markers of Fatty Acid Oxidation in Skeletal Muscle and Mitochondria. Neither LPS injection nor supplementation with VSL-3 probiotics had an effect on total, complete or incomplete fatty acid

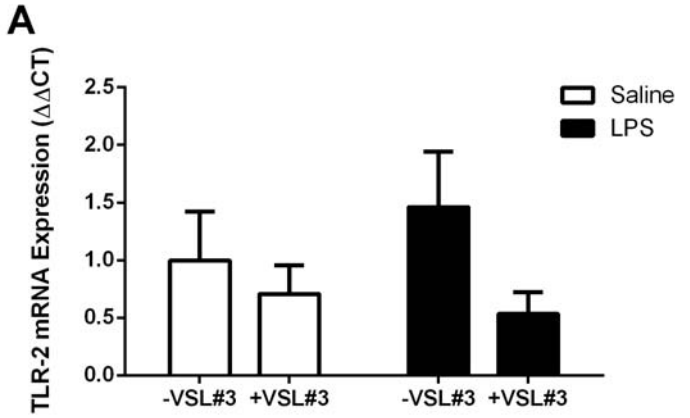
oxidation in skeletal muscle or isolated mitochondria (Table 2). There were no differences in mitochondrial biogenesis of isolated mitochondria (Table 2).

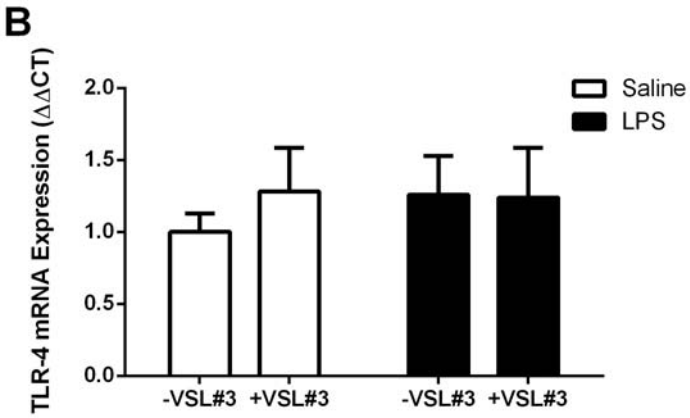
Markers of Skeletal Muscle Metabolism				
	-VSL#3		+VSL#3	
	Saline	LPS	Saline	LPS
White Skeletal Muscle				
Total Fatty Acid Oxidation	34.42±5.05	33.56±8.33	31.24±3.97	25.58±3.79
CO2 Production	8.79±1.08	8.21±1.21	8.40±1.66	6.97±1.51
ASM Production	25.63±4.35	17.93±1.92	22.84±3.01	18.56±2.82
Red Skeletal Muscle				
Total Fatty Acid Oxidation	74.40±8.65	67.14±5.08	83.97±11.65	73.26±5.24
CO2 Production	20.6±2.21	19.63±2.02	23.99±3.63	21.34±2.36
ASM Production	56.53±7.28	47.52±3.72	59.98±8.23	51.91±3.78
Mitochondria				
Total Fatty Acid Oxidation	200.81±27.43	188.65±25.43	198.70±24.31	191.38±14.62
CO2 Production	11.99±1.86	7.80±1.64	9.57±1.94	8.80±1.44
ASM Production	188.82±26.17	158.75±10.93	189.14±23.8	182.54±14.21
Baseline OCR	153.50±31.96	103.75±18.45	112.00±26.90	119.50±20.15
ADP-Stimulated/State III	1,377.00±214.26	118.14±212.77	1,298.00±192.16	1,347.50±161.67
Oligo-Inhibited/State IV	188.82±26.17	158.75±10.93	189.14±23.80	182.54±14.2
FCCP	1,590.43±194.45	1,673.86±305.01	1,744.33±216.62	1,841.75±192.82
Respiratory Control Ratio	2.23±.38	2.20±0.41	2.34±.39	2.57±.38

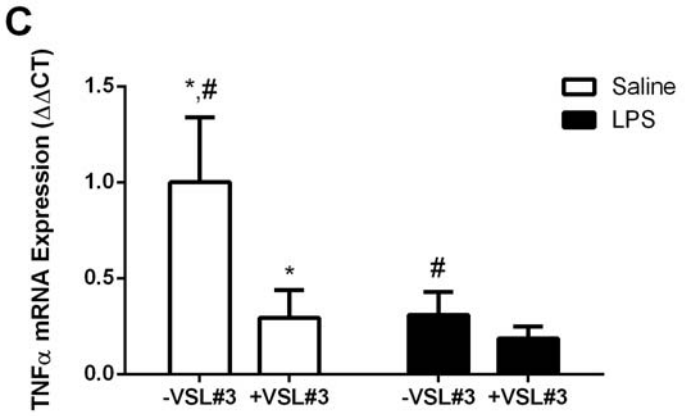
Table 2. Markers of skeletal muscle metabolism in mice injected with saline or low-dose LPS (0.1μg/kg BW) after 4 weeks with or without VSL#3 supplementation. Fatty acid oxidation, CO2 production, and acid-soluble metabolite (ASM) production are expressed as μmol/mg protein/hr. Baseline oxygen consumption rate (OCR), ADP-stimulated/State III respiration, Olgo-Inhibited/State IV respiration, FCCP-induced uncoupled respiration, and respiratory control ratio are expressed as pmol/min. There were no differences among any groups in red skeletal muscle (n=8-9), white skeletal muscle (n=8-10), or isolated mitochondria (n=6-10).

Markers of Inflammation in White Skeletal Muscle. Expression levels of TNFα were significantly reduced in saline+VSL#3 mice and LPS-VSL#3 mice compared to saline-VSL#3 mice; levels in LPS+VSL#3 mice were not significantly different from any group (Figure 6C). There were no differences in TLR-2 (Figure 6A) or TLR-4 (Figure 6B) mRNA expression levels.

Figure 6







LPS x VSL#3 Interaction, p=.13
LPS Effect, p<.05
VSL#3 Effect, p<.05
*=Saline -VSL#3 v. Saline +VSL#3, p<.05
#=Saline -VSL#3 v. LPS -VSL#3, p<.05

Figure 6. mRNA expression levels of TLR-2 (A), TLR-4 (B), and TNFα (C) in white skeletal muscle of mice injected with saline or low-dose LPS (0.1 μg/kg BW) after 4 weeks with or without VSL#3 supplementation (n=8-9). Expression levels are corrected to β-actin.

Markers of Inflammation in Red Skeletal Muscle. Expression of TLR-2 was significantly different in saline-VSL#3 mice compared to saline+VSL#3, but in mice that received an LPS injection these levels did not differ from any group (Figure 7A). TLR-4 expression levels were significantly lower in saline+VSL#3 mice and LPS-VSL#3 mice compared to saline-VSL#3 mice (Figure 7B). Expression levels of TLR-4 in mice that received both LPS and VSL#3 supplementation were not significantly different from any group. There were no changes in TNFα expression (Figure 7C).

Figure 7

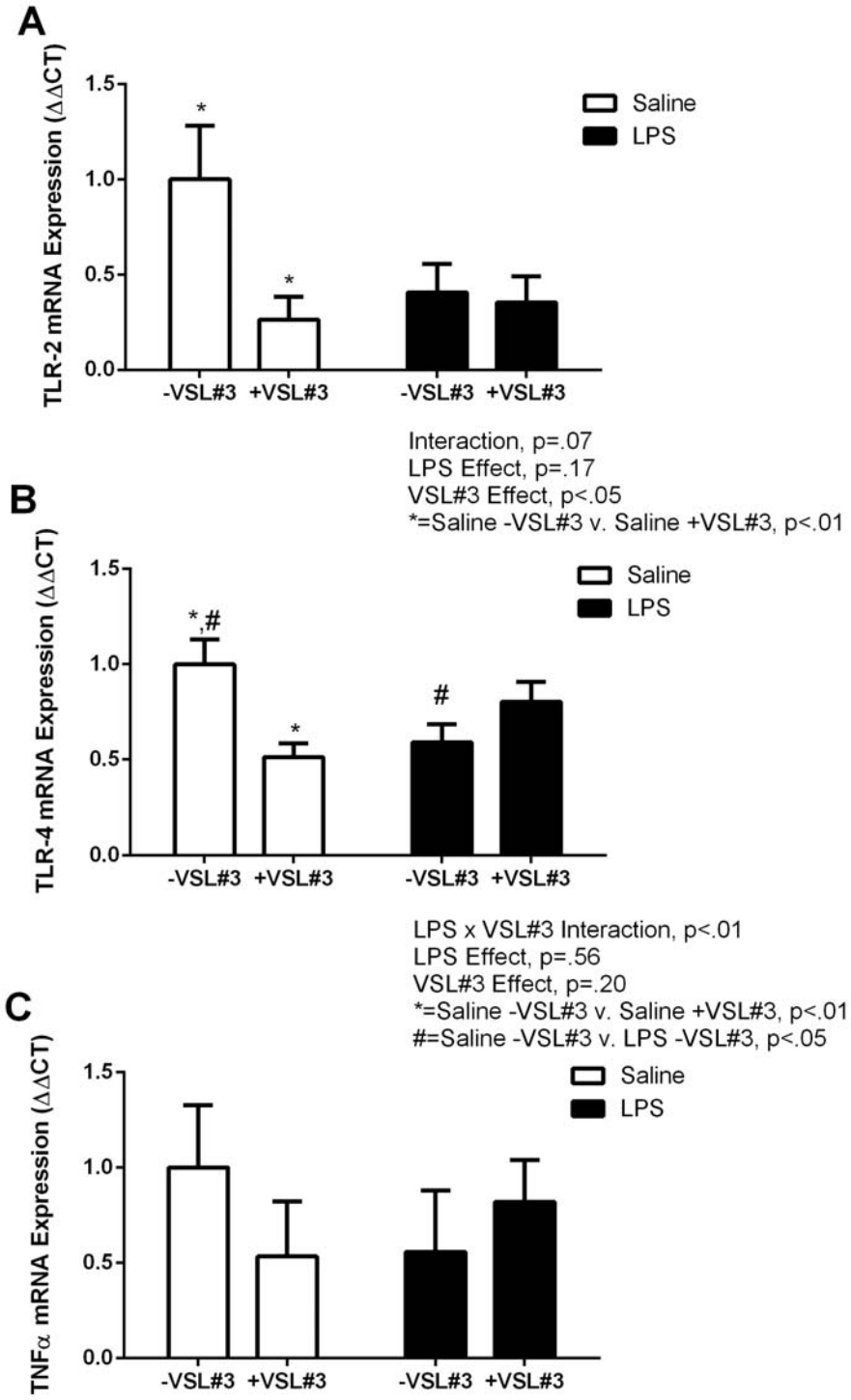


Figure 7. mRNA expression levels of TLR-2 (A), TLR-4 (B), and TNFα (C) in red skeletal muscle of mice injected with saline or low-dose LPS (0.1 μg/kg BW) after 4 weeks with or without VSL#3 supplementation (n=8-10). Expression levels are corrected to β-actin.

Markers of Inflammation in Liver. TNFα (Figure 8A) and IL-10 (Figure 8B) expression levels were significantly reduced in saline+VSL#3 mice compared to LPS+VSL#3 mice. Levels of expression between saline-VSL#3 and LPS-VSL#3 mice were not significantly different. There were no differences within the groups that received saline or within the groups that received LPS.

Figure 8

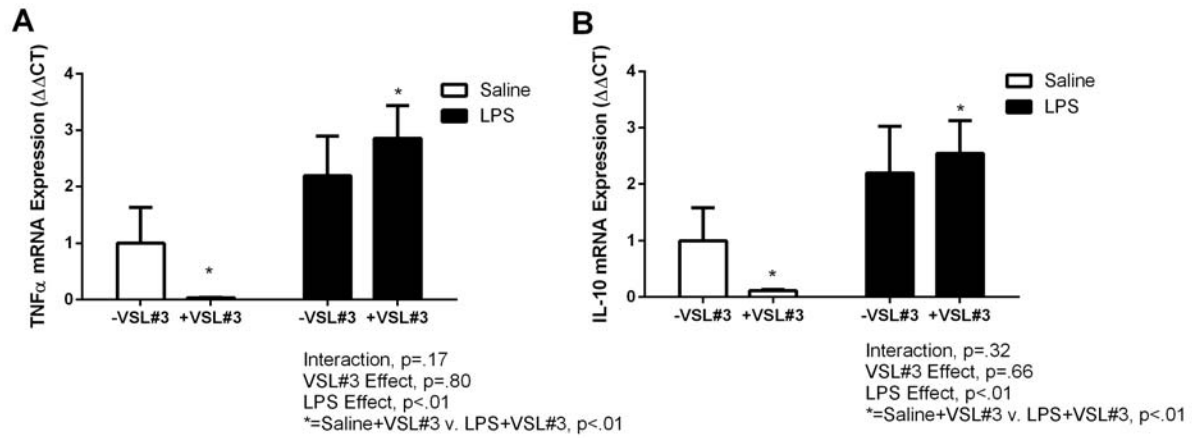


Figure 8. mRNA expression levels of TNFα (A) and IL-10 (B) in liver of mice injected with saline or low-dose LPS (0.1 μg/kg BW) after 4 weeks with or without VSL#3 supplementation (n=7-10). Expression levels are corrected to β-actin.

6.5 Discussion

The results of this study are novel as it is the first to utilize VSL#3 as an intervention against metabolic endotoxemia. They are indeed in agreement with previous data regarding the use of probiotics to reduce inflammation [86, 118, 120-122]. Notably, VSL#3 supplementation reduced baseline mRNA expression levels of TLR-2 and TLR-4 in red skeletal muscle, and TNFα in white skeletal muscle. Additionally, while VSL#3 supplementation did not abrogate the inflammatory effects of LPS injection in the liver, there was a greater difference in TNFα and IL-10 expression between the saline- and LPS-injected mice receiving VSL#3 compared to those that did not receive probiotic supplementation. These findings could indicate increased activation of the immune system with VSL#3 probiotics.

VSL#3 supplementation resulted in significantly reduced mRNA expression levels of TLR-2 and TLR-4 in red muscle and TNFα in the white muscle of saline-injected mice. Interestingly, in mice not receiving VSL#3 supplementation, a low-dose LPS injection did not increase mRNA expression of TLR-4 or TNFα compared to saline-injected mice given VSL#3 supplementation. Given our low dosage and time course, it is difficult to ascertain whether this could be due LPS-induced downregulation of these receptors or a latent decrease in mRNA expression in response to elevated protein levels. Other studies have shown that various forms of LPS administration can reduce TLR-4 protein levels in adipose tissue while eliciting only minor changes in TLR-2 or TLR-4 in skeletal muscle [47, 123]. Regardless, it can be extrapolated that VSL#3 probiotic supplementation may have similar effects on the immune system as an acute low-dose LPS challenge, which may be beneficial. Studies have shown that cells treated with low doses of LPS (1μg/mL) can become endotoxin resistant, leading to reduced inflammatory responses to subsequent LPS challenges [124]. Alternatively, the lipoteichoic acid or peptidoglycan found on the gram-positive bacteria in VSL#3 could have entered circulation and activated TLR-2, causing release of TNFα and NFκB in a similar fashion to TLR-4 after LPS treatment [125]. By activating the immune system, VSL#3 supplementation could reduce the inflammatory response to metabolic endotoxemia.

The liver is a main site of LPS clearance [126]. There, it is taken up primarily by Kupffer cells which release inflammatory cytokines such as TNFα and interleukins. Our results illustrate that the low-dose LPS injection resulted in a significant increase in TNFα as well as the anti-inflammatory cytokine IL-10 which is activated in Kupffer cells to abrogate inflammatory responses [127]. However, in contrast to previous studies, VSL#3 supplementation did not reduce LPS-induced liver inflammation. Interestingly, mice who received VSL#3

supplementation exhibited a greater difference in TNFα and IL-10 mRNA expression between saline and LPS conditions compared to mice who did not receive the probiotic. This supports our theory that basal levels of inflammation may have been lower with VSL#3 supplementation. The elevated inflammatory response in the liver also indicates that LPS may have been cleared by the four-hour time point at which the tissues were collected, which may explain the lack of LPS effect in skeletal muscle metabolism and mitochondrial biogenesis.

The 4-hour low-dose LPS treatment elicited no changes in skeletal muscle metabolism or mitochondrial biogenesis. Previous studies have shown that a low-dose LPS challenge reduced fatty acid uptake and oxidation in both cell culture and animal models [5, 128]. This is a limitation of the current study because it indicates that the LPS dose may not have been sufficient to induce metabolic dysregulation, therefore we could not observe VSL#3 as a potential protective intervention. Similarly, previous studies have shown increased mitochondrial biogenesis after a high dose of LPS, but we did not observe this, perhaps due to the low dosage administered [129, 130]. The lack of metabolic effects indicates that the model may have better represented the acute endotoxemia that may follow a high-fat meal rather than the chronic metabolic endotoxemia linked to insulin resistance and obesity [24, 41].

The original purpose of this study was to determine whether VSL#3 supplementation could protect against the deleterious effects of an LPS challenge modelling metabolic endotoxemia during high-fat feeding. However, due to the lack of diet effect in any parameter, the diet factor was removed in order to simplify the model. Therefore, the study examined instead the potential protective effects of VSL#3 against an acute, low-dose LPS challenge modelling acute metabolic endotoxemia. Our results showed that VSL#3 supplementation may reduce basal inflammatory tone in skeletal muscle, which could protect against metabolic dysregulation associated with

chronic inflammation caused by metabolic endotoxemia.

CHAPTER VI: IMPLICATIONS & FUTURE DIRECTIONS

High-fat diets and obesity have been linked to metabolic endotoxemia and metabolic dysregulation characterized by the inability of skeletal muscle to utilize appropriate substrate [5, 24]. Rates of obesity have reached epidemic proportions while obesity-related medical costs are estimated to reach over $209 billion annually [131]. The gut microbiome has been shown to influence fatty acid uptake, oxidation, and synthesis, as well as adipogenesis and lipogenesis [11, 65, 87, 99]. In both humans and mice, an obesogenic bacterial profile has been identified, and the term ‘dysbiosis’ now refers to the overabundance of non-beneficial bacteria in conjunction with a loss of bacterial variety and a predisposition to increased energy harvesting from the diet [4]. Recent discoveries showing the link between high-fat diets, obesity and the gut microbiome have led to new studies examining the potential role of probiotics in preventing or treating diet-induced obesity. Probiotic supplementation is a non-invasive, dietary intervention that has been safely used in children and adults with some promising results.

The current study tested whether supplementation with VSL#3, a multi-strain probiotic, would prevent inflammation, metabolic inflexibility, and loss of intestinal tight junction proteins during high-fat feeding or metabolic endotoxemia. This was the first study to utilize VSL#3 as a dietary intervention during high-fat feeding or metabolic endotoxemia, so the results are novel, but they are largely in agreement with previous studies utilizing single or double probiotic strains during high-fat feeding. Overall, these results demonstrate that VSL#3 supplementation 1) significantly lowered fasting blood glucose, 2) increased FIAF and decreased GLP-2 expression during high-fat feeding, and 3) reduced basal expression levels of TLR-2, TLR-4 and TNFα in skeletal muscle. VSL#3 supplementation was not shown to protect against an increase in percent body fat during high-fat feeding or LPS-induced inflammation in skeletal muscle or liver. In this model, 4

weeks of high-fat feeding did not induce metabolic inflexibility in skeletal muscle, so potential protection through VSL#3 supplementation could not be observed.

Because of the intervention design of this study, mechanisms of influence were examined in breadth but not depth, opening a great number of avenues for future directions. The major limitation of this study was due to the short feeding protocol, so future studies aiming to intervene in a model of high-fat diet-induced metabolic dysregulation should extend their protocol until metabolic dysregulation is observed through glucose or insulin tolerance tests. In addition, future studies aiming to utilize VSL#3 in models of metabolic endotoxemia should measure plasma endotoxin and markers of inflammation in living mice at regular intervals post-injection to determine peak inflammatory response and LPS clearance rates. This would ensure a model of metabolic endotoxemia rather than a latent inflammatory response.

Additionally, mechanistic studies must be performed to determine which pathways are being affected by VSL#3 supplementation. A decrease in 12-hour fasting blood glucose indicates that supplementation has chronic effects, perhaps at the level of the liver where glycogenolysis and gluconeogenesis take place during a fasted state to maintain euglycemia. Recently, Kim, et al. illustrated decreased expression of genes that regulate gluconeogenesis in addition to increased GLUT4 expression after supplementation with a single-strain probiotic [65]. Future studies should measure changes in these regulatory genes after supplementation with VSL#3 to determine mechanistic action. In the current study, VSL#3 supplementation increased FIAF mRNA expression and decreased GLP-2 receptor mRNA expression, findings that are complementary to other studies that have shown an increase in circulating FIAF and GLP-2 [60, 87]. Future studies using VSL#3 supplementation should quantify the protein levels of these genes to determine their activity. Similarly, mRNA expression levels of TLR-2, TLR-4 and

TNFa were reduced, but this may or may not translate to reductions in protein levels, and that in turn does not necessarily translate to the inflammatory state. A previous study by Raso, et al. illustrated a simultaneous decrease in mRNA expression and protein levels of TLR-4 after intervention with a single-strain probiotic and prebiotic fibers [86]. In order to better determine the inflammatory state, future studies to measure levels of membrane-bound TLR-2 and TLR-4 and nuclear TNFα.

VSL#3 has been safely used in many clinical trials to treat inflammation during inflammatory bowel diseases and steatotic liver diseases. Here, we have shown that VSL#3 supplementation can also reduce fasting hyperglycemia and insulin resistance during high-fat feeding while enhancing nutrient sensing in the gut in a murine model. Given the limited but compelling data showing the anti-inflammatory and protective mechanisms of VSL#3 during high-fat feeding, future studies should explore its mechanisms and potential influence in preventing diet-induced obesity and its co-morbidities. VSL#3 supplementation could serve as a non-invasive method for combating the obesity epidemic.

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