Effect of a Probiotic Supplement on Insulin Sensitivity and Skeletal Muscle Substrate Oxidation during High Fat Feeding.

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Dissertation submitted to the faculty of Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

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July 2, 2014

Blacksburg, VA

Keywords: Probiotic, High Fat Diet, Insulin Resistance, Substrate Oxidation

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Background: Modifying the gut microbiota through the administration of probiotics during high fat feeding has been shown to attenuate weight gain and body fat accretion while improving insulin sensitivity in animal models.

Objective: Our objective was to determine the effects of the probiotic VSL#3 on body weightand composition, skeletal muscle substrate oxidation, and insulin sensitivity and during 4 weeks of high-fat, hypercaloric feeding. We hypothesized that the probiotic would attenuate the body weight and fat gain and adverse changes in insulin sensitivity and substrate oxidation following high fat, hypercaloric feeding in young, non-obese males.

Methods: Twenty non-obese males (18-30 y) volunteered to participate in the present study. Following a 2-week eucaloric control diet, subjects underwent a dual x-ray absorptiometry (DXA) to determine body composition, an intravenous glucose tolerance test (IVGTT) to determine insulin sensitivity, a skeletal muscle biopsy for measurement of substrate oxidation. Serum endotoxin was also measured. Subsequently, subjects were randomized to receive either VSL#3 (2 satchets) or placebo during 4 weeks of consuming a high fat (55% fat), hypercaloric diet (+1,000 kcal/day). Macronutrient composition of the high fat diet was 55% fat, 30% carbohydrate, and 15% protein.

Results: There were no differences between the groups in subject characteristics or in the dependent variables at baseline. Body weight and fat mass increased less ($P<0.045$) following the high fat diet with VSL#3 compared to placebo. Insulin sensitivity (and other IVGTT variables) and both glucose and fat oxidation did not change significantly with time or VSL#3 treatment. Serum endotoxin concentration was not different between groups following the high-fat diet.

Conclusions: VSL#3, a multi-strain probiotic, attenuated body weight and fat gain following a 4-week high fat, hypercaloric diet compared with a placebo. There were no differences between the VSL and control in circulating endotoxin, insulin sensitivity (and other IVGTT variables) or in skeletal muscle substrate oxidation.

Key words: Probiotic, gut microbiota, high-fat feeding, endotoxin

ACKNOWLEDGEMENTS

I'm very grateful to Kevin and Brenda Davy for encouraging me to start this journey and to each of my committee members for their inspiration, support, and guidance throughout the process. I'm also indebted to the students and staff within the Davy / Hulver/ Frisard laboratories for their help with this study. It wouldn't have happened without a strong team. Finally, I'm thankful for my family – Lon, Maddie, Rusty, and Molly for their eternal optimism, steadfast friendship, and unconditional love. Thank you for being my soft place to fall.

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CHAPTER I

Introduction

Nearly 10% of the global population [1] and more than one third of adults in the United States [2] are obese. If the current trends continue, it is projected that 51% of the US population will be obese by the year 2030 [3]. Obesity-associated risks include osteoarthritis, cancer, cardiovascular disease, and Type 2 Diabetes Mellitus (T2DM). Statistics from the American Diabetes Association report that 8.3% of the US population has been diagnosed with diabetes estimating that another 7 million people are undiagnosed and 79 million people have prediabetes [4]. The economic burden of obesity and diabetes is substantial with the total estimated costs in the US of $245 billion including $176 billion in direct medical costs and $69 billion in reduced productivity [4]. As the population ages, the prevalence of diabetes and the associated costs will only continue to rise unless effective strategies are implemented. Though lifestyle changes promoting weight loss through diet and exercise are highly effective, few people experience long-term success. Results from the LOOK AHEAD trial found that intensive lifestyle modification resulted in, on average, a modest 6% weight loss and 7.3% T2DM remission after 4 years [5].

A growing body of evidence suggests that the gut microbiota may be an effective target for the treatment of obesity-related conditions. The term “dysbiosis” has recently been defined as “any change to the composition of resident commensal communities relative to the community

found in healthy individuals" [6]. In the last decade, animal [7-9] and human studies [10, 11] indicate that obesity is associated with alterations in gut microbial communities. Animal experiments have revealed that ob/ob mice and high fat diet-induced obese mice have changes in gut microbiota which are associated with increased gut permeability, higher levels of circulating lipopolysaccharide (LPS), and decreased insulin sensitivity [8]. Humans with T2DM also have altered gut microbiota [12-14], increased gut permeability [15, 16] and higher circulating endotoxin [17, 18]. Altering the gut microbiota by the administration of prebiotics [9, 19, 20] has been shown to ameliorate these negative consequences in animal models, however human studies have not been as clear. Accumulating evidence supporting the potential benefits warrant further investigation.

Though obesity is an established risk factor for the development of T2DM, research over the last 20 years indicates there are multiple mechanisms at work. A Western diet, high in fat and calories, can cause obesity, but can also increase gut permeability and circulating LPS [21]. Excessive adipose tissue [22] and LPS [23] can each cause inflammation, while LPS [24, 25], inflammation [26] and high circulating fatty acids [27] independently lead to insulin resistance. The following literature review will evaluate the mechanisms by which obesity, high fat diets, and inflammation can disrupt insulin signaling and lead to the development of T2DM. Targeting the gut microbiota may be a cost-effective and simple strategy to reduce the risk and severity of T2DM.

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CHAPTER II

Review of Literature

A. Obesity, Inflammation, and T2DM

A-1. Adipose tissue and inflammation:

The discovery in the 1990's that adipose tissue secretes cytokines such as tumor necrosis factor-alpha (TNF-α) [1] and hormones such as leptin [2] provided insight into the mechanisms by which obesity is a risk factor for cardiovascular disease and T2DM. The relationship between over nutrition, adipose tissue expansion, macrophage infiltration, and adipokine production helps to explain, at least in part, the development of T2DM in some obese individuals.

Adipose tissue, in particular adipose stored in the viscera (VAT), secretes a number of peptides that have paracrine and endocrine actions. Adipose tissue expansion produces an abundance of inflammatory cytokines contributing to the low-grade inflammation often associated with excess adiposity. These adipokines influence energy balance [3], vascular health [3], and insulin signaling [4-7].

Adipocyte number is believed to be established in early adolescence and the expansion of adipose tissue in adulthood is due to increased size of the adipose cells [8]. This is notable because the secretion of several proinflammatory cytokines is elevated in very large adipocytes compared to smaller cells [9]. Furthermore, the frequency of adipocyte death is positively correlated with increased adipocyte size [10]. An estimated 40% of adipose tissue in obese

humans is infiltrated with macrophages [11] which accumulate around dead adipocytes in crown-like structures (CLS) [10, 12, 13]. Although there are likely several mechanisms by which macrophages hone to adipocytes, chronic hypoxia and cellular necrosis is believed to be the primary mechanism [14]. Macrophages accumulate in VAT in response to increased chemokines and cytokines released from adipocytes and other immune cells [15] and also secrete a host of inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) [16]. Greater expression of these cytokines from visceral adipose tissue is related to higher fasting glucose and impaired insulin action [17].

A-2. *Inflammation and Insulin sensitivity:*

Acute [18] and chronic [19] inflammation can cause insulin resistance through the disruption of the insulin signaling pathway. The discovery that TNF-α was elevated in several rodent models of obesity and diabetes was the first to link inflammation with these conditions [1, 19]. When TNF-α was neutralized in these animals, glucose uptake and insulin action was significantly improved [1]. Subsequently, others have confirmed that TNF-α impairs insulin signaling and reduces insulin sensitivity in animal models [20, 21] and numerous studies have found that TNF-α is elevated in obese humans [19, 22-24]. Insulin resistance in adipose tissue may develop through the release of TNF-α by macrophages within the CLS [1]. Though the mechanism in which TNF-α interferes with insulin signaling is not entirely clear, it is likely through a defect in the phosphorylation of the insulin receptor. Hotamisligil et al. [25] found that there were no differences in GLUT-4 mRNA expression between TNF-α knockout mice compared to wild type mice. There was, however, a 2-fold increase in insulin-stimulated tyrosine phosphorylation of

the insulin receptor in the muscle and adipose tissue of the knockout mice [25]. Other mechanisms include increased serine activation of IRS-1, activation of extracellular signal-regulated kinase 1 / 2 (ERK 1 / 2), JNK, and increased transcription of NFκB [26].

IL-6 receptor belongs to a class 1 family of cytokine receptors that use the Janus kinases (JAK) as an intracellular signaling pathway. IL-6 is released by a number of tissues including endothelial cells, fibroblasts, skeletal muscle and adipocytes. Although IL-6 is released from subcutaneous fat depots [27], 2-3 fold more IL-6 is released from visceral stores [28] and may be one of the links between visceral adiposity, inflammation, and insulin resistance. In extremely obese individuals (BMI > 50 kg/m^2), IL-6 was found to be 50% higher in the portal circulation compared to the radial artery [29]. This elevation in IL-6 was directly correlated to systemic C-reactive protein (CRP) concentrations. Serum concentrations of highly sensitive-CRP (hs-CRP) has been found to be significantly higher in individuals with impaired glucose tolerance and T2DM [30, 31]. Although IL-6 is thought to be an inflammatory cytokine impairing insulin signaling [32], it can also have insulin sensitizing effects [33]. IL-6 appears to potentiate the action of insulin acutely (in response to exercise), however, chronic exposure contributes to insulin resistance [33, 34]. With increased IL-6 production, there is a corresponding increase in the expression of suppressor of cytokine signaling (SOCS)-3, a protein that binds to and inhibits the insulin receptor and targets IRS proteins for degradation [35].

Resistin and adiponectin are both associated with glucose disposal with resistin decreasing [36, 37] and adiponectin increasing [38, 39] insulin sensitivity. Circulating levels of resistin are

higher in rodent models of obesity and have been shown to induce insulin resistance [40]. Conversely, adiponectin is reduced in obesity and T2DM [41] with waist circumference strongly and inversely associated with adiponectin concentrations [42]. Adiponectin has potent anti-inflammatory effects inhibiting LDL-oxidation and suppressing superoxide production [43]. A recent study created an index calculated from adiponectin-resistin (AR) and insulin resistance (IR_{AR}) and found it to be a reliable measure of predicting T2DM [44].

Though the evidence supporting a link between excess adiposity, inflammation, and insulin resistance is strong, a number of human studies have found conflicting results [45-47] . A comparison of patients with T2DM and healthy, age-matched subjects found that IL-6 concentrations were related to fat mass but not insulin sensitivity. Further, when the data was corrected for BMI, neither IL-6 nor TNF-α was related to glucose disposal rate [46]. Conversely, Daniele [45] found that inflammatory score calculated from plasma cytokines was predictive of fasting glucose and insulin resistance, however, this score did not correlate to BMI or fat mass. Moreover, when overweight individuals lost approximately 10% of their body mass there was no change in TNF-α, IL-6, or adiponectin despite a significant improvement in insulin sensitivity [47]. It is likely that there is a complex relationship between adipocytes, cytokines, and metabolic state, all of which interact to produce metabolic perturbations.

B. Gut Microbiota, Obesity, and T2DM

Commensal bacteria in the gut outnumber the cells in the human body by a factor of 10 and perform a wide variety of important metabolic and biologic functions. Several factors influence the composition of the microbes present in the gastro-intestinal tract including genetics [48, 49] geographic location [50, 51], age [52], antibiotic use [53], and diet [54]. Growing evidence implicates the microbiome in obesity, diabetes, and cardiovascular disease [55]. The gut microbiota on a phyla level are very similar between individuals with estimates of over 90% coming from Firmicutes and Bacteriodetes [56]. Though not all agree [57, 58], the many show that obesity is associated with increased proportions of Firmicutes and decreased numbers of Bacteriodetes [56, 59-62]. The following chapter reviews the role of the gut microbiota may play in the development of obesity and T2DM.

B-1. Obesity, T2DM, and Dysbiosis:

While a healthy microbiota is marked by high microbial diversity and stability [63], dysbiosis is defined as a reduction in beneficial bacteria (Lactobacilli, Bifidobacteria) and a proliferation of pathogenic bacteria (E. Coli, Proteobacteria) [64]. The consequences of dysbiosis include altered metabolite production and reduced intestinal barrier function [64]. Larsen, et al. [65] found that class Betaproteobacteria were highly enriched in patients with T2DM compared to healthy controls. Likewise, Qin et al. [66] found that T2DM was associated with fewer numbers of butyrate-producing bacteria with greater numbers of E. Coli. Prebiotics increase the populations of Bifidobacteria [67-69] as well as Lactobacilli [70, 71] in both human and animal studies. Prebiotic fibers, when fed in conjunction with a high fat diet, stimulate the

proliferation of Bifidobacteria, improve gut barrier function, and reduce inflammation in obese mice [68, 72, 73]. Whether dysbiosis is a cause or consequence of obesity is not clear. However, targeting the gut microbiota through the administration of prebiotics or probiotics may prove to be effective strategy to reduce cardiometabolic risk.

B-2. Contribution of the Gut Microbiota to Obesity:

The gut microbiome carries out many important functions including energy and nutrient extraction [60, 74]. Germ free mice, mice with no microbiota, are resistant to weight gain when fed a Western-style diet high in fat and calories [75]. Transplantation of the microbiota from conventionalized mice into the germ free mice resulted in significant increases in adiposity and insulin resistance despite reduced food intake [76]. Further, fecal transplant from either obese or lean mice into germ-free resulted in greater weight gain and adiposity from the obese transplant [61].

The evidence that gut microbiota contribute to energy harvesting appears evident, however, it is not clear which bacteria are responsible. Ley, et al [59] compared ob/ob, ob/+, and wild type mice and found that ob/ob mice had significantly more Firmicutes bacteria and 50% less Bacteriodetes with reduced bacterial diversity compared to wild-type [59]. Additionally, Ob/ob mice lacking the gene for leptin have a greater number of Firmicutes compared to Bacteriodetes and display a greater number of enzymes capable of degrading indigestible carbohydrate [61]. These mice also have lower fecal energy loss demonstrating an ability to absorb and utilize more dietary carbohydrate [77]. A short-term feeding study in lean and

obese adults found that overfeeding lean subjects resulted in a 20% increase in Firmicutes bacteria and a corresponding 150 kcal of energy extraction [60]. Firmicutes bacteria produce short-chain fatty acids which may increase the energy available for absorption. Overweight and obese humans had a higher Firmicutes : Bacteriodetes ratio as well as a greater number of short-chain-fatty acids in their feces compared to lean [78]. However, animal studies have not found increased energy harvest despite similar shifts in gut microbiota and increased fecal SCFA [79]. The extent to which the energy-harvesting capabilities of the microbiota contribute to energy balance is unknown but studies in germ-free mice provide some mechanisms by which the microbiota may contribute to lipid metabolism and adiposity.

Bäckhed, et al, [75, 76, 80] have conducted a series of experiments uncovering the mechanisms by which gut microbiota increases lipid storage in mice. Germ-free mice have high levels of fasting-induced adipocyte factor (FIAF), an inhibitor of lipoprotein lipase (LPL). In contrast, conventionalized mice have suppressed (FIAF) and increased LPL leading to increased storage of triglyceride and expansion of adipose tissue [76]. Germ free mice also have 40% higher levels of AMPK in skeletal muscle compared to conventionalized mice suggesting that germ-free mice have an increased capacity for fat oxidation compared to conventionalized mice [75]. Although, these studies provide important insight into the mechanisms by which germ-free mice are resistance to weight gain, the translation of these findings to humans remains unexplored.

C. High Fat Diet, Gut Permeability, and Lipopolysaccharide

High fat diets leading to excessive energy intake are strongly linked with the development of obesity. Though the organoleptic properties and satiating effects of fat certainly contribute to overconsumption and energy surplus [81], animal models suggest that a high fat diet also increases intestinal permeability [62] allowing for the translocation of LPS from the intestinal lumen into circulation. LPS is a portion of the cell wall found in gram negative bacteria and through binding its receptor, toll-like receptor-4 (TLR-4), induces an inflammatory response. High levels of circulating LPS has been termed "endotoxemia" and has been implicated as a cause for the low-grade systemic inflammation associated with obesity [82]. Higher levels of circulating LPS have been shown to initiate obesity and insulin resistance in animal models [83] and is positively correlated to insulin resistance [84] and incident diabetes [85] in humans. The following chapter will focus on the link between a high fat diet and gut permeability and the roles that both LPS and fatty acids play in the development of inflammation and insulin resistance.

C-1. LPS, Intestinal Permeability, and Inflammatory Response:

LPS enters circulation in two ways; one of which renders LPS inert, the other results in the stimulation of the innate immune response. High fat diets increase the incorporation of LPS into chylomicrons in the Golgi apparatus of intestinal cells [86]. Once bound to chylomicrons, LPS enters lymphatic circulation and is transferred to lipoproteins (HDL and VLDL) through LPS binding protein (LBP) [87]. LPS has a high affinity for phospholipids [88] and when bound to

lipoproteins is less likely to stimulate the immune response as the lipid A portion is sequestered within the lipoprotein micelle [89].

The secondary route passes LPS through the intestinal epithelium. High fat diets [62, 90, 91] and T2DM [92] increase intestinal permeability allowing LPS to pass directly into circulation [62, 82]. This results in "free" LPS and is found in the plasma as LPS aggregates, bacterial membrane fragments, or loosely bound to albumin, soluble CD14 (sCD14) or other proteins [89]. TLR-4 receptors are widely expressed [93] and the binding of LPS induces the transcription of NFκ-B, increasing the gene expression of inflammatory cytokines, specifically TNF-α and IL-1β [94, 95] .

Animal experiments have shown that a high fat diet increases the proportion of gram negative bacteria in the gut and raises plasma LPS concentrations [83, 96]. Subcutaneous infusion of LPS in normal weight mice resulted in increased fat mass and similar disturbances in glycemic control as the high fat diet [83]. Interestingly, TLR-4 knockout mice do not develop intestinal permeability, inflammation or peripheral insulin resistance in response to a high fat diet [82, 96-98] but may not be immune to hepatic insulin resistance [100]. Though LPS is the primary ligand for TLR-4, some evidence suggests that TLR-4 receptors may also be activated by saturated fatty acids [101, 102], however, these findings are controversial [103].

Improving metabolic health by enhancing intestinal barrier function and reducing plasma LPS has shown promise. Patients with T2DM have increased gut permeability [92] and higher plasma LPS [84, 104, 105]. When mice are fed prebiotic fibers in conjunction with a high fat

diet, plasma LPS, inflammation, and insulin resistance are attenuated. These changes were attributed to increased levels of glucagon-like peptide-1 (GLP-1) and greater protein synthesis of intestinal tight junction proteins (occluden and ZO-1) [68, 90]. Prebiotics have also been shown to decrease plasma LPS and inflammatory markers, and improve glucose control in women with T2DM [106, 107]. Though these findings are promising, other mechanisms associated with insulin resistance in obesity including ectopic fat accumulation and substrate oxidation require closer investigation.

D. High Fat Diet, Obesity, and Insulin Resistance

D.1. High Fat Diet and Insulin Resistance:

High fat, hypercaloric diets increase the concentration of free fatty acids in circulation. Most excess fat is stored in white adipose tissue, however, in the face of dietary fat overload, other organs and tissues become ectopic storage depots. These tissues are not well suited for fat accumulation and some hypothesize that the resulting lipotoxicity may underlie insulin resistance and T2DM [108]. In support of this hypothesis is evidence showing that intramuscular lipid (IMCL) content is elevated in obesity [109-111] and associated with insulin resistance [112-114]. The balance of the chapter will focus on the problems associated with impaired fatty acid oxidation in obesity and altered insulin signaling associated with the incomplete oxidation of IMCL.

D-2. Substrate Oxidation in Obesity

Whole-body lipid oxidation under resting conditions is important for the maintenance of body weight. High resting respiratory exchange ratio (RER), indicative of glucose rather than fat oxidation, has been found to be a predictor of weight gain [115]. The ability of the muscle to switch from oxidizing fatty acids to glucose and back again has been termed "metabolic flexibility" [116]. In the early 1960's, Philip Randle hypothesized that insulin resistance in obesity and Type 2 diabetes was due to increased fat oxidation in skeletal muscle which reduced oxidation of glucose resulting in hyperglycemia [117].

More recent findings that IMCL is elevated in obesity and T2DM indicate that lipid oxidation may actually be reduced in this population [107, 116]. Though not all have found differences in intramyocellular lipid content between obese and T2DM [118, 119], the presence of IMCL in skeletal muscle is most likely not as problematic as the ability to oxidize lipid. This is evidenced by the high IMCL in endurance trained athletes who remain highly insulin sensitive [120]. A comparison study found that the myotubes of physically active subjects had greater lipid content compared to lean sedentary or subjects with T2DM and lipid content was inversely correlated with insulin sensitivity [121]. Diacylglycerol (DAG) and ceramide were inversely correlated with insulin sensitivity [121]. The specific mechanisms by which these intermediates disrupt insulin signaling will be discussed at the end of this chapter.

The potential for IMCL to be oxidized is dependent upon the muscle's oxidative capacity. Type 1 fibers have high mitochondrial density and oxidative capacity [122] and predominate in lean,

endurance-trained athletes while some have reported a lower percentage of Type 1 fibers in obese individuals [123]. A comparison between lean, obese, and type 2 diabetic subjects, found that lean individuals had significantly greater oxidative enzyme capabilities whereas obese and diabetic subjects had greater lipid accumulation [110]. Hulver, et al found fatty acid oxidation in the skeletal muscle of extremely obese subjects was substantially lower compared to normal weight or mildly obese subjects [124].

Though a number of studies have found reduced skeletal muscle fat oxidation in obesity [110, 124, 125] and T2DM [126], it is not clear if this is due to decreased mitochondrial density or diminished oxidative capacity [127]. Studies in insulin resistant first-degree relatives of diabetes patients have shown reduced oxidative capacity [128] while a 38% lower mitochondrial density was found in insulin resistant children of T2DM parents compared to healthy controls [129]. Though family history is a risk factor for the development of T2DM, many believe that environmental factors, most notably low aerobic capacity [130, 131], remain a major contributor to the development of mitochondrial defects and insulin resistance [127]. Obese, insulin resistant women who underwent a 12-week endurance training program, significantly increased mitochondrial oxidative capacity despite no change in body weight. Further, IMCL within the muscle was relocated increasing the percentage of IMCL in direct contact with mitochondria [132]. Similarly, a 12-week training study in men with T2DM improved glucose disposal and metabolic flexibility despite increased IMCL [133]. Conversely, ten days of endurance training in lean, obese, and T2DM women was not enough to increase oxidative capacity in any of the groups but did lower IMCL in the women with T2DM [134].

D.3. Intramyocellular Lipid and Insulin Signaling:

High fat diets and the accumulation of IMCL in skeletal muscle may disrupt insulin signaling in a number of ways including lipid intermediates (fatty acyl-CoAs, DAGs, and ceramide) and oxidative stress. DAGs activate a number of protein kinase C (PKC) isoforms which are serine/threonine kinases [135]. Phosphorylation of the insulin receptor with serine induces a conformational change in the receptor inhibiting tyrosine phosphorylation. IRS-1 is also serine-phosphorylated by PKC at a number of residues [136] which may lead to the dissociation of IR/IRS-1 and/or IRS-1/PI3 kinase, preventing PI3 kinase activity [137]. Inhibition of IRS-1 decreases insulin action and GLUT-4 translocation. Though an inverse relationship has been found between DAG and insulin sensitivity , not all have found higher concentrations of DAG in the skeletal muscle of obese subjects [138].

Ceramides are generated either from de novo synthesis involving two saturated fatty acids and serine [139] or by the action of sphingomyelinase which hydrolyzes sphingomyelin, a phospholipid found in the cell membrane [140]. Enzymes involved in de novo ceramide synthesis are induced by inflammatory events which, as mentioned earlier, are upregulated in obesity [141]. Ceremades have been found to disrupt insulin signaling in both skeletal muscle and adipose tissue [142]. The accumulation of these products induces lipotoxicity and, like DAG, can disrupt insulin signaling by serine phosphorylation of insulin receptor and insulin receptor substrate (IRS)-1 [143]. In vitro, ceramide has also been found to inhibit the phosphorylation of Akt causing reduced insulin signaling [144].

A high fat diet has been shown to cause mitochondrial DNA damage [145], endoplasmic reticulum stress [146], and induce mitochondrial oxidative stress [147]. Though strong evidence suggests that production of reactive oxygen species (ROS) is associated with insulin resistance, the mechanisms have not been clearly elucidated. Hydrogen peroxide (H_2O_2) is a key signaling molecule and is involved in many redox signaling pathways [148]. Anderson, et al has shown that a high fat diet increases the potential of the mitochondria to release H_2O_2 creating an environment within the cell that more oxidized [147]. Attenuating the production of H_2O_2 with antioxidants may preserve insulin sensitivity despite a high fat intake [147]. Many kinases involved in insulin signaling are redox-sensitive including several serine/threonine kinases [149] such as ERK1, JNK-1, inhibitor of NF-κB kinase β (IKKβ), and p70-S6 kinase 1 (S6K1) [149]. Increasing lipid oxidation and decreasing the accumulation of lipid intermediates through exercise [150] or improving antioxidant status within the cell [147] may potentially improve insulin sensitivity.

E. Probiotics in the Treatment of Insulin Resistance

Probiotics are defined by the World Health Organization as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the consumer.' [151]. The discovery of probiotics as agents of health was first proposed in the 19th century by Elie Metchnikoff, known as the "Father of Probiotics" who linked the well-being of Bulgarian populations to their consumption of fermented milk products [152]. Since that time, probiotic strains, specifically Lactobacillus and Bifodobacterium species, have been shown to influence the gut microbiota in a number of ways including the improvement of gut barrier integrity

through the increase in tight junction proteins [153], reduction in pathogenic bacteria [154] ,as well as reduced intestinal inflammation [155].

As mentioned previously, obesity is associated with dysbiosis of the gut microbiota and patients with T2DM have also been found to have altered gut microbial communities [65, 66, 156]. Obese and diabetic animal models show that altering the gut microbiota by increasing bifidogenic bacteria improves gut barrier function [68, 90], reduces circulating LPS [83], and improves insulin sensitivity [62, 157]. VSL#3, a commercial multispecies probiotic, was found to attenuate body weight gain and insulin resistance in mice fed a high fat diet. This effect was found to be associated with an increase in butyrate production and GLP-1 secretion leading to improvements in gut barrier integrity [158]. Similarly, oral doses of Lactobacillus casei Shirota improved glucose handling and decreased lipopolysaccharide binding protein (LBP) [159] and Lactobacillus rhamnosus GG improved insulin sensitivity by increasing adiponectin and reducing lipid accumulation in adipocytes [160] in mice.

Despite these positive findings in rodents, the effect of probiotics on intestinal permeability, inflammation, and insulin sensitivity in humans have, so far, been equivocal. Diabetic patients receiving a multi-species probiotic supplement 8 weeks had a lower rise in fasting plasma glucose, a decrease in high-sensitivity C-reactive protein (hs-CRP) and an increase in plasma glutathione (GSH) [161], compared to placebo. Healthy overweight adults supplementing with VSL#3 for 6 weeks improved insulin sensitivity and decreased hsCRP [162]. Because many of the co-morbidities of obesity and T2DM are associated with inflammation, these findings are of

particular importance. Likewise, obese adults who were being treated with an herbal supplement for weight loss (Bofutsushasan) were randomized to receive a probiotic or placebo. Though no differences in body composition or metabolic markers were found, Bifidobacterium breve was negatively correlated with LPS level [163] perhaps indicating an improvement in gut barrier function. Conversely, patients with metabolic syndrome were compared to healthy controls for measures of gut permeability, circulating LPS, lipopolysaccharide-binding protein, and sCD14. Though gut permeability was significantly greater in subjects with metabolic syndrome, supplementation for 3 months with L casei Shirota did not change intestinal permeability, inflammation, endothelial function, or insulin sensitivity [92, 164]. Likewise, obese adolescents supplemented with Lactobacillus salivarius for 12 weeks had no changes in any cardiovascular, metabolic, or inflammatory measure [165], despite alterations to gut microbial groups [166].

The promising results of animal research have not, so far, translated to humans. One possibility is that the dietary composition of the animals is tightly controlled, however, none of the human studies mentioned were controlled feeding studies and instead added a probiotic to the existing diet. While practical, it may allow a great deal of variation between subjects masking potential differences Other inconsistencies lie in the strain or strains of probiotic administered, the dosages given, and the length of treatment, all potential confounders in the interpretation of the data.

F. Summary

Low-grade inflammation is thought to underlie many of the co-morbidities associated with obesity including cardiovascular disease and T2DM. Research in the last decade has elucidated two sources of inflammatory cytokines; the adipose tissue, and circulating LPS. Adipose tissue expansion attracts M1 macrophages which release inflammatory cytokines such as TNF-α, IL-6, and IL-1β. LPS binds to TLR-4 receptors, stimulates the transcription of NFκB, and the induction of many of the same inflammatory cytokines. These cytokines are all capable of interfering with insulin signaling through disruption of the IR and IRS-1 and inhibiting GLUT-4 translocation. High fat diets can lead to obesity, expansion of adipose tissue, and increased circulating LPS but can also impair insulin signaling though accumulation in liver and skeletal muscle. Studies in obese and high-fat fed rodents have shown that targeting the gut microbiota reduces adipose tissue accumulation, circulating LPS, and insulin resistance. Altering the gut microbiome through the administration of pre and probiotics may be a safe and low-cost way to reduce the risk or severity of cardiometabolic disease in humans, however more research is needed to determine the efficacy of these treatments.

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Chapter III: Probiotic Supplementation Attenuates Increases in Body Mass and Fat Mass during High Fat Feeding in Humans.

Abstract

Background: Modifying the gut microbiota through the administration of probiotics during high fat feeding has been shown to attenuate weight gain and body fat accretion while improving insulin sensitivity in animal models.

Objective: Our objective was to determine the effects of the probiotic VSL#3 on body weightand composition, skeletal muscle substrate oxidation, and insulin sensitivity and during 4 weeks of high-fat, hypercaloric feeding. We hypothesized that the probiotic would attenuate the body weight and fat gain and adverse changes in insulin sensitivity and substrate oxidation following high fat, hypercaloric feeding in young, non-obese males.

Methods: Twenty non-obese males (18-30 y) volunteered to participate in the present study. Following a 2-week eucaloric control diet, subjects underwent a dual x-ray absorptiometry (DXA) to determine body composition, an intravenous glucose tolerance test (IVGTT) to determine insulin sensitivity, a skeletal muscle biopsy for measurement of substrate oxidation. Serum endotoxin was also measured. Subsequently, subjects were randomized to receive either VSL#3 (2 satchets) or placebo during 4 weeks of consuming a high fat (55% fat), hypercaloric diet (+1,000 kcal/day). Macronutrient composition of the high fat diet was 55% fat, 30% carbohydrate, and 15% protein.

Results: There were no differences between the groups in subject characteristics or in the dependent variables at baseline. Body weight and fat mass increased less ($P<0.045$) following the high fat diet was less with VSL#3 compared to placebo. Insulin sensitivity (and other IVGTT variables) and both glucose and fat oxidation did not change significantly with time or VSL#3 treatment. Serum endotoxin concentration was not different between groups following the high-fat diet.

Conclusions: VSL#3, a multi-strain probiotic, attenuated body weight and fat gain following a 4-week high fat, hypercaloric diet compared with a placebo. There were no differences between the VSL and control in circulating endotoxin, insulin sensitivity (and other IVGTT variables) or in skeletal muscle substrate oxidation.

Key words: Probiotic, gut microbiota, high-fat feeding, endotoxin

INTRODUCTION

Obesity and type 2 diabetes are associated with dysbiosis of the gut microbiota [1, 2]. High fat diets cause a shift in gut bacterial communities, and circulating endotoxin concentrations [3]. This "metabolic endotoxemia" [3], has been implicated as a cause for the low-grade systemic inflammation observed in obesity. In animal models, elevated circulating endotoxin has been shown to initiate obesity and insulin resistance [4]. In addition, elevated endotoxin concentrations have been are associated with insulin resistance [5] and increase the risk for incident diabetes [6] in humans. Therefore, therapeutic modulation of the gut microbiota may be a promising strategy for prevention and treatment of metabolic diseases.

Probiotic species, such as *Lactobacillus* and *Bifodobacterium*, improve intestinal health by discouraging growth of gram negative bacteria [7], improving gut barrier integrity [8], and reducing intestinal inflammation VSL#3, a commercial multispecies probiotic, has been reported to attenuate body weight gain and insulin resistance in mice fed a high fat diet [9]. However, the relevance of these findings to humans is unclear.

Therefore, we tested the hypothesis that the probiotic VSL#3 would attenuate the adverse effects of high fat feeding on body mass and composition, insulin sensitivity, and skeletal muscle metabolism in healthy, young males.

MATERIALS AND METHODS

Subjects:

Twenty healthy, non-obese males volunteered to participate in this study. Subjects were between 18-40 years of age with a body mass index (BMI) <30 kg/m^2. A thorough health screening ensured that all subjects were free of overt disease and not taking any medications that might impact the dependent variables. Furthermore, none of the subjects had taken antibiotics within the past 6 months, or had a history of gastrointestinal conditions or overt disease. Subjects were also screened for lactose intolerance and food allergies. Subjects were required to meet the following criteria: fasting blood glucose <100 mg/dl, total cholesterol <200 mg/dl, triglycerides <150 mg/dl and resting blood pressure <140 / 90 mmHg. All subjects were sedentary and consumed a diet containing less than 40% of total energy from fat as assessed from a 4-day food record. The Institutional Review Board at Virginia Polytechnic Institute and State University approved the study protocol. The purpose, risks, and benefits of participating were explained to each subject prior to obtaining written consent.

Controlled Feeding:

Diets were planned using Nutrition Data System for Research (NDSR) software (University of Minnesota) and utilized a 7-day rotating menu. Energy requirements were estimated based on height, weight, age, and activity level using the Institutes of Medicine equation [10]. Food modules (250 kcal) with the same macronutrient composition as the rest of the diet allowed for further refinement of energy requirements. The subjects reported to the eating laboratory each morning and were weighed to ensure weight stability. Breakfast was prepared each day

and upon finishing, subjects received a cooler with food for the remainder of the day. All subjects consumed a eucaloric control diet (55% carbohydrate, 30% fat, 15% protein) for 2 weeks prior to the baseline measurements.

The composition of the 4 week high fat diet was designed to provide 55% fat, 30% carbohydrate, 15% protein and was 1,000 kcal above energy requirements. The extra calories were in the form of a high fat milkshake (1,017 kcal, 15 g protein, 60 g carbohydrate, 81 g fat) that contained 2 satchets of either VSL#3 (450 billion bacteria per satchet) or placebo (cornstarch). The strains of bacteria contained in VSL#3 is as follows: Streptococcus thermophilus DSM24731, Lactobacillus acidophilus DSM24735, Lactobacillus delbrueckii ssp. bulgaricus DSM24734, Lactobacillus paracasei DSM24733, Lactobacillus plantarum DSM24730, Bifidobacterium longum DSM24736, Bifidobacterium infantis DSM24737, Bifidobacterium breve DSM24732. Milkshakes were given to the subjects each day for breakfast under supervision to confirm that it was completely consumed. Subjects were required to return all containers unwashed along with any food that was not eaten. Energy and macronutrient intake was tightly controlled and was within ±5 grams of the target for carbohydrate, protein, fat, saturated fat, and fiber each day.

Experimental Design:

The study utilized a double-blind, placebo controlled, randomized design. After the 2-week control diet, the participants underwent baseline testing consisting of dual energy x-ray

absorptiometry (DXA) (General Electric, Lunar Digital Prodigy Advance, Madison, WI) to determine body composition, an intravenous glucose tolerance test (IVGTT) to assess insulin sensitivity, and a skeletal muscle biopsy to measure substrate oxidation. Following baseline testing, subjects were randomized to receive either placebo or probiotic for the 4-week high fat diet. Following the high-fat diet, all tests were repeated and samples collected.

Endotoxin:

Serum endotoxin was measured with a Recombinant Factor C Endotoxin Detection Assay (Pyrogene, Lonza, Walkersville, MD) with a minimal detection limit of 0.01 EU/ml and a measureable endotoxin range of 0.01 – 10.0 EU/ml.

Intravenous Glucose Tolerance Test (IVGTT):

Whole body insulin sensitivity was estimated using Bergman's minimal model (MINMOD Millenium software) using a modified frequently sampled IVGTT [11]. Following an overnight fast, subjects were placed on a bed in a recumbent position and an intravenous catheter was placed in each arm. Two baseline blood samples were drawn at (t) = -10 and -1 min and glucose (0.3 g/kg) was injected at time 0. 10 mls of blood were drawn at (t) = 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 18 min. Insulin was injected (0.025 U/kg) at (t) = 20 min with blood drawn at (t) = 22, 23, 24, 25, 27, 30, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 min. Samples were centrifuged at 4°C and 2500 rpm for 15 minutes. Plasma glucose was measured immediately in duplicate using a YSI Glucose Analyzer (Yellow Springs, OH). Serum insulin concentrations were measured at the end of the study using a microparticle immunoassay (Abbott Laboratories,

Abbott Park, IL). Variable obtained from the MINMOD software include insulin sensitivity (SI), acute insulin response to glucose (AIRg), disposition index (DI), and glucose effectiveness (Sg).

Skeletal Muscle Biopsy:

Subjects arrived at the laboratory following an overnight fast and having abstained from exercise and anti-inflammatory medications (aspirin, NSAIDS) for at least 72 hours. Additionally, subjects were free of infections or inflammatory conditions. The skin was prepared for the biopsy by cleaning with a povidone-iodine solution and a sterile fenestrated covering was placed over the leg. Lidocaine HCl (1%) was injected into the skin and the fascia of the vastus lateralis. A small (1/4 inch) incision was made with a scalpel and samples were collected using a modified Bergström needle (Cadence, Staunton, VA) with suction.

Skeletal Muscle Homogenate Preparation:

Each homogenate sample was minced 200 times with scissors, transferred to a glass homogenization tube and homogenized on ice using a Teflon pestle (12 passes at 150 RPM). The sample was rested on ice for ~30 seconds and the homogenization steps were repeated. The homogenate was transferred to an Eppendorf tube and fresh sample was used to measure glucose oxidation, fatty acid oxidation, and enzyme activity. Homogenate protein concentrations were determined spectrophotometrically using the bicinchoninic acid (BCA) assay (Thermo Scientific, Pittsburg, PA).

Palmitate and pyruvate oxidation rates were determined in the fresh muscle homogenates prepared as previously described [18]. The oxidation rate in muscle homogenates was measured by counting the $^{14}CO_2$ produced from [1-^{14}C] palmitic acid or [1-^{14}C] pyruvate during incubation. Briefly, 80 ml of a 20-fold (wt:vol) diluted muscle homogenates were incubated with 320 ml of reaction media (pH 7.4). Final concentrations of the reaction media were in mmol per liter: sucrose, 100; Tris-HCl, 10; potassium phosphate, 5; potassium chloride, 80; magnesium chloride, 1; L-carnitine, 2; malate, 0.1; ATP, 2; coenzyme A, 0.05; dithiothreitol, 1; EDTA, 0.2; and bovine serum albumin, 0.3%. After 3 hours of incubation at 37°C, 200 ul of 70% perchloric acid was injected to stop the reaction and evolve $^{14}CO_2$ from the reaction media. $^{14}CO_2$ produced during the 3 hour incubation was trapped with 400 ml of 1M sodium hydroxide. Trapped $^{14}CO_2$ was determined by liquid scintillation counting by use of 5 ml EcoLite liquid scintillation cocktail (MP Biomedicals, Santa Ana, CA) in the LS 6500 scintillation counter (Beckman Coulter, Pasadena, CA). Total fatty acid oxidation was determined by measuring and summing the production of ^{14}C-labeled CO_2 and ^{14}C-labeled acid soluble metabolites (ASM's). All samples were run in triplicate and data is normalized to total protein content and expressed in nmol/mg prot/h. ASM's were collected from the reaction media (after a 1hr acidification) and centrifuged at 14,000 rpm at 4C for 15 minutes. A 300ul sample of the supernatant was placed in a scintillation vial and ^{14}C-labeled ASM's were counted on a scintillation counter.

Statistical Analysis:

All data was analyzed by two-way repeated measures ANOVA (time and treatment). Post hoc analyses were performed on variables of interest using independent samples t-tests. The comparison of changes from baseline in dependent variables between groups were made with one-tailed, independent t-tests. Significance was set *a priori* at $P < 0.05$. Values in the text are mean ± SE

RESULTS

Subject Characteristics, Body Composition and Endotoxin Concentrations

Subject characteristics are shown in Table 1. There were no differences between VSL#3 and placebo in any of the subject characteristics at baseline. There was no significant differences between the groups in total energy and macronutrient intake, saturated fat intake, or dietary fiber intake as assessed from 4 day food records (data not shown). Total energy and macronutrient composition of the control and high fat diet are shown in Table 2. By design, the high fat diet was higher in total energy, fat, and saturated fat, and lower in carbohydrate and fiber (all $P < 0.001$). Serum endotoxin concentration was similar (VSL: 1.89 ± 0.47 vs Placebo: 1.36 ± 0.33 EU/ml, $P>0.05$) in the two groups at baseline and was not affected by the high fat diet or VSL#3 treatment (VSL: 1.80 ± 0.21 vs. Placebo: 1.64 ± .35 EU/ml; $P<0.05$).

Body Mass and Body Composition

Body mass did not change during the lead-in period (VSL: 74.2 ± 3.9 kg and 73.7 ± 4.0 kg; Placebo: 74.6 ± 2.5 kg and 74.7 ± 2.6 kg). Body mass and composition were similar ($P > 0.05$) in

the two groups at baseline. Following the high fat diet, body mass increased ($P < 0.001$), in both groups. However, the increase in body mass was smaller ($P<0.045$) in the VSL#3 compared with placebo (1.42 ± 0.42 kg vs. 2.30 ± 0.28 kg, respectively (Figure 1). The increase ($P=0.023$) in body fat mass that occurred in the placebo group during the high fat diet was not observed in VSL#3 group (Figure 2). Fat-free mass increased ($P = 0.001$) in both groups but there was no significant effect of VSL#3 treatment. Change in body weight attributed to lean versus fat mass was not different between groups with 44.7 ± 7.6% lean versus 55.3 ± 7.6% fat and 42.1 ± 10.5% lean versus 57.9 ± 10.5% fat for placebo and VSL#3, respectively.

Insulin Sensitivity and Substrate Oxidation:

Fasting glucose and insulin concentrations were similar at baseline in VSL#3 and placebo. Insulin sensitivity and the other IVGTT variables were not different ($P>0.05$) between the groups at baseline. There was no effect of the high fat diet or VSL3#3 treatment on any of the IVGTT variables ($P > 0.05$). There were no differences between groups in FAO-CO_2, FAO-ASM, total FAO, or pyruvate oxidation nor were these variables affected by the high fat diet or VSL#3 treatment ($P > 0.05$).

DISCUSSION

To our knowledge, our study is the first to determine if probiotic supplementation attenuates the effect of a high fat feeding on body mass and fat mass, insulin sensitivity, and skeletal muscle substrate oxidation. The major finding of the present study was that VSL#3 supplementation was associated with significantly less body weight and fat gain compared with

placebo. We did not observe any significant impact of VSL#3 on insulin sensitivity or skeletal muscle substrate metabolism.

Our findings are consistent with the observation that rodents fed probiotics gain less weight and adipose tissue during high fat feeding [16-20]. In addition, probiotic supplementation has been reported to increase weight loss during energy restriction in animal models (21) and overweight or obese humans [22-24]. Our study was not designed to determine the mechanism(s) responsible for the attenuated body mass and fat gain with VSL#3 treatment during high fat feeding. However there are several plausible mechanism including reduced energy harvesting, reduced dietary fat absorption [18], and increased energy expenditure and fat oxidation [25] associated with VSL3# stimulated GLP-1 secretion [9].

There are some limitations of the present study that should be discussed. First, our sample size was relatively small and included only healthy, nonobese young male subjects. As such, it is unknown whether VSL would attenuate the increase in body and fat mass during high fat feeding in females. Second, it is possible that the amount of fat in the diet provided, the duration of the intervention, or amount of body mass/fat gain was too little to impair insulin sensitivity and substrate oxidation in healthy, nonobese young males. Future studies will be necessary to better understand this issue. Third, we did not assess enrichment of probiotic bacteria in the gut or attempt to identify specific probiotic strains that may have contributed to the attenuated increase in body and fat mass with high fat feeding in the present study. Future studies will be necessary to replicate our findings and determine the mechanisms responsible.

In conclusion, supplementation with the probiotic VSL#3 attenuated in the increase in body mass and body fat mass during high fat feeding in healthy, nonobese males. Neither endotoxin concentration, insulin sensitivity nor substrate oxidation were influenced by the high fat diet in the present study. Future studies will be necessary to determine the mechanisms responsible for the smaller increase in body mass and fat mass with probiotic supplementation with VSL#3 during high fat feeding.

Acknowledgements: We're grateful to our research subjects for taking part in this study.

Source of Funding: Funding for this study was provided by VSL Pharmaceuticals, Inc.

Disclosures: None

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FIGURE LEGEND

Figure 1.

Change in body mass following the high fat diet. Values are mean ± SE. *P < 0.05 placebo versus VSL#3.

Figure 2.

Change in body fat mass following the high fat diet. Values are mean ± SE. *P < 0.05 versus placebo versus VSL#3.

Table 1: Subject characteristics at baseline

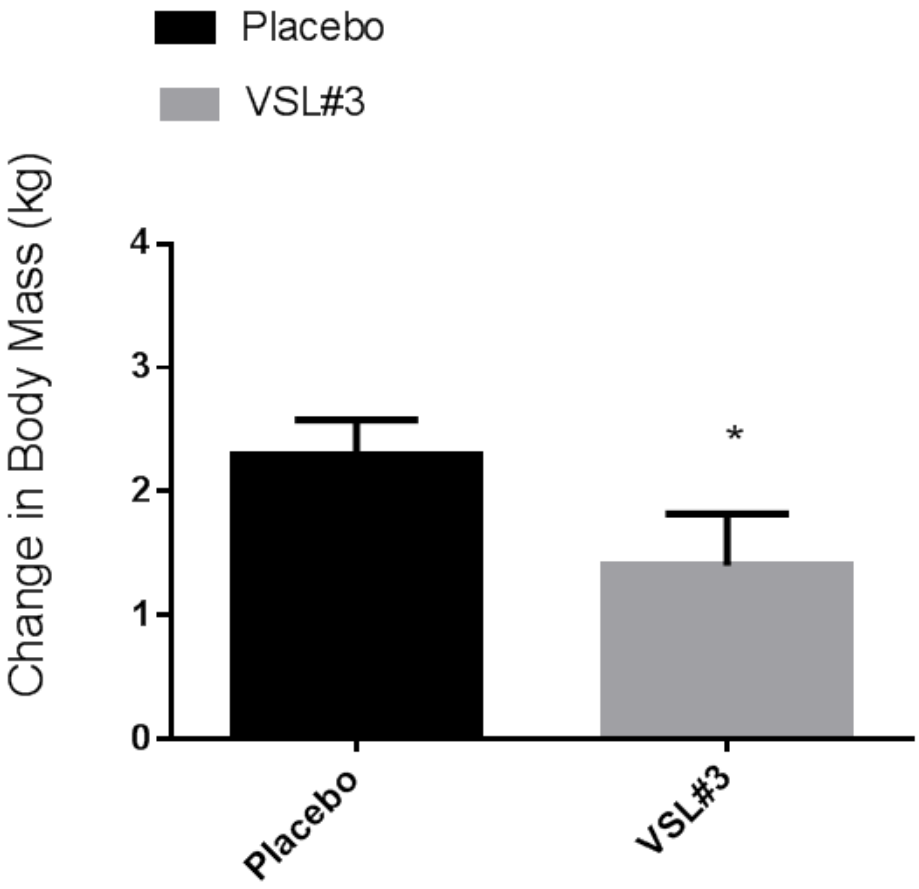
	Placebo (N = 11)	VSL#3 (N = 9)
Age (years)	22.9 ± 0.9	22.4 ± 1.4
Height (inches)	70.5 ± 1.2	68.3 ± 0.8
BMI (kg/m^2)	23.2 ± 0.6	23.9 ± 0.9
% Body Fat	18.5 ± 2.4	21.0 ± 2.3
Lean Mass (kg)	58.01 ± 2.64	55.64 ± 1.64
Fat Mass (kg)	13.17 ± 1.82	15.59 ± 2.78

Table 2: Energy and macronutrient composition of the control and high fat diets.

Diet Condition	Kcal	Protein (g)	Carbohydrate (g)	Fat (g)	Saturated Fat (g)	Fiber (g)
Control	2902.9 ± 78.2	107.8 ± 2.7	407.5 ± 10.6	97.8 ± 3.9	27.5 ± 1.1	18.4 ± 0.7
High Fat*	3946.6 ± 78.8	125.0 ± 3.2	282.4 ± 5.8	255.5 ± 4.7	139.8 ± 2.0	14.1 ± 0.4

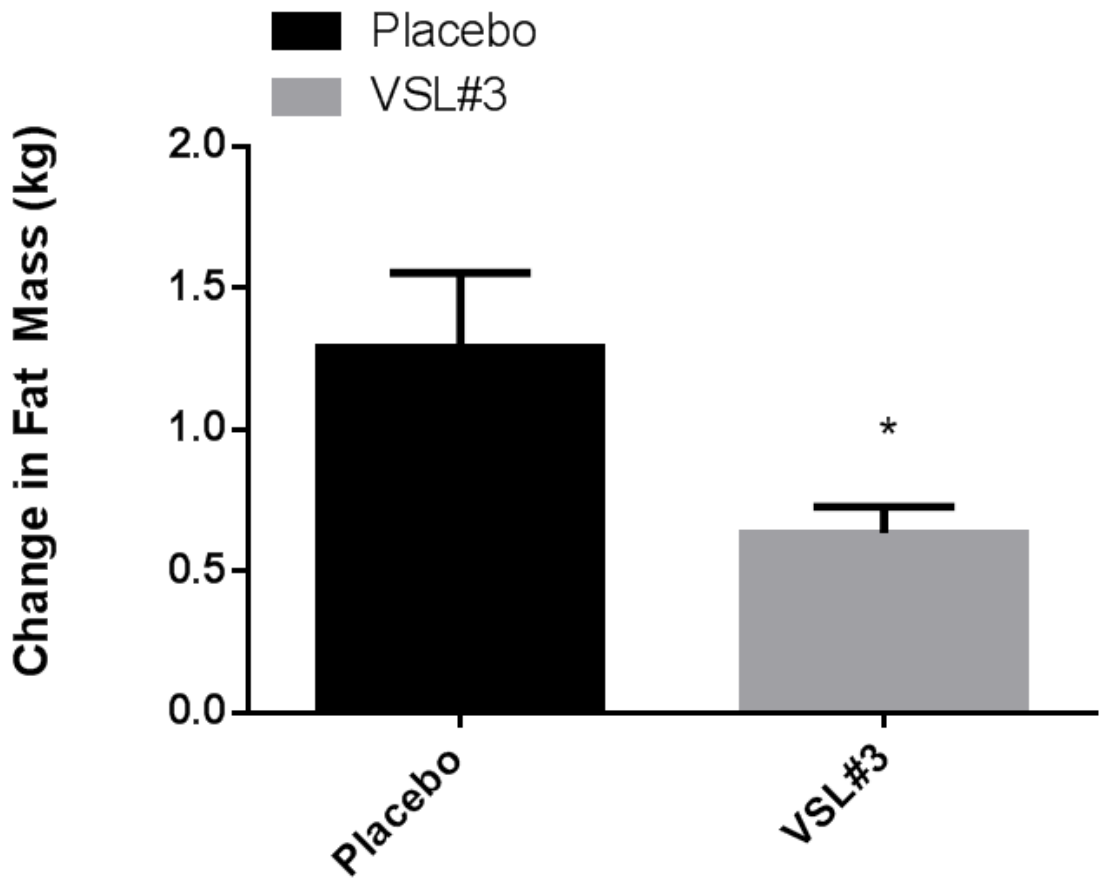
*Values reflect the addition of the high fat milkshake.

Figure 1



* P = 0.045 compared with placebo.

Figure 2



* P = 0.023 compared with placebo.

Table 3: Insulin sensitivity before and after the 4 week high fat diet.

Variable	Placebo		VSL#3	
	Pre High Fat Diet	**Post High Fat Diet**	**Pre High Fat Diet**	**Post High Fat Diet**
Fasting Glucose (mg/dl)	79.8 ± 3.5	81.4 ± 2.2	81.7 ± 2.3	81.8 ± 3.5
Fasting Insulin (uIU/ml)	5.22 ± 1.4	4.22 ± 0.8	6.15 ± 1.5	5.97 ± 1.4
SI [(mU/L)/min]	5.8 ± 0.9	5.9 ± 0.6	5.9 ± 1.3	5.5 ± 0.9
AIRg [mU/L) 10 min]	526.0 ± 71.9	561.5 ± 82.6	480.9 ± 102.1	523.1 ± 90.1
DI	2604.0 ± 300.9	2916.0 ± 478.7	2159.6 ± 293.2	2717.7 ± 470.9
Sg (%/min)	0.027 ± 0.003	0.037 ± 0.007	0.259 ± 0.003	0.042 ± 0.009

Values are mean ± SE. SI=insulin sensitivity, AIRg = acute insulin response, DI = disposition index, Sg = glucose effectiveness

Table 4: Skeletal muscle substrate oxidation before and after the 4 week high fat feeding.

	Placebo		VSL#3	
	Pre High Fat Diet	**Post High Fat Diet**	**Pre High Fat Diet**	**Post High Fat Diet**
FAO-CO_2	0.663 ± 0.073	0.579 ± 0.071	0.598 ± 0.113	0.484 ± 0.044
FAO-ASM	7.40 ± 0.98	5.83 ± 0.68	5.52 ± 0.79	4.99 ± 0.36
FAO-Total	8.06 ± 1.02	6.41 ± 0.72	6.11 ± 0.92	5.48 ± 0.38
CO_2/ASM	0.0957 ± 0.009	0.089 ± 0.008	0.101 ± 0.009	0.097 ± 0.008
PDH Activity	157.5 ± 24.7	133.4 ± 14.8	157.2 ± 21.5	142.6 ± 22.7

Values are means ± SE. FAO-CO_2 = complete fatty acid oxidation, FAO-ASM = incomplete fatty acid oxidation (acid soluble metabolites), FAO-Total = Total fat oxidation, CO2-ASM = ratio of complete to incomplete fatty acid oxidation, PDH =pyruvate dehydrogenase activity.

CHAPTER IV:

Conclusions and Future Directions

Probiotics have been associated with both weight gain and weight loss in humans. We found that probiotics attenuated weight gain and body fat accretion in subjects fed a high-fat, hypercaloric diet for 4 weeks. The mechanisms by which probiotics may contribute to energy balance are poorly understood. A number of hypotheses have been suggested including an increase in GLP-1, decreased fat absorption, reduced energy harvest, and production of CLA. Though there is evidence to support each, research is limited. GLP-1 is released in response to a meal and because all our testing occurred in a fasting condition, we did not measure it. Supplementation with prebiotics and probiotics has been shown to increase GLP-1 and there is some evidence that GLP-1 increases energy expenditure and fat oxidation. Future studies may include measurements of energy expenditure, resting metabolic rate and / or whole body substrate oxidation to determine if some species of probiotics have this effect.

Gut microbiota in humans is assessed via stool sample. Although this is undoubtedly the most practical method, it is not reflective of the entire gastrointestinal (GI) tract. The vast majority of nutrients entering the GI tract are absorbed in the ilium and jejunum. Understanding microbial communities inhabiting the small intestine may provide additional insight into energy harvest capabilities and microbial by-products including CLA.

Animal research supporting the role of the gut microbiota in obesity and insulin resistance is intriguing however, human investigations have not been as convincing. There are a number of

potential confounders in the translation of the research to humans. First, it is unclear the impact that diet has on gut microbial communities. Though dietary components may influence the proliferation of certain bacterial species, the influence on the greater microbiome has not been elucidated. Second, the definition of a healthy microbiota has not been characterized. Currently, a "healthy" gut microbiota is defined as having a high microbial diversity which is stable over time. Due to the high inter-individual variability in humans, it is difficult to determine if there is an "ideal" microbial composition for health. Finally, "metabolic endotoxemia" in humans has not been defined. Endotoxin increases acutely in response to a high fat meal and chronically with high energy and fat intake. Endotoxin concentrations are also associated with insulin resistance and incident diabetes. However, to date, there is no consensus regarding the concentration of circulating endotoxin required to increase risk. Future studies may provide more clarity into the role the gut microbiota plays in energy balance and metabolic health.

Appendices

Appendix A: Informed Consent Form

Informed Consent for Participants of Investigative Projects

Department of Human Nutrition, Foods and Exercise

Virginia Tech

TITLE: High Fat Feeding, Gut Microflora, and Skeletal Muscle Substrate Metabolism

INVESTIGATORS: **Kevin P. Davy, Ph.D.**
Mathew W. Hulver, Ph.D.
Madlyn I. Frisard, Ph.D.
Brenda M. Davy, Ph.D., R.D.

MEDICAL DIRECTOR: Jose Rivero, M.D.

Sponsor: VSL Pharmaceuticals, Inc.

PURPOSE:
Too much fat in the diet is a risk for diabetes and cardiovascular disease. Too much fat in the diet can increase inflammation (a response of your immune system to defend your body against harmful substances) and change metabolism (how the body gets energy from food in the diet). Probiotics are good bacteria found in the intestines. Probiotic supplements can be found in grocery stores and health food stores. They have been used to help with some gastrointesintestinal disorders such as irritable bowel syndrome. Probiotics have also been shown to reduce inflammation. We do not know if probiotic supplements improve metabolism in muscle. The first purpose of this study is to determine if a probiotic supplement change the effects of eating a high fat diet on skeletal muscle metabolism. Inflammation can also influence the health of your blood vessels. Therefore, a second purpose is to see if probiotics reduce the effects of a high fat diet on the function of your blood vessels. Thirty males will be included in this study.

METHODS:
You are being asked to be involved in a study that involves eating a diet similar to your usual diet for two weeks. You will then follow a 4-week high fat diet with or without a probiotic supplement. VSL#3 is a probiotic supplement that is made of probiotic bacteria. The placebo powder contains maltose (a type of sugar). You will be asked to take 2 packets (4.4 grams each) of either the VSL#3 or placebo powder with juice or water in the morning and another 2 packets in the evening. VSL#3 should be stored in your refrigerator and not in your freezer. You should not mix VSL#3 with hot beverages or food or keep in clothes pockets close to your body. If you want to take the VSL#3 or placebo away from home, you should store it in the cooler that we will provide. If you agree to be involved in this study you will first have to fill out a health history questionnaire. The additional tests are described below under Session 1. Your results may be discussed with the study medical director to determine if you can be a subject. You may be able to be a subject if you are between 18 and 40 years of age. Your body mass index (a measure of obesity) must be less than or equal to 30. If you smoke or have high blood pressure, heart disease or diabetes then you cannot be in this study. You will not be able to participate if your cholesterol is too high or have other health problems that would make it unsafe for you to be in the study. Your body mass index (a measure of fatness) must be less than 30 kg/m^2. You will not be able to participate

if you have lost or gained more than 5 pounds in the last 6 months or exercise three or more times a week at a moderate to hard level (e.g., exercise that causes you to breathe hard and sweat). If you use any medication or nutritional supplements that might influence the study variables or have taken antibiotics in the last month then you will not be able to be in this study. You will not be able to be a subject if you have an allergy to lidocaine or bipivicaine, have food allergies (for example, gluten allergy), or are allergic to silicon dioxide (a food additive used to reduce moisture in the VSL#3 or placebo packet). The amount of silicon dioxide is less than 0.1 % (or 0.44 grams) of each 4.4 gram packet.

During the first two-week period you will be given all your food. This food will have the same number of calories you usually eat. The food given to you will have 55% of the calories from carbohydrates, 30% from fat, and 15% from protein. After this 2 week period, you will be randomized (a process similar to flipping a coin) to either a high fat diet with VSL#3 probiotic supplement powder or high fat diet with a placebo (sugar powder).

You will be provided all of your food during this period so that 50-60% of all the calories you eat come from fat. In addition, your calorie intake will be increased by 1000 calories during the 4-week high fat diet portion of the study. Although most subjects will gain weight approximately 3-4 pounds during the study, some may gain less and some may gain more. However, you will meet with a registered dietitian upon completion of the study to help you lose the weight you gained through diet and exercise.

Blood samples and muscle biopsies will be taken at three time points during the 6 week study, once in the beginning of the study, once immediately following the 2 weeks of eating a diet similar to your normal, habitual diet period, and once immediately following the 4-week high fat diet. You will also be required to obtain stool sample the day prior to your laboratory visit and bring it with you so we can measure how the VSL#3 influences the bacteria in your intestine. You will need to come to the laboratory each day during the initial two weeks and the 4-week high fat diet period to have your bodyweight measured as well as to pick up your food and return any uneaten foods from the previous day.

There will be about 50 visits if you participate in the study. This will require approximately 25 hours of time commitment. The real number and order of visits will depend on your and the study staffs schedule. In addition, the order may differ from the order of appearance in this document. You will undergo Session 1 one time and session 2 and 3 three times (before and after 2 weeks of eating your typical diet and again following the high fat diet).

Session 1: Approximate time required: 1.5 hour

(You will complete this session only one time at the beginning of the study)

- **Overnight Fast:** You will have to avoid eating for 12 hours prior to this visit so that the test results will not be influenced by the food you eat.

- **Medical History:** You will be asked to complete a medical history questionnaire. This questionnaire is used to screen for health problems or reasons you should not participate in this study. Your height and weight will also be measured at this time. Your body weight will be measured on a bathroom scale. Your height will be measured with a kind of ruler. Your waist, hip, and neck circumference will be measured using a measuring tape.

- **Blood Pressure:** You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor.

- **Physical Activity Questionnaire:** You will need to answer questions so we estimate your physical activity level. This will take about 15 minutes.

- **Urine Test:** You will be asked to urinate in a small cup that we provide to you. We will measure the amount of sodium and other electrolytes, glucose, protein, pH and whether there are blood cells present to determine whether it is safe for you to participate in the study.
- **Blood Draw:** A small needle will be put in your arm to draw blood (about 3 tablespoons). We will measure glucose, cholesterol, and other factors to determine if you can be a subject.

Session 2: Approximate time required: 1 hour

(You are being asked to complete this session three times; before and after the 2 week habitual diet period and once following the 4 week high fat diet trial).

- **Overnight Fast:** You will have to avoid eating for 12 hours prior to this visit so that the test results will not be influenced by the food you eat.

- **Urine Test:** You will be asked to urinate in a small cup that we provide to you. We will measure the byproducts of metabolism that may change after you take the probiotic supplement.

- **Body Weight and Composition:** These tests are to measure your body weight and body fat. Your body weight will measured without shoes on a hospital scale. Then you will lie on a hospital-type bed and a small amount of x-ray will be passed through your body to determine the amount of bone, muscle and fat in your body. This unit is called a DEXA scan. This test takes approximately 5 minutes and there is no pain associated with the procedure. This procedure will be performed once at the beginning of the study and a second time at the end of the study. Your weight and height will also be measured at this time.

- **Blood Pressure:** You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor.

- **Arterial Stiffness**: To measure arterial stiffness, the blood flow and diameter in the arteries in your chest, neck and leg will be measured with an ultrasound machine. An ultrasonic machine is sort of like radar – a low frequency radio wave that bounces off the tissues and sends a picture back to a “TV-like” screen. A mobile hand unit used will be pressed gently against an artery in your neck and leg.

Session 3: Approximate time required: 4.5 hours

(You are being asked to complete this session three times; before and after the 2 week habitual diet period and once following the 4 week high fat diet trial).

- **Overnight Fast:** You will need to avoid eating or drinking for 12 hours and having caffeine-containing foods or drinks for 24 hours before to this visit. This is to make sure that your eating does not influence the test results.

- **Infection/Inflammation Questionnaire:** You will be asked to complete a questionnaire about any recent illnesses or infections that you may have had in the past month.

- **Physical Activity Questionnaire:** You will be asked a series of questions to estimate your usual physical activity level, which will require about 15 minutes to complete.

- **Muscle Biopsy:** You should not take aspirin, ibuprofen or other non-steroidal, anti-inflammatory medication (such as Advil, Motrin, Celebrex, or Vioxx, or other medication or anything that may affect bleeding or bruising, for 72 hours prior and after this procedure. This procedure is used to sample a small amount of muscle (about 450 mg) from below the skin on your thigh. The actual biopsy site will be on the top of either the right or left leg between your knee and hip.

  This procedure will be performed by a study investigator (Kevin P. Davy, Ph.D.) or co-investigator (Mathew Hulver, Ph.D.) who has been trained to perform the biopsy. A Physician or a Nurse will be on site and available if needed, but may not be present during the procedure itself. You will be lying down and your skin will be cleansed with iodine-type solution (Providine or Betadine). If you are allergic to iodine, we will use chlorhexadine, which does not contain iodine. A sterile drape will be placed over the area and your skin and muscle tissue will be numbed by injecting numbing medication (lidocaine/bipivicaine) into the area with a small needle. If you allergic to lidocaine or bipivicaine, you cannot participate in this study. Then, a small incision (about 1/4 of an inch) will be made in the skin and a needle (a little thinner than a pencil) will be inserted to remove a small amount of muscle (about 450 mg). Some suction may be applied to the other end of the needle to help remove the muscle.

  After the biopsy is completed, pressure will be applied and the skin will be closed with sterile tape. To ensure cleanliness, the skin will be cleaned with saline and will be covered with gauze and a clear adhesive dressing. The site will then be wrapped with an ACE bandage. You will be asked to keep the ACE bandage on for at least 10-15 minutes. You may take Tylenol for any discomfort you may experience following the biopsy. We will use the biopsy samples to measure factors which contribute to inflammation. The biopsy will take place at either the Human Integrative Physiology Laboratory (228 War Memorial Hall) or Dr. Jose Rivero's medical office in Christiansburg. You will be asked to return to the physiology laboratory within 5 days after the biopsy to have the site checked to ensure proper healing.

  You will be provided with instructions on how to care for the biopsy sites as well as what to look for if a problem were to occur.

- **Intravenous Glucose Tolerance Test (IVGTT).** You will be need to fast 12 hours prior to your visit to the lab. Two small plastic tubes (intravenous catheters) will be placed in each of two arm veins (different arms) and about 3 tablespoons of blood will be taken to measure hormones or proteins that influence your metabolism and cardiovascular system. We will then

inject a small amount of glucose (0.3 mg/kg body weight) and insulin (0.03 unit/kg body weight) into your veins (insulin is a hormone which helps your body's cells metabolize glucose). We will draw a small amount of blood (less than one half teaspoon) about 28 times over a 3-hour period. A registered nurse will be present to perform this test with the assistance of investigators.

Take-Home Tests

- **Diet Records:** To get an idea of what and how much food you eat, you will be asked to record all of the food you eat for 4 days (3 weekdays and one weekend day). You will also be asked to keep track of the food you eat for the 5 days you are in the habitual-diet phase of the study.

- **Stool Collection:** You will be asked to collect a stool sample to bring to the laboratory on three occasions (each time you come in for session two).

  Collection Instructions:

  - Collect stool sample on the day prior to, or day of, your scheduled visit.
  - Place plastic stool hat onto toilet seat for stool collection.
  - Tightly close (seal) the white container and put in the clear plastic bag (zip lock), which was provided. Close the bag tightly.
  - Place a freezer pack in the cooler with your sample, and return the items to the research study staff.

SUMMARY OF SUBJECT RESPONSIBILITIES

- Provide an accurate history of any health problems or medications you use before the study begins.

- Inform the investigators of any discomfort or unusual feelings before, during or after any of the study sessions.

- Be on time and attend all of the scheduled experiment.

- Follow all participant instructions for each session.

- Collect your stool samples and follow the instructions for returning in the cooler provided.

- Record any food you eat that has not been provided by the investigators.

- Return any uneaten food that has been provided by the investigators.

- Follow physical activity instructions provided by the investigators.

- Carefully read the instructions on consuming any food provided to you.

RISKS OF PARTICIPATION

- VSL#3 Probiotic Supplement: VSL#3 has been shown to be a safe. There is small risk of flatulence (gas), bloating, and/or a change in bowel habits while taking VSL#3. You should not be in the study if you are allergic to silicon dioxide, a food additive used to absorb moisture during the packaging and storage of VSL#3 and the placebo.

- Catheter and Blood Draw: Some pain or discomfort may be experienced when the catheter is inserted in the vein, but this should persist for only a short time. During the blood draws, you may have pain and/or bruising at the place on your arm where the blood is taken. In about 1 in 10 or 10% of the cases, a small amount of bleeding under the skin will cause bruising. The risk of a blood clot forming in the vein is about 1 in 200, while the risk of infection or significant blood loss is 1 in 1000. There is a small risk of the vein becoming inflamed and/or painful in the hours or days after the catheter is removed. If you feel faint during or after a blood draw, you should notify the study doctor or study staff immediately and lie down right away to avoid falling down. Having staff who are experienced in catheter placement and blood draws will minimize these risks.

- Intravenous Glucose Tolerance Test: Because this procedure requires the placement of a catheter in an arm vein, the risks here are identical to that stated above. In addition, there is a small risk of low blood sugar occurring during or after the test. We will be monitoring your blood sugar frequently and can usually anticipate this before your blood sugar drops too low. If this happens, orange juice (with table sugar) or some other simple carbohydrate containing food will be given to you. We will monitor your glucose until it returns to normal. A registered nurse will perform the test with the assistance of the investigators.

- HIV/AIDS: Your blood will be tested for the presence of HIV if one of the study investigators is exposed to your blood. There will not be any cost to you for this test. The results will be sent to your primary care physician or the study medical director, Dr. Jose Rivero, if you do not have a primary care physician. He/she will discuss them with you and provide you with the necessary referral for further evaluation and/or counseling if your results are positive. The results of your test will remain confidential.

- Muscle Biopsies: If you are allergic to lidocaine, you will not be allowed to participate in this study. There may be slight discomfort and burning when the local anesthetic is injected prior to the biopsy, but you are not expected to experience discomfort during the biopsy procedure. Bruising in the area of the muscle biopsy for 1-2 weeks will likely occur, but local pressure and ice are applied to the site immediately after the procedure to limit this potential effect and its accompanying tenderness. There is a slight risk of infection at the biopsy site. There is a small risk that you will become lightheaded, dizzy, or anxious before or during the procedures. All of these reactions are temporary and resolve within a short time after completing or stopping the procedure. These risks are minimized by having a trained individual perform the procedure. You will be asked to return to the physiology laboratory within 5 days after the biopsy to have the site checked to ensure proper healing.

  You will likely receive a scar from each of the biopsies performed but these are expected to be very small. These scars usually turn a purple color in the weeks to months following the biopsy and then fade considerably over time. The study staff will show you several pictures of examples of the scarring (greater than 1 year old) that can occur following similar biopsy procedures. It is important that you understand that these are just examples of the scarring that can occur. The actual scar you receive may be smaller or larger or differ in coloring. Individuals with darker skin (e.g., African Americans, Hispanics and Asians) tend to scar more than those with lighter skin. You should consider this before you agree to participate.

- DEXA Scan: The amount of radiation that you will receive in the DEXA exam (combined with the CT scan) is less than the amount permitted by the Food and Drug Administration (FDA) per year. The amount you will receive is equal to 1/20 of a chest x-ray. The more radiation you receive over the course of your lifetime, the more likely your risk increases in developing cancerous tumors. The radiation in this study is not expected to greatly increase these risks; however the exact increase in such risk is not known.

- Arterial Stiffness: There are no known risks associated with these procedures.

- Weight gain: It is expected that you will gain 3-4 pounds during the study but some individuals will gain less and some will gain more. You should know that your weight can fluctuate 1-2 pounds over the course of 1-2 days even without changing your diet. At the end of the study you will meet with a dietitian and receive instructions on how to modify your diet and increase your physical activity to return to your original body weight.

- It is not possible to identify all potential risks in an experiential study. However, the study doctors and study staff will take all possible safeguards to minimize any known and potential risks to your well-being. We believe the overall risks of participation are minimal. All of the procedures are well established and used routinely in the study investigators laboratory.

- Side effects are possible in any research study despite high standards of care, and could occur through no fault of your own or the study doctors or study staff.

BENEFITS OF PARTICIPATION

Your participation will provide you with:

- Information on your body composition.
- Information on your blood pressure, cholesterol and glucose tolerance
- A primary goal of this study is to obtain generalizable medical knowledge related to the benefits of this probiotic supplement.

COMPENSATION

You will be compensated $100 for completing each muscle biopsy. Muscle biopsies will be performed during the three session 3 visits and will be performed before and after your typical diet and again after the high fat diet ($300 total). You can receive an additional $200 for your participation in the high fat feeding period. Total compensation for your participation in the study is $500.

CONFIDENTIALITY

The data from this study will be kept strictly confidential. No data will be released to anyone but those working on the project without your written permission. Data will be identified by a code, without anything to identify you by name. In the event that any of your tests indicate a problem, your results may be shared with the medical director, Dr. Rivero, and your personal physician.

FREEDOM TO WITHDRAW

You are free to withdraw from the study at any time for any reason. Simply inform the experimenters of your intention to cease participation. In addition, circumstances could arise which would lead to your exclusion from the study. For example, lack of compliance to instructions, failure to attend testing sessions, and illness could be reasons for the researchers to stop your participation in the study. Other reasons include an inability by the researchers to obtain an

adequate muscle sample or other measurements that are necessary for the study. All of the sessions and measurements are required components.

INJURY DURING PARTICIPATION IN THIS STUDY
Neither the researchers nor the University have money set aside to pay for medical treatment that would be necessary if injured as a result of your participation in this study. Any expenses that you incur including emergencies and long term expenses would be your own responsibility. You should consider this limitation before you consider participating in this study.

APPROVAL OF RESEARCH
This research has been approved, as required, by the Institutional Review Board for Research Involving Human Subjects at Virginia Tech. You will receive a copy of this form to take with you.

SUBJECT PERMISSION

I have read the informed consent and fully understand the procedures and conditions of the project. I have had all my questions answered, and I hereby give my voluntary consent to be a participant in this research study. I agree to abide by the rules of the project. I understand that I may withdraw from the study at any time.

If you have questions, you may contact:
- Principal Investigator: Kevin Davy, Professor, Department of Human Nutrition, Foods, and Exercise. (540) 231-3487; After hours: 540-230-0486

- Chairman, Institutional Review Board for Research Involving Human Subjects:
David Moore, Associate Vice President for Research (540) 231-4991

Name of Subject (please print) _____

Signature of Subject_____ Date_____

Once complete, upload this form as a Word document to the IRB Protocol Management System: https://secure.research.vt.edu/irb

Section 1: General Information

1.1 DO ANY OF THE INVESTIGATORS OF THIS PROJECT HAVE A REPORTABLE CONFLICT OF INTEREST? (http://www.irb.vt.edu/pages/researchers.htm#conflict)

☒ **No**
☐ **Yes,** explain:

1.2 WILL THIS RESEARCH INVOLVE COLLABORATION WITH ANOTHER INSTITUTION?

☐ **No,** go to question 1.3
☒ **Yes,** answer questions within table

IF YES
Provide the name of the institution [for institutions located overseas, please also provide name of country]**:** **Heart Specialists of Southwest Virginia**
Indicate the status of this research project with the other institution's IRB: ☐ Pending approval ☐ Approved ☒ Other institution does not have a human subject protections review board ☐ Other, explain:
Will the collaborating institution(s) be engaged in the research? (http://www.hhs.gov/ohrp/policy/engage08.html) ☐ No ☒ Yes
Will Virginia Tech's IRB review all human subject research activities involved with this project? ☐ No, provide the name of the primary institution: ☒ Yes *Note: primary institution = primary recipient of the grant or main coordinating center*

1.3 IS THIS RESEARCH FUNDED?

☐ **No,** go to question 1.4
☒ **Yes,** answer questions within table

IF YES
Provide the name of the sponsor [if NIH, specify department]: VSL Pharmaceuticals Inc.
Is this project receiving federal funds? ☒ No ☐ Yes **If yes,**

Does the grant application, OSP proposal, or "statement of work" related to this project include activities involving human subjects that are <u>not</u> covered within this IRB application?

☒ No, all human subject activities are covered in this IRB application
☐ Yes, however these activities will be covered in future VT IRB applications, these activities include:
☐ Yes, however these activities have been covered in past VT IRB applications, the IRB number(s) are as follows:
☐ Yes, however these activities have been or will be reviewed by another institution's IRB, the name of this institution is as follows:
☐ Other, explain:

Is Virginia Tech the primary awardee or the coordinating center of this grant?

☐ No, provide the name of the primary institution:
☒ Yes

1.4 DOES THIS STUDY INVOLVE CONFIDENTIAL OR PROPRIETARY INFORMATION (OTHER THAN HUMAN SUBJECT CONFIDENTIAL INFORMATION), OR INFORMATION RESTRICTED FOR NATIONAL SECURITY OR OTHER REASONS BY A U.S. GOVERNMENT AGENCY?

For example – government / industry proprietary or confidential trade secret information

☒ **No**
☐ **Yes,** describe:

1.5 DOES THIS STUDY INVOLVE SHIPPING ANY TANGIBLE ITEM, BIOLOGICAL OR SELECT AGENT OUTSIDE THE U.S?

☒ **No**
☐ **Yes**

Section 2: Justification

2.1 DESCRIBE THE BACKGROUND, PURPOSE, AND ANTICIPATED FINDINGS OF THIS STUDY:

Sixty-two percent of the American population exceeds the recommended guidelines for total lipid intake while fifty-nine percent exceeds the guidelines for saturated fatty acid (SFA) intake. Consumption of a high fat, Westernized diet is largely responsible for the prevalence of obesity and type II diabetes among Americans. Saturated fatty acid consumption is positively and significantly correlated to body mass index (BMI) and it has long been established that consumption of SFA is closely correlated to skeletal muscle insulin resistance and metabolic disease. Replacing five percent of energy from SFA consumption with energy from polyunsaturated fatty acids (PUFA) reduces the risk of type II diabetes by 35%. The mechanism(s) underlying the connection between high saturated fat intake and disease are not entirely understood.

In response to short term, high fat feeding, some, but not all individuals are able to up shift energy metabolism to burn off excess fat, a phenomenon now known as metabolic flexibility. Since skeletal muscle comprises approximately 30-50% of an individual's body mass and therefore accounts for a significant contribution to overall energy metabolism, most of the changes occurring in response to high fat feeding are occurring in muscle. However, the ability of skeletal muscle to facilitate this shift varies significantly between individuals and this variation may be the reason why some people gain weight following short term high fat feeding and some people don't. In fact, many obese individuals contain a metabolic defect within their skeletal muscle that reduces their capacity to shift substrate metabolism. While this defect is more prevalent in obese individuals, it is unclear whether this is a cause or effect of the metabolic syndrome. Following a year after weight loss surgery, previously morbidly obese individuals retained impaired skeletal muscle metabolic flexibility despite significant weight loss. Studies have also documented impaired

metabolic flexibility in previously obese women as compared to weight-matched controls. These findings suggest that some individuals are predisposed to excessive lipid storage in response to elevated consumption of dietary fats.

Growing evidence suggests that fatty acids induce insulin resistance though a mechanism involving chronic low-grade stimulation of the immune system. Exposing skeletal muscle to saturated fatty acids increases plasma levels and peripheral tissue accumulation of the inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha) in vitro and in vivo. In addition, excessive consumption of SFA increases gut production and circulating levels of lipopolysaccharide (LPS), a ligand for toll-like receptor 4, an integral component of the immune system. Once activated, TLR4 initiates a signaling cascade that results in liberation of nuclear factor kappa B (NF-kB), allowing it to translocate to the cellular nucleus where it stimulates the activation of pro inflammatory pathways. Data from our own laboratory has previously shown that SFA's heighten LPS-stimulated TLR4 activation of the immune system in cell culture and animal models. Furthermore, protein and mRNA expression of TLR4 is significantly increased in skeletal muscle of obese and insulin resistant individuals suggesting a link between high saturated fat intake and inflammation in skeletal muscle and disease. Our own data has shown that short term high fat feeding (5 days) results in increased skeletal muscle inflammation that is associated with defective substrate metabolism. Individuals that were metabolically flexible did not have increased skeletal muscle inflammation following high fat feeding, however those that were not able to respond to the diet became "inflamed." However, whether abnormal metabolism is caused by inflammation or vice versa is not known. As stated above, recent evidence has indicated that the gut and the gut microbiome may play a significant role in the development of metabolic disease. In mice, high fat feeding results in an alteration of the gut microbiome, which allows for increased energy extraction from the diet as well as potentially increasing inflammation through low-grade endotoxemia. However, whether alterations in the gut microbiome following high fat feeding can effect skeletal muscle is not known. In the current study, we propose to test whether alteration of the gut microbiome through probiotic supplementation can alter the effects of high saturated fat feeding on substrate metabolism and inflammation in skeletal muscle.

Probiotics are cultures of beneficial bacteria that are normally present in a healthy digestive tract. Increasing evidence shows that the activity of probiotic bacteria in the human GI tract plays an important role in the dietary management of metabolic health. Probiotic bacteria, specifically the Lactobacilli and Bifidobacteria species, have been recognized as potential therapeutic agents for over a century but only recently has attempts been made to use probiotics to promote health. This renewed interest in the use of probiotics is in part due to the recent publication of numerous clinical studies demonstrating the use of probiotics is associated with a reduction in chronic inflammation in a number of conditions. In addition, there has been a considerable expansion in the commercial availability of probiotics in yogurt, capsule and powder form. However, it is unknown whether probiotic use can alter the effects of saturated fat feeding on skeletal muscle substrate metabolism and inflammation. Therefore the current study will test whether supplementation of VSL#3, a probiotic supplement, can alter the effects of 4 weeks of high saturated fat feeding on skeletal muscle substrate metabolism and inflammation. We expect that individuals supplemented with VLS#3 for weeks will not development skeletal muscle inflammation or abnormal substrate metabolism while on a high fat diet. Because vascular health is also impacted by inflammation, a secondary aim will be to determine if individuals supplemented with VSL#3 will be protected from the development of arterial stiffening while on a high fat diet.

2.2 EXPLAIN WHAT THE RESEARCH TEAM PLANS TO DO WITH THE STUDY RESULTS:

For example - publish or use for dissertation

The results will be published in peer reviewed journals. The study may be used for dissertation research as well.

Section 3: Recruitment

3.1 DESCRIBE THE SUBJECT POOL, INCLUDING INCLUSION AND EXCLUSION CRITERIA AND NUMBER OF SUBJECTS:

Examples of inclusion/exclusion criteria - gender, age, health status, ethnicity

Non-smoking males between the ages of 18-40 who do not have diabetes or high cholesterol as assessed by medical history and blood tests with body mass index less than 30 kg/m2 and blood pressure less than 140/90mmHg. Subjects will be weight stable (+/-2.5 kg) for the last 6 months. Subjects will be sedentary to recreationally active (less than 2 d/wk, 20 min/d). Subjects will have a total cholesterol that is less than 200 mg/dl, and a triglyceride concentration of less than 150 mg/dl. Subjects will have no past or current history of coronary heart disease, stroke or major cardiovascular disease events, respiratory diseases, endocrine or metabolic, neurological, or hematological disorders that would compromise the study or the health of the subject. Subjects will also have no evidence of liver diseases or persistant unexplained elevation in transaminases. Subjects should not be on any medication that could influence the outcome of the study including cholesterol lowering medication (including fibric acid derivatives and niacin), erythromycin, immunosuppressive drugs, or antioxidant/ supplements or taken antibiotics in the last month. Subjects should not have food allergies, allergy to silicon dioxide, or lidocaine/bipvucaine. Subjects should not have had recent surgery, or history of alcohol or drug abuse. Females will not be included in this study because many of the variables we are measuring change during the menstrual cycle and performing measurements during the early follicular phase, for example, is impractical for our initial study on this topic. More importantly, we do not yet know how gut bacteria fluctuate during the menstrual cycle in females. This will need to be determined before an intervention such as proposed is performed. Parenethetically, we have had very few females volunteer for similar studies in the past. Thus, future studies with special efforts to oversample females will be necessary.

3.2 WILL EXISTING RECORDS BE USED TO IDENTIFY AND CONTACT / RECRUIT SUBJECTS?

Examples of existing records - directories, class roster, university records, educational records

☒ **No,** go to question 3.3
☐ **Yes,** answer questions within table →

IF YES
Are these records private or public? ☐ Public ☐ Private, describe the researcher's privilege to the records:
Will student, faculty, and/or staff records or contact information be requested from the University? ☐ No ☐ Yes, visit the following link for further information: http://www.policies.vt.edu/index.php (policy no. 2010)

3.3 DESCRIBE RECRUITMENT METHODS, INCLUDING HOW THE STUDY WILL BE ADVERTISED OR INTRODUCED TO SUBJECTS:

Subjects will be recruited through advertisement. We anticipate recruting through posted fliers, emails, and internet surveys.

3.4 PROVIDE AN EXPLANATION FOR CHOOSING THIS POPULATION:

Note: the IRB must ensure that the risks and benefits of participating in a study are distributed equitably among the general population and that a specific population is not targeted because of ease of recruitment.

Recruiting through the general poplulation. We are recruiting men ages 18-40 years of age of all races and ethnic backgrounds. We are not targeting a specicic population.

Section 4: Consent Process

For more information about consent process and consent forms visit the following link: http://www.irb.vt.edu/pages/consent.htm

If feasible, researchers are advised and may be required to obtain signed consent from each participant unless obtaining signatures leads to an increase of risk (e.g., the only record linking the subject and the research would be the consent document

and the principal risk would be potential harm resulting in a breach of confidentiality). Signed consent is typically not required for low risk questionnaires (consent is implied) unless audio/video recording or an in-person interview is involved. If researchers will not be obtaining signed consent, participants must, in most cases, be supplied with consent information in a different format (e.g., in recruitment document, at the beginning of survey instrument, read to participant over the phone, information sheet physically or verbally provided to participant).

4.1 CHECK ALL OF THE FOLLOWING THAT APPLY TO THIS STUDY'S CONSENT PROCESS:

☐ Verbal consent will be obtained from participants
☒ Written/signed consent will be obtained from participants
☐ Consent will be implied from the return of completed questionnaire. Note: The IRB recommends providing consent information in a recruitment document or at the beginning of the questionnaire (if the study only involves implied consent, skip to Section 5 below)
☐ Other, describe:

4.2 PROVIDE A GENERAL DESCRIPTION OF THE PROCESS THE RESEARCH TEAM WILL USE TO OBTAIN AND MAINTAIN INFORMED CONSENT:

Those who respond will be told the general plan for the study and asked to complete a brief online screening to confirm basic eligibility requirements (e.g., age, body mass index, medications). Those still interested and eligible to participate will be invited to a group or individual session to hear the details of participation and potential risks. They will be given a chance to ask any questions. Those still interested will receive a copy of the informed consent to take home with them to read and consider further. Those who return this signed document will proceed with screening and testing.

4.3 WHO, FROM THE RESEARCH TEAM, WILL BE OVERSEEING THE PROCESS AND OBTAINING CONSENT FROM SUBJECTS?

Kevin Davy Ph. D. will be responsible for this and all aspects of the study.

4.4 WHERE WILL THE CONSENT PROCESS TAKE PLACE?

Human Integrative Physiology Laboratory and the metabolic kitchen/laboratory in Wallace Hall on Virginia Tech Campus

4.5 DURING WHAT POINT IN THE STUDY PROCESS WILL CONSENTING OCCUR?

Note: unless waived by the IRB, participants must be consented before completing any study procedure, including screening questionnaires.

In the initial contact with the subject the study will be explained to them and they will receive a copy of the informed consent.

4.6 IF APPLICABLE, DESCRIBE HOW THE RESEARCHERS WILL GIVE SUBJECTS AMPLE TIME TO REVIEW THE CONSENT DOCUMENT BEFORE SIGNING:

Note: typically applicable for complex studies, studies involving more than one session, or studies involving more of a risk to subjects.

Subjects will be allowed to take a copy of the informed consent home with them to review. They will return at a later date with the consent to ensure they have had enough time to review the consent and have any questions answered.

☐ Not applicable

Section 5: Procedures

5.1 PROVIDE A STEP-BY-STEP THOROUGH EXPLANATION OF ALL STUDY PROCEDURES EXPECTED FROM STUDY PARTICIPANTS, INCLUDING TIME COMMITMENT & LOCATION:

Subjects are being asked to participate in a study involving a high fat diet with or without VSL supplementation. As part of their participation, subjects will undergo a two week habitual diet lead-in period that will be followed by a four week high fat diet feeding where subjects will either be randomized to VSL supplementation or placebo. During the two week of the lead-in period, subjects will be provided with all of their meals, which will be similar in composition and amount to their habitual diet. After completion of the 2 week habitual diet lead-in period, subjects will be randomized to one of two groups: high fat diet with VSL supplementation and high fat diet without VSL supplementation. During the four week high fat diet period, subjects will be provided with all of their meals that will contain 1000 kcals more than their habitual diet. The composition of the "extra" calories will be 50-60% of calories as fat, 20-30% as carbohydrate, and 10-20% as protein. Subjects will be provided all of their meals throughout the study. Food will be purchased and/or prepared and packaged for subjects to take home with them in the metabolic kitchen in the Department of Human Nutrition, Foods, and Exercise. Uneaten items will be returned and weighed. Subjects will be asked to return to Wallace Hall once daily during all of the feedings periods for the food to be weighed, receive more food, and turn in any uneaten food to be weighed. Subjects will also be weighed daily and asked to report any intake of food not provided to them. During the second week of the lead-in period and the 4 week high fat feeding period subjects will be asked to come to Wallace Hall each morning to eat breakfast and pick up the two remaining meals for the day.

Testing Sessions in War Memorial Hall
Participants will participate in all testing sessions (except session 1) on three occasions, once before the two week controlled/ habitual diet feeding period, once immediately following the two week controlled/ habitual diet feeding period, and once immediately following the four week high fat feeding period.

Session One: (Approximately 1.5 hour)
Overnight Fast: Subjects will be asked to avoid eating for 12 hours prior to this visit so that the test results will not be influence by the food they eor or by the normal digestion process.

Medical History: Subjects will be asked to complete a medical history questionnaire, which will be used to screen for health problems or any reason and individual shoud be excluded from the study.

Resting Blood Pressure and Heart Rate. Blood pressure measurements will be made under quiet, comfortable ambient laboratory conditions via mercury sphygmomanometry. Measurements will conform strictly to American Heart Association guidelines. Heart rate will be determined from a standard electrocardiographic signal.

Physical Acticvity Questionnaire: Subjects will be asked to fill out a questionnaire concerning their previous physical activity level.

Blood Draw: A small needle will be inserted into the subjects arm to draw blood (approximately 3 table spoons). The blood will be used to measure fasting glucose, insulin, lipids, and other factors that may affect a subjects health.

Urine Test. A small cup of urine will be collected to measure electrolytes, glucose, protein, pH, and blood cells.

Session 2: (Approximately 1 hr)

Overnight Fast: Subjects will be asked to avoid eating for 12 hours prior to this visit so that the test results will not be influence by the food they eat or by the normal digestion process.

Urine Test. A small cup of urine will be collected to measure byproducts of metabolism that may change as a result of the probiotic supplement.

Body Mass and Composition. Body weight will be measured on a digital scale accurate to +0.01 kg. Height will be measured with a standard stadiometer. Percent body fat and fat-free mass will be measured in all subjects using dual-energy x-ray absorptiometry (DEXA) (Prodigy Advance, GE Healthcare).

Resting Blood Pressure and Heart Rate. Blood pressure measurements will be made under quiet, comfortable ambient laboratory conditions via mercury sphygmomanometry. Measurements will conform strictly to American Heart Association guidelines. Heart rate will be determined from a standard electrocardiographic signal.

Arterial Stiffness: Carotid Ultrasonography: Common carotid artery diameters will be masured from the imaged obtained from an ultrasound unit (HP Sonos 7500, Phillips Medical Systems) equipped with a high resolution linear array transducer.

Applanation Tonometry: The carotid, brachial, radial and femoral artery pressure waveform and amplitude will be obtained finger tip probe incorporating a high fidelity strain guage transducer.

Session 3: (Approximately 4.5 hours)

Overnigt Fast: Subjects will be asked to avoid eating 12 hours prior to this visit so that the test results will not be influenced bvy the food they eat or by the normal digestion process.

Skeletal Muscle Biopsy. This procedure is used to sample a small amount of muscle (~450 mg) from the Vastus Lateralis. The actual biopsy site will be on the top of thigh, midway between the knee and hip. Volunteers will be placed in the supine position and the skin will be cleansed with an iodine-type solution (Providine or Betadine). If the subject is allergic to iodine, chlorhexadine will be used. A sterile drape will be placed over the area and the skin, fat tissue, and skeletal muscle fascia will be anesthetized by injecting a local anesthetic (lidocaine/bupivacaine) into the area. If the subject is allergic to either lidocaine or bupivacaine, s/he will not be allowed to take part in the study. Then, a small incision (about 1/4 of an inch) will be made in the skin. A needle (a little thinner than a pencil) will be inserted to remove a small amount of muscle. Some suction may be applied to the other end of the needle to help remove the muscle. After the biopsy is completed, pressure will be applied and the skin will be closed with sterile tape. To ensure cleanliness, the skin will be cleansed with saline. The biopsy site will be covered with gauze and a clear adhesive dressing. The site will then be wrapped with an ACE wrap. The participant will be encouraged to leave the ACE wrap on for at least 10-15 minutes. This procedure will be performed by the principal investigator (Kevin P. Davy, Ph.D.) or co-investigator (Mathew Hulver, Ph.D.) of the study. Subjects will be provided with instructions on how to care for the biopsy site as well as what to look for if a problem were to occur. We will measure factors which are involved in metabolism or contribute to inflammation in these samples. This test will take place at either Dr Jose Rivero's medical office in Christiansburg or the Human Integrative Physiology Laboratory at Virginia Tech (228 War Memorial Hall). Directions will be provided.

Intravenous Glucose Tolerance Test (IVGTT): Two small plastic tubes (catheters) will be placed in each of two arm veins (different arms). The test involves injecting small amounts of glucose (0.3mg/kg body weight)and insulin (0.03 units/kg body weight) into your veins and blood stream (insulin is a hormone which helps your body's cells metabolize glucose). We will draw a small amount of blood (less than one half teaspoon) approximately 28 times over a 3 hour period. A regeistered nurse will be present to perform this test with the assistance of investigators.

Infection/Inflammation Questionnaire: Subjects will be asked to complete a questionnaire about any recent illnesses or infections that they may have had in the prior month.

Take Home Tests:

Food records: Subjects will be asked to record all the food that they eat during a four day period. They will also be asked to record all of the food they eat during the baseline or lead-in period during which they consume their typical diet.

Stool Collection: Participant will be given the stool collection container, commode hat, and a Zip loc bag. They will also be given a stool collection cooler and freezer pack. Study ID number and collection date will be written on the collection container along with the assessment point (e.g., baseline, post-lead in, or post intervention). Subjects will be instructed to collect one fecal sample in the day prior to or day of their scheduled lab visit. After securely covering the sample with the lid, the subject should place the sample (not hat) in the Zip loc bag, seal it, and place it in the cooler with an ice pack. Upon return, sample will be verified for proper labeling information and placed in the freezer.

5.2 DESCRIBE HOW DATA WILL BE COLLECTED AND RECORDED:

Study data will be collected on data sheets (see attached) and manually entered into a database (excel format).

5.3 DOES THE PROJECT INVOLVE ONLINE RESEARCH ACTIVITES (INCLUDES ENROLLMENT, RECRUITMENT, SURVEYS)?

View the "Policy for Online Research Data Collection Activities Involving Human Subjects" at http://www.irb.vt.edu/documents/onlinepolicy.pdf

☐ **No,** go to question 6.1
☒ **Yes,** answer questions within table

IF YES

Identify the service / program that will be used:

☒ www.survey.vt.edu, go to question 6.1
☐ Blackboard, go to question 6.1
☐ Center for Survey Research, go to question 6.1
☐ Other

IF OTHER:

Name of service / program:
URL:
This service is…

☒ Included on the list found at: http://www.irb.vt.edu/pages/validated.htm
☐ Approved by VT IT Security
☐ An external service with proper SSL or similar encryption (https://) on the login (if applicable) and all other data collection pages.
☐ None of the above (note: only permissible if this is a collaborative project in which VT individuals are only responsible for data analysis, consulting, or recruitment)

Section 6: Risks and Benefits

6.1 WHAT ARE THE POTENTIAL RISKS (E.G., EMOTIONAL, PHYSICAL, SOCIAL, LEGAL, ECONOMIC, OR DIGNITY) TO STUDY PARTICIPANTS?

The potential risks include the following:

• VSL#3 Probiotic Supplement: VSL#3 has been shown to be a safe. However, there is small risk of flatulence, bloating, and/or a change in bowel habits while taking VSL#3. Individuals should not be in the study if you are allergic to silicon dioxide, a food additive used to absorb moisture during the packaging and storage of VSL#3 and the placebo.

Blood Draw: There is some pain and discomfort that may be experienced when the catheter is inserted in the vein. There may be pain and/or bruising at the place on the arm where the blood is taken. In about 1 in 10 or 10% of the cases, a small amount of bleeding under the skin will cause bruising. The risk of a blood clot forming in the vein is about 1 in 200 (0.005%), while the risk of infection or significant blood loss is 1 in 1000 (0.001%). There is a small risk of the vein becoming inflamed and/or painful in the hours or days after the catheter is removed.

HIV/AIDS: Blood from a subject will be tested for HIV if one of the study investigators are exposed. There will not be any cost to the subject. The results will be sent to the individuals primary care physician or the study medical director, Dr. Jose Rivero. He/she will discuss the results and provide referral for further evaluation or counseling if the results are positive. The results will remain confidential.

Arterial Stiffness: There are no known risks associated with this procedure.

DEXA Scan: The amount of radiation that subjects will receive in the DEXA exam (combined with the CT scan) is less than the amount permitted by the Food and Drug Administration (FDA) per year. The amount subjects will receive is equal to 1/20 of a chest x-ray. The more radiation an individual receives over the course of their lifetime, the more likely that individual's risk increases in developing cancerous tumors. The radiation in this study is not expected to greatly increase these risks, however the exact increase in such risk is not known.

Muscle Biopsy: For the muscle biopsy, there may be slight discomfort and burning when the local anesthetic is injected prior to the biopsy, but the subject should not feel significant discomfort during the actual biopsy procedure. There is a small risk of bleeding during the procedure but this will be minimized by placing immediate pressure over the incision site. Bruising in the area of the biopsy for 1-2 weeks will likely occur, but local pressure and ice are applied to the site immediately to limit this potential and its accompanying tenderness. There is a slight risk of infection at the biopsy site, however subjects will be required to return to the lab within 5 days following the biopsy to have the site checked to ensure proper healing.

Subjects will be shown pictures of a typical biopsy scar. It will also be explained that the pictures are just one example of scarring and that individuals will scar differently.

Intravenous Glucose Tolerance Test: Because this procedure requires the placement of a catheter in an arm vein, the risks here are identical to that stated above. In addition, there is a small risk of low blood sugar occurring during or after the test. We will be monitoring your blood sugar frequently and can usually anticipate this before your blood sugar drops too low. If this happens, orange juice (with table sugar) or some other simple carbohydrate containing food will be provided. We will monitor the individuals glucose until it returns to normal. A registered nurse will perform the test with the assistance of the investigators.

Weight gain: There is a risk of weight gain of approximately 3 to 4 pounds. Some subjects may gain a less and some may gain more. Upon completion of the study subjects will meet with a registered dietician who will provide them with a weight loss program to restore them to their original weight. NOTE: We have done this successfully in the past at Virginia Tech (IRB# WG_05-457).

It is not possible to identify all potential risks. However, the study doctors and staff will take all possible safeguards to mimimize any known and potential risks to their well being. All of the procedures are will established and used routinely in the investigators laboratory.

6.2 EXPLAIN THE STUDY'S EFFORTS TO REDUCE POTENTIAL RISKS TO SUBJECTS:

Blood Draw: Dr. Kevin Davy, a registered nurse, or a trained technician will perform all blood draws. Aseptic conditions will be followed during all of the procedures. Universal precautions will be taken in collection and handling of all blood samples. Subjects will be told that their blood will be analyzed for presence of HIV if an experimenter is exposed to their blood.

Muscle Biopsy: The muscle biopsies will be performed by a trained investigator or technician under the supervision of Dr Jose Rivero. The possible risk involved with the biopsies are minimized by having trained individuals use aseptic techniques. In addition, subjects will be asked to return to lab within 5 days following the biopsy in order to ensure proper healing.

6.3 WHAT ARE THE DIRECT OR INDIRECT ANTICIPATED BENEFITS TO STUDY PARTICIPANTS AND/OR SOCIETY?

There are no direct benefits of participation. Subjects will receive health information including blood pressure, fasting glucose, insulin, and lipids, and body composition.

Section 7: Full Board Assessment

7.1 DOES THE RESEARCH INVOLVE MICROWAVES/X-RAYS, OR GENERAL ANESTHESIA OR SEDATION?

☐ **No**
☒ **Yes**

7.2 DO RESEARCH ACTIVITIES INVOLVE PRISONERS, PREGNANT WOMEN, FETUSES, HUMAN IN VITRO FERTILIZATION, OR MENTALLY DISABLED PERSONS?

☒ **No,** go to question 7.3
☐ **Yes,** answer questions within table →

IF YES
This research involves: ☐ Prisoners ☐ Pregnant women ☐ Fetuses ☐ Human in vitro fertilization ☐ Mentally disabled persons

7.3 DOES THIS STUDY INVOLVE MORE THAN MINIMAL RISK TO STUDY PARTICIPANTS?

Minimal risk means that the probability and magnitude of harm or discomfort anticipated in the research are not greater in and of themselves than those ordinarily encountered in daily activities or during the performance of routine physical or psychological examinations or tests. Examples of research involving greater than minimal risk include collecting data about abuse or illegal activities. Note: if the project qualifies for Exempt review (http://www.irb.vt.edu/pages/categories.htm), it will not need to go to the Full Board.

☐ **No**
☒ **Yes**

IF YOU ANSWERED "YES" TO ***ANY ONE*** OF THE ABOVE QUESTIONS, 7.1, 7.2, OR 7.3, THE BOARD MAY REVIEW THE PROJECT'S APPLICATION MATERIALS AT ITS MONTHLY MEETING. VIEW THE FOLLOWING LINK FOR DEADLINES AND ADDITIONAL INFORMATION: http://www.irb.vt.edu/pages/deadlines.htm

Section 8: Confidentiality / Anonymity

For more information about confidentiality and anonymity visit the following link: http://www.irb.vt.edu/pages/confidentiality.htm

8.1 WILL PERSONALLY IDENTIFYING STUDY RESULTS OR DATA BE RELEASED TO ANYONE OUTSIDE OF THE RESEARCH TEAM?

For example – to the funding agency or outside data analyst, or participants identified in publications with individual consent

☒ **No**
☐ **Yes,** to whom will identifying data be released?

8.2 WILL ANY STUDY FILES CONTAIN PARTICIPANT IDENTIFYING INFORMATION (E.G., NAME, CONTACT INFORMATION, VIDEO/AUDIO RECORDINGS)?

Note: if collecting signatures on a consent form, select "Yes."

☐ **No,** go to question 8.3
☒ **Yes,** answer questions within table →

IF YES
Describe if/how the study will utilize study codes:
If applicable, where will the key [i.e., linked code and identifying information document (for instance, John Doe = study ID 001)] **be stored and who will have access?** *Note: the key should be stored separately from subjects' completed data documents and accessibility should be limited.*
The IRB strongly suggests and may require that all data documents (e.g., questionnaire responses, interview responses, etc.) do not include or request identifying information (e.g., name, contact information, etc.) from participants. If you need to link subjects' identifying information to subjects' data documents, use a study ID/code on all data documents.

8.3 WHERE WILL DATA BE STORED?

Examples of data - questionnaire, interview responses, downloaded online survey data, observation recordings, biological samples

They will be stored in a locked cabinet in the human integrative physiology laboratory and Dr. Hulvers laboratory/office in ILSB, which is also locked.

8.4 WHO WILL HAVE ACCESS TO STUDY DATA?

Investigators and graduate students involved in the study.

8.5 DESCRIBE THE PLANS FOR RETAINING OR DESTROYING THE STUDY DATA

De-identified data may be kept indefinitely

8.6 DOES THIS STUDY REQUEST INFORMATION FROM PARTICIPANTS REGARDING ILLEGAL BEHAVIOR?

☒ **No,** go to question 9.1
☐ **Yes,** answer questions within table

IF YES
Does the study plan to obtain a Certificate of Confidentiality? ☐ No ☐ Yes (Note: participants must be fully informed of the conditions of the Certificate of Confidentiality within the consent process and form) *For more information about Certificates of Confidentiality, visit the following link:* *http://www.irb.vt.edu/pages/coc.htm*

Section 9: Compensation

For more information about compensating subjects, visit the following link: http://www.irb.vt.edu/pages/compensation.htm

9.1 WILL SUBJECTS BE COMPENSATED FOR THEIR PARTICIPATION?

☐ **No,** go to question 10.1
☒ **Yes,** answer questions within table

IF YES
What is the amount of compensation? $200
Will compensation be prorated? ☒ Yes, please describe: **The total amount of compensation is $500.00. Subjects will be compensated $150 for completing each muscle biopsy during the three session 2 visits which take place before and after the baseline period and following the high fat diet ($450 total). Additional compensation of $50 will be provided for successful completion of high fat diet period.** ☐ No, explain why and clarify whether subjects will receive full compensation if they withdraw from the study? *Unless justified by the researcher, compensation should be prorated based on duration of study participation. Payment must not be contingent upon completion of study procedures. In other words, even if the subject decides to withdraw from the study, he/she should be compensated, at least partially, based on what study procedures he/she has completed.*

Section 10: Audio / Video Recording

For more information about audio/video recording participants, visit the following link: http://www.irb.vt.edu/pages/recordings.htm

10.1 WILL YOUR STUDY INVOLVE VIDEO AND/OR AUDIO RECORDING?

☒ **No,** go to question 11.1
☐ **Yes,** answer questions within table →

IF YES
This project involves: ☐ Audio recordings only ☐ Video recordings only ☐ Both video and audio recordings
Provide compelling justification for the use of audio/video recording:
How will data within the recordings be retrieved / transcribed?
How and where will recordings (e.g., tapes, digital data, data backups) be stored to ensure security?
Who will have access to the recordings?
Who will transcribe the recordings?
When will the recordings be erased / destroyed?

Section 11: Research Involving Students

11.1 DOES THIS PROJECT INCLUDE STUDENTS AS PARTICIPANTS?

☐ **No,** go to question 12.1
☒ **Yes,** answer questions within table →

IF YES
Does this study involve conducting research with students of the researcher? ☒ No ☐ Yes, describe safeguards the study will implement to protect against coercion or undue influence for participation: *Note: if it is feasible to use students from a class of students not under the instruction of the researcher, the IRB recommends and may require doing so.*
Will the study need to access student records (e.g., SAT, GPA, or GRE scores)? ☒ No ☐ Yes

11.2 DOES THIS PROJECT INCLUDE ELEMENTARY, JUNIOR, OR HIGH SCHOOL STUDENTS?

☒ **No,** go to question 11.3
☐ **Yes,** answer questions within table →

IF YES
Will study procedures be completed during school hours? ☐ No ☐ Yes **If yes,** **Students not included in the study may view other students' involvement with the research during school time as unfair. Address this issue and how the study will reduce this outcome:** **Missing out on regular class time or seeing other students participate may influence a student's decision to participate. Address how the study will reduce this outcome:**
Is the school's approval letter(s) attached to this submission? ☐ Yes ☐ No, project involves Montgomery County Public Schools (MCPS) ☐ No, explain why: *You will need to obtain school approval (if involving MCPS, click here: http://www.irb.vt.edu/pages/mcps.htm). Approval is typically granted by the superintendent, principal, and classroom teacher (in that order). Approval by an individual teacher is insufficient. School approval, in the form of a letter or a memorandum should accompany the approval request to the IRB.*

11.3 DOES THIS PROJECT INCLUDE COLLEGE STUDENTS?

☐ **No,** go to question 12.1
☒ **Yes,** answer questions within table →

IF YES
Some college students might be minors. Indicate whether these minors will be included in the research or actively excluded: ☐ Included ☒ Actively excluded, describe how the study will ensure that minors will not be included: **Date of Birth**

Will extra credit be offered to subjects?

☒ No
☐ Yes

If yes,

What will be offered to subjects as an equal alternative to receiving extra credit without participating in this study?

Include a description of the extra credit (e.g., amount) to be provided within question 9.1 ("IF YES" table)

Section 12: Research Involving Minors

12.1 DOES THIS PROJECT INVOLVE MINORS (UNDER THE AGE OF 18 IN VIRGINIA)?

Note: age constituting a minor may differ in other States.

☒ **No,** go to question 13.1
☐ **Yes,** answer questions within table →

IF YES

Does the project reasonably pose a risk of reports of current threats of abuse and/or suicide?

☐ No
☐ Yes, thoroughly explain how the study will react to such reports:

Note: subjects and parents must be fully informed of the fact that researchers must report threats of suicide or suspected/reported abuse to the appropriate authorities within the Confidentiality section of the Consent, Assent, and/or Permission documents.

Are you requesting a waiver of parental permission (i.e., parent uninformed of child's involvement)?

☐ No, **both** parents/guardians will provide their permission, if possible.
☐ No, **only one** parent/guardian will provide permission.
☐ Yes, describe below how your research meets **all** of the following criteria (A-D):
Criteria A - The research involves no more than minimal risk to the subjects:
Criteria B - The waiver will not adversely affect the rights and welfare of the subjects:
Criteria C - The research could not practicably be carried out without the waiver:
Criteria D - (Optional) Parents will be provided with additional pertinent information after participation:

Is it possible that minor research participants will reach the legal age of consent (18 in Virginia) while enrolled in this study?

☐ No
☐ Yes, will the investigators seek and obtain the legally effective informed consent (in place of the minors' previously provided assent and parents' permission) for the now-adult subjects for any ongoing interactions with the subjects, or analysis of subjects' data? If yes, explain how:

For more information about minors reaching legal age during enrollment, visit the following link:
http://www.irb.vt.edu/pages/assent.htm

*The procedure for obtaining assent from minors and permission from the minor's guardian(s) must be described in **Section 4** (Consent Process) of this form.*

Section 13: Research Involving Deception

For more information about involving deception in research and for assistance with developing your debriefing form, visit our website at http://www.irb.vt.edu/pages/deception.htm

13.1 DOES THIS PROJECT INVOLVE DECEPTION?

☒ **No,** go to question 14.1
☐ **Yes,** answer questions within table

IF YES
Describe the deception:
Why is the use of deception necessary for this project?
Describe the debriefing process:
Provide an explanation of how the study meets <u>all</u> the following criteria (A-D) for an alteration of consent: Criteria A - The research involves no more than minimal risk to the subjects: Criteria B - The alteration will not adversely affect the rights and welfare of the subjects: Criteria C - The research could not practicably be carried out without the alteration: Criteria D - (Optional) Subjects will be provided with additional pertinent information after participation (i.e., debriefing for studies involving deception): *By nature, studies involving deception cannot provide subjects with a complete description of the study during the consent process; therefore, the IRB must allow (by granting an alteration of consent) a consent process which does not include, or which alters, some or all of the elements of informed consent.*
The IRB requests that the researcher use the title "Information Sheet" instead of "Consent Form" on the document used to obtain subjects' signatures to participate in the research. This will adequately reflect the fact that the subject cannot fully consent to the research without the researcher fully disclosing the true intent of the research.

Section 14: Research Involving Existing Data

14.1 WILL THIS PROJECT INVOLVE THE COLLECTION OR STUDY/ANALYSIS OF EXISTING DATA DOCUMENTS, RECORDS, PATHOLOGICAL SPECIMENS, OR DIAGNOSTIC SPECIMENS?

Please note: it is not considered existing data if a researcher transfers to Virginia Tech from another institution and will be conducting data analysis of an on-going study.

☒ **No,** you are finished with the application
☐ **Yes,** answer questions within table

IF YES
From where does the existing data originate?
Provide a detailed description of the existing data that will be collected or studied/analyzed:
Is the source of the data public?

☐ No, continue with the next question
☐ Yes, you are finished with this application

Will any individual associated with this project (internal or external) have access to or be provided with existing data containing information which would enable the identification of subjects:

- **Directly** (e.g., by name, phone number, address, email address, social security number, student ID number), or
- **Indirectly through study codes** even if the researcher or research team does not have access to the master list linking study codes to identifiable information such as name, student ID number, etc or
- **Indirectly through the use of information that could reasonably be used in combination to identify an individual** (e.g., demographics)

☐ No, collected/analyzed data will be completely de-identified
☐ Yes,

If yes,

Research will not qualify for exempt review; therefore, if feasible, written consent must be obtained from individuals whose data will be collected / analyzed, unless this requirement is waived by the IRB.

Will written/signed or verbal consent be obtained from participants prior to the analysis of collected data? Yes, signed consent will be obtained

This research protocol represents a contract between all research personnel associated with the project, the University, and federal government; therefore, must be followed accordingly and kept current.

Proposed modifications must be approved by the IRB prior to implementation except where necessary to eliminate apparent immediate hazards to the human subjects.

Do not begin human subjects activities until you receive an IRB approval letter via email.

It is the Principal Investigator's responsibility to ensure all members of the research team who interact with research subjects, or collect or handle human subjects data have completed human subjects protection training prior to interacting with subjects, or handling or collecting the data.

----------END----------

Appendix B: Screening Materials

B-1: Health History Form

Virginia Tech
Department of Human Nutrition, Foods, and Exercise

HEALTH HISTORY QUESTIONNAIRE

STUDY_____ **DATE**_____

SUBJECT ID #_____

PLEASE PRINT

1. Address:_____

   City:_____ State:_____ Zip Code_____

   Home Phone:_____ Work Phone:_____

   E-mail address:_____

   Emergency Contact:_____ Phone:_____

   Relation to you:_____

2. Employer:_____ Occupation:_____

3. Age:_____ Sex:___

   Race and/or Ethnic Origin

   ☐ American Indian or Alaskan Native ☐ Asian or Pacific Islander ☐ Black, not of Hispanic Origin

   ☐ Hispanic ☐ White, not of Hispanic Origin

   ☐ Other

4. **GENERAL MEDICAL HISTORY**

Do you have any current medical conditions? YES ☐ NO ☐ If Yes, please explain:
Are you allergic to any medications? YES ☐ NO ☐ If Yes, please explain:

Have you had any major illnesses in the past? YES ☐ NO ☐ If Yes, please explain:

Have you ever been hospitalized or had surgery? YES ☐ NO ☐ If Yes, please explain: (include date and type of surgery, if possible)

Are you currently taking any medications or supplements, including aspirin, hormone replacement therapy, or other over-the-counter products?

YES ☐ NO ☐ If Yes, please explain:

Medication/Supplement	**Reason**	**Times taken per Day**	**Taken for how long?**

Have you ever had an EKG? YES ☐ NO ☐ If Yes, please explain:

Have you been diagnosed with diabetes? YES ☐ NO ☐ If Yes, please explain:

Age at diagnosis_____

FAMILY HISTORY

	Age (if alive)	Age of Death	Cause of Death
Father			
Mother			
Brothers/Sisters			

Do you have a family history of any of the following: (Blood relatives only, please give age at diagnosis if possible)

	YES	NO	Relation	Age at Diagnosis
a. High blood pressure	☐	☐		
b. Heart Attack	☐	☐		
c. Coronary bypass surgery	☐	☐		
d. Stroke	☐	☐		
e. Diabetes	☐	☐		
f. Obesity	☐	☐		

5. **TOBACCO/ALCOHOL HISTORY** (check one)
6. **CURRENT TOBACCO USE**

			(if applicable) **# per day**
None	☐		
Quit	☐	(when)_____	
Cigarette	☐		
Cigar	☐		
Pipe	☐		
Chew Tobacco	☐		
Snuff	☐		

Total years of tobacco use_____

Do you consume alcohol? Drinks per day ____ Drinks per week ____

10. **CARDIORESPIRATORY/METABOLIC HISTORY**

	YES	**NO**
Are you presently diagnosed with heart disease?	☐	☐
Do you have any history of heart disease?	☐	☐
Do you have a heart murmur?	☐	☐
Occasional chest pain or pressure?	☐	☐
Chest pain or pressure on exertion?	☐	☐
Episodes of fainting?	☐	☐
Daily coughing?	☐	☐
High blood pressure?	☐	☐
Shortness of breath?		
At rest?	☐	☐
lying down?	☐	☐
After 2 flights of stairs?	☐	☐
Do you have asthma?	☐	☐
Do you have a history of bleeding disorders?	☐	☐

Do you have a history of problems with blood clotting?	☐	☐
Do you have high cholesterol? Or, low good (HDL) cholesterol?	☐	☐
Do you have thyroid problems?	☐	☐

If you checked YES to any of the above, you will be asked to clarify your response by an investigator so we can be sure to safely determine your ability to participate.

11. **MUSCULOSKELETAL HISTORY**

	YES	NO
Any current muscle injury or illness?	☐	☐
Any muscle injuries in the past?	☐	☐
Do you experience muscle pain at rest?	☐	☐
Do you experience muscle pain on exertion?	☐	☐
Any current bone or joint (including spinal) injuries?	☐	☐
Any previous bone or joint (including spinal) injuries?	☐	☐
Do you ever experience painful joints?	☐	☐
Do you ever experience swollen joints?	☐	☐
Do you ever experience edema (fluid build up)?	☐	☐
Do you have pain in your legs when you walk?	☐	☐

If you checked YES to any of the above, you will be asked to clarify your response by an investigator so we can be sure to safely determine your ability to participate.

12. **NUTRITIONAL HABITS**

Do you have any food allergies? YES ☐ NO ☐
If Yes, please explain:

Have you ever dieted? YES ☐ NO ☐

If YES, have you dieted within the past 12 months or are you currently on a diet?

YES ☐ NO ☐

If YES, please describe the diet:

a). Name (if applicable):_____

b). Prescribed by a Physician/nutritionist? YES ☐ NO ☐

c). Have you lost weight? YES ☐ NO ☐

d). Duration of diet_____

13. **NUTRITIONAL HABITS (CON'T)**

What was your weight 24 months ago? _____ 12 months ago? _____ 6 months ago? _____

Have you dieted other than in the past 12 months? YES ☐ NO ☐

If YES, please answer the following:

a). How many times have you dieted?

b). How old were you?

c). Weight loss (amount)?

You may be asked to complete a more detailed diet survey if you are volunteering for a research study.

9. **PHYSICAL ACTIVITY SURVEY**

Compared to a year ago, how much regular physical activity do you get? (Check one)

- Much less ☐
- Somewhat less ☐
- About the same ☐
- Somewhat more ☐
- Much more ☐

Have you been exercising regularly for the past three months? YES ☐ NO ☐

If YES, what type of exercise do you regularly participate in? (check those that apply)

		Days per week	Minutes per session	Intensity (1=easy, 10=very hard)
Walking	☐	_____	_____	_____
Running	☐	_____	_____	_____
Cycling	☐	_____	_____	_____
Swimming	☐	_____	_____	_____
Aerobics	☐	_____	_____	_____
Weight Training	☐	_____	_____	_____
Martial Arts	☐	_____	_____	_____
Other (describe)		_____	_____	_____

14. **OBSTETRIC/GYNECOLOGICAL HISTORY**

	YES	NO
Do you have a normal menstrual cycle (1 menses each ~1 month)?	☐	☐

If no, please indicate frequency_____

How long has it been since your last menstrual period_____

Do you take any kind of contraceptive (oral, injectable, implant)? ☐ ☐

If yes, please indicate type and name_____

How many full term pregnancies have you had? ____
How long ago was your more recent pregnancy?_____

Have long since you have last breast fed?____

15. **SLEEP HISTORY**

Please answer yes/no or circle appropriate answer.

	YES	NO	
Do you snore?	☐	☐	**Don't Know** ☐

Snoring loudness
- Loud as breathing
- Loud as talking
- Louder than talking
- Very loud. Can be heard in nearby rooms.

Snoring frequency
- Almost every day
- 3-4 times per week
- 1-2 times per week
- 1-2 times per month
- Never or almost never

Does your snoring bother other people? ☐ ☐

Has anyone told you that you quit breathing during your sleep? ☐ ☐

How often have your breathing pauses been noticed?
- Almost every day
- 3-4 times per week
- 1-2 times per week
- 1-2 times per month
- Never or almost never

Are you tired after sleeping?
- Almost every day
- 3-4 times per week

1-2 times per week
1-2 times per month
Never or almost never

Are you tired during wake time?

Almost every day
3-4 times per week
1-2 times per week
1-2 times per month
Never or almost never

Have you ever fallen asleep while driving?

Almost every day
3-4 times per week
1-2 times per week
1-2 times per month
Never or almost never

Sleepiness Assessment

0 (zero) = would never doze off
1 (one) = slight chance of dozing
2 (two) = moderate chance of dozing
3 (three) = high chance of dozing

Situation	**Chance of Dozing**
Sitting and reading	
Watching TV	___
Sitting, inactive in a public place (e.g., a theatre or meeting)	___
As a passenger in a car for an hour without a break	___
Lying down to rest in the afternoon when circumstances permit	___
Sitting 78uietly after lunch without alcohol	___
Sitting and talking to someone	___
In a car, while stopped for a few minutes in traffic	___

16. **EDUCATION**

Please check the highest degree obtained:

Grade School	☐
Junior High	☐
High School	☐
College Degree	☐
Master's Degree	☐
Doctorate	☐

13. FAMILY PHYSICIAN

Name:_____

Address:_____

_____

Phone: _____

Should it be necessary, may we send a copy of your results to your physician?

YES☐ NO☐

Signature:_____ Date:_____

Witness: _____ Date:_____

Print Name Signature

Reviewer:_____ Date:_____

Print Name Signature

Appendix B-2

Godin Leisure-Time Exercise Questionnaire

INSTRUCTIONS

In this excerpt from the Godin Leisure-Time Exercise Questionnaire, the individual is asked to complete a self-explanatory, brief four-item query of usual leisure-time exercise habits.

CALCULATIONS

For the first question, weekly frequencies of strenuous, moderate, and light activities are multiplied by nine, five, and three, respectively. Total weekly leisure activity is calculated in arbitrary units by summing the products of the separate components, as shown in the following formula:

Weekly leisure activity score = (9 × Strenuous) + (5 × Moderate) + (3 × Light)

The second question is used to calculate the frequency of weekly leisure-time activities pursued "long enough to work up a sweat" (see questionnaire).

EXAMPLE

Strenuous = 3 times/wk

Moderate = 6 times/wk

Light = 14 times/wk

Total leisure activity score = (9 × 3) + (5 × 6) + (3 × 14) = 27 + 30 + 42 = 99

Godin, G., Shephard, R. J.. (1997) Godin Leisure-Time Exercise Questionnaire. Medicine and Science in Sports and Exercise. 29 June Supplement: S36-S38.

Godin Leisure-Time Exercise Questionnaire

1. During a typical **7-Day period** (a week), how many times on the average do you do the following kinds of exercise for **more than 15 minutes** during your free time (write on each line the appropriate number).

Times Per Week

a) STRENUOUS EXERCISE
(HEART BEATS RAPIDLY)
(e.g., running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, vigorous long distance bicycling) _____

b) MODERATE EXERCISE (NOT EXHAUSTING)
(e.g., fast walking, baseball, tennis, easy bicycling, volleyball, badminton, easy swimming, alpine skiing, popular and folk dancing) _____

c) MILD EXERCISE (MINIMAL EFFORT)
(e.g., yoga, archery, fishing from river bank, bowling, horseshoes, golf, snow-mobiling, easy walking) _____

2. During a typical **7-Day period** (a week), in your leisure time, how often do you engage in any regular activity **long enough to work up a sweat** (heart beats rapidly)?

OFTEN	SOMETIMES	NEVER/RARELY
1.0	2.0	3.0

Appendix C

INFECTION/INFLAMMATION QUESTIONNAIRE

Evaluator Script: I would like you to think if you had a cold, the flu, a dental infection or other infection during the past month. I am going to ask you about some symptoms that may have accompanied those types of conditions.

1) Did you have a cold, the flu, a dental infection or other infection in the past month?
   ( ) Yes ( ) No ( ) Refused ( ) Don't Know
   If yes, ( ) Within 1 week ( ) 2 weeks prior ( ) 3 weeks prior ( ) 4 weeks prior

In the prior month did you experience any of the following symptoms? [Note to examiner: If symptom was present, the timing of symptom onset and resolution (# days) prior to interview is recorded. If symptom is still present on the day of interview, place 0 in "Resolved___days ago".]

2) Did you feel feverish or have a fever? ( ) Yes ( ) No
   If Yes, Symptom Started ___days ago. Resolved_____days ago.
   Did you take your temperature? ( ) Yes ( ) No

3) Chills? ( ) Yes ( ) No
   If Yes, Started____days ago. Resolved____days ago.

4) Sore throat ? ( ) Yes ( ) No
   If Yes,.Started____days ago. Resolved____days ago.

5) Coughing? ( ) Yes ( ) No
   If Yes, Started____days ago. Resolved____days ago.

6) Sputum? ( ) Yes ( ) No
   If Yes,.Started____days ago. Resolved____days ago.

7) Sneezing? ( ) Yes ( ) No
   If Yes, Started____days ago. Resolved____days ago.

8) Runny nose or nasal congestion? ( ) Yes ( ) No
If Yes, Started____days ago. Resolved____days ago.

If Yes to (5), (6), (7), or (8). Do you have seasonal allergies? ( ) Yes ( ) No
Do you have a chronic lung or sinus condition? ( ) Yes ( ) No
If Yes, are these symptoms typical for your chronic lung or sinus condition?
( ) Yes ( ) No

9) Ear pain or discharge? ( ) Yes ( ) No
If Yes, Started____days ago. Resolved____days ago.

10) Run down feeling or achy muscles you feel may have been due to a cold or flu?
( ) Yes ( ) No
If Yes, Started____days ago. Resolved____days ago.

11) Tooth/Gum pain? ( ) Yes ( ) No
If Yes, Started____days ago. Resolved____days ago.
If Yes, did you seek dental care?( ) Yes ( ) No
If Yes, did a Dentist find a cavity or other dental infection? ( ) Yes ( ) No

12) Mouth/gum (Y N), Skin (Y N), or Joint (Y N) redness or swelling?
If Yes, Started____days ago. Resolved____days ago.

13) Skin infection? ( ) Yes ( ) No
If Yes, Started____days ago. Resolved____days ago.

14) Nausea/Vomiting? ( )Yes( )No
If Yes, Started____days ago. Resolved____days ago.

15) Diarrhea? ( )Yes ( )No
If Yes, Started____days ago. Resolved____days ago.

16) Pain upon urination or urgency? ( )Yes ( )No
If Yes, Started____days ago. Resolved____days ago.

17) Cloudy discolored urine? ( )Yes ( )No
Urinalysis showing evidence of infection? ( )Yes ( )No
If Yes, Started____days ago. Resolved____days ago.

18) Did you seek medical care for any sort of cold, flu, or infection in the prior month?
( )Yes ( )No
If yes, diagnosis given_____

19) Did you take any over the counter or prescription medications for a cold, flu, or any infection in the prior month?
( )Yes ( )No
If yes, names of medication_____