

IMPACT OF A SYNBIOTIC SUPPLEMENT ON BODY COMPOSITION
AND SELECTED METABOLIC BIOMARKERS
IN HEALTHY OVERWEIGHT SUBJECTS:
A RANDOMIZED PILOT STUDY

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DEDICATION

For Tyler, Mom, Dad, Sandy and James

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CHAPTER I: INTRODUCTION

Obesity

The prevalence of overweight and obesity has increased in the United States and worldwide for the past few decades. According to data obtained as part of the 2009-2010 National Health and Nutrition Examination Survey (NHANES), more than one-third of adults, 35.5% of men and 35.8% of women, in the U.S. were obese (Flegal et al., 2012). Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health and classified as a body mass index (BMI) greater than 25 kg/m² for overweight and greater than 30 kg/m² for obesity (World Health Organization, 2013). Overweight and obesity are major health concerns because of the severe health consequences that often accompany excess body weight and fat mass, including type 2 diabetes mellitus, cardiovascular disease, hypertension, musculoskeletal disorders, and various cancers.

Obesity is a complex disease with diverse causes, including genetic and environmental factors. The dramatic increase in overweight and obesity rates is thought to be the result of the effect that the obesogenic environment has on genes (Wangenstein et al., 2005). For example, some human genes may have evolved as a protective mechanism against starvation when energy consumption was low (Korner et al., 2003). Now that there is an abundance of energy in the typical diet from energy dense foods and beverages, these protective systems are thought to be responsible for excess storage of

energy in the form of adipose tissue. There are also some rare heritable traits, such as a mutation in the melanocortin-4 receptor, that contribute to obesity by negatively affecting appetite regulation by the brain (Loos et al., 2008). Other genetic mutations, such as those occurring in the fat mass and obesity (FTO) gene, contribute to increased fat mass and can increase an individual's risk for developing T2DM (Fischer, 2009).

Regardless of the cause, excess weight gain in the form of fat in most cases results from an imbalance of energy consumed via diet versus energy expended through physical activity and basal metabolism. Caloric restriction and increasing physical activity are traditionally recommended as approaches to balance the energy equation. However, a majority of individuals are unable to sustain these changes over the long term.

Kraschnewski et al. (2010) found that approximately 17.3% of individuals could maintain long-term weight loss management of at least 10% reduction in body weight. A quest for new modifiable targets of obesity has thus become a high research priority. A novel environmental factor with a potentially significant role in obesity is an individual's 'gut flora' or 'gut microbiota.' The complexity and variety of microorganisms that reside in the human gut may impact obesity through interactions between diet and the intestinal habitat of these bacteria.

Human Gut Microbiota

Within the human gastrointestinal (GI) tract reside trillions of bacteria that make up the most complex bacterial ecosystem in the human body (Gill et al., 2006; DiBaise et al., 2008; Saulnier et al., 2009). Several hundred bacterial strains are able to thrive and grow in this environment as a result of a favorable pH and readily available nutrients for survival and colonization (Saulnier et al., 2009). This complex and diverse microbial

composition of the human GI tract plays an important role in nutrient absorption and energy regulation, as well as in immune function. Over 90% of the bacteria colonizing the human GI tract belong to two major phyla, Firmicutes and Bacteroidetes (Eckburg et al., 2005). These two phyla, along with other bacterial groups, including bacteria from the phylum Actinobacteria, contribute to the overall functioning of the intestinal ecosystem (Table 1) (DiBaise et al., 2008).

Table 1. Major Bacteria and Archaea Phyla and Genera Found in the Human Gut Microbiome (DiBaise et al., 2008).

Phyla	Representative genera
Bacteria	
Firmicutes	<i>Ruminococcus</i> <i>Clostridium</i> <i>Peptostreptococcus</i> <i>Lactobacillus</i> <i>Enterococcus</i>
Bacteroidetes	<i>Bacteroides</i>
Proteobacteria	<i>Desulfovibrio</i> <i>Escherichia</i> <i>Helicobacter</i>
Verrucomicrobia	
Actinobacteria	<i>Bifidobacterium</i>
Cyanobacteria	
Synergistes	
Archaea	
Euryarchaeota	<i>Methanobrevibacter</i>

Within each of these phyla are bacterial strains that can be classified as having harmful or beneficial effects on human health or those that may have both (Gibson & Roberfroid, 1995). Some of the predominant bacterial species that have been identified from human stool samples and their influence on human health are illustrated in Figure 1 (Gibson & Roberfroid, 1995; Meyer et al., 2009).

Human Intestinal Bacteria

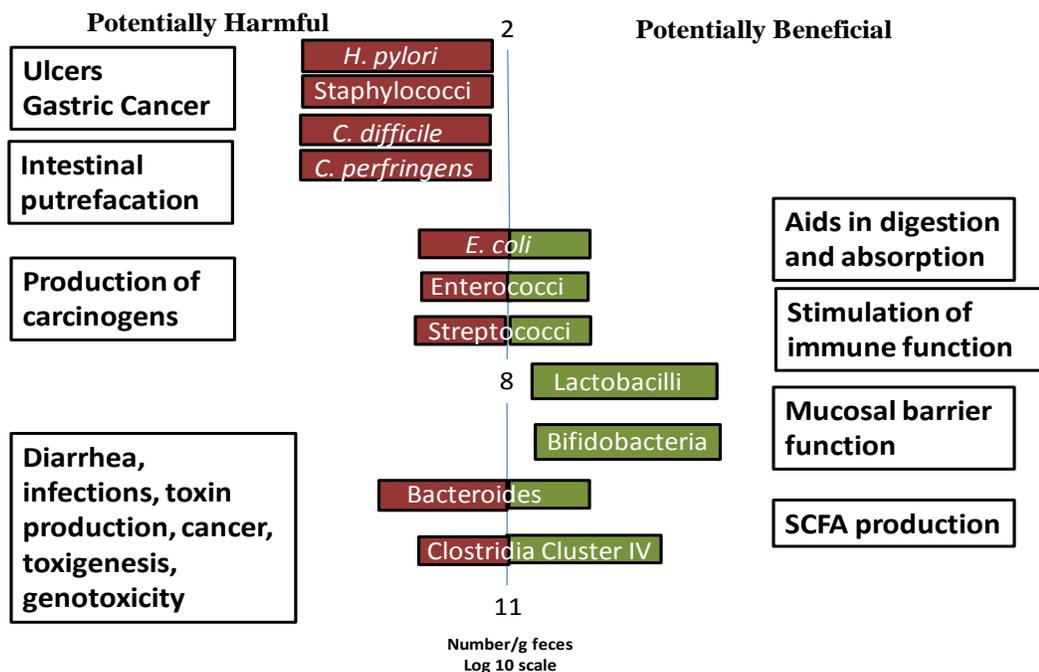


Figure 1. General Composition of Human Gut Bacteria and Associated Health Effects. The figure shows approximate numbers of bacteria in various regions of the GI tract (Gibson & Roberfroid, 1995; Meyer et al., 2009).

Gut Flora and Obesity Link

Bacteria colonizing the intestinal tract have the ability to ferment non-digestible dietary carbohydrates, including some types of starch compounds, non-starch saccharides, oligosaccharides, and non-absorbable sugar alcohols, which are unable to be broken down by pancreatic enzymes for digestion, as well as other food components that may have reached the large intestine unabsorbed (Saulnier et al., 2009). Once these compounds are broken down via bacterial fermentation and degradation, some of the products are then absorbed so that additional energy from the diet is absorbed that can lead to weight gain (Cani et al., 2009). Not all gut microbiota breakdown these undigestible compounds in the same way. Some strains may promote weight gain while

others may play a different role in nutrient metabolism and beneficially influence physiological processes that result in weight loss (Cani et al., 2009).

Ley et al. (2005) investigated the differences in the composition of gut microbiota via gene sequencing of distal gut bacterial strains obtained from the intestines of genetically obese *ob/ob* mice, lean *ob/+* and wild-type siblings. This model was chosen because mouse and human microbiota are comparable at the superkingdom level, with bacteria from the phyla Firmicutes and Bacteroidetes dominating (Ley et al., 2005). Results from this study revealed a distinct difference in bacterial composition between lean and obese mice (Ley et al., 2005). Lean phenotype was associated with a 50% higher proportion of bacteria from the phylum Bacteroidetes, while an obese phenotype was associated with a higher proportion of bacteria from the phylum Firmicutes (Ley et al., 2005). This finding was confirmed in a follow up study investigating the effect of weight change on bacterial composition in humans. Ley et al. (2006) determined that an obese phenotype in humans was also associated with an increased proportion of Firmicutes with regards to relative numbers of Bacteroidetes. In this study, weight loss was achieved through the implementation of calorie-restricted diets. Weight reduction was associated with an increase in bacterial strains from the phylum Bacteroidetes and a decrease in the number of Firmicutes, revealing a link between weight and gut microbiota composition (Ley et al., 2006). The direct impact of weight change on gut bacteria and the association between lean and obese phenotypes and gut bacteria composition has lead researchers to consider the possibility that gut microflora manipulation may be an additional means of obtaining and maintaining a healthy body weight, in addition to caloric restriction and physical activity.

Gut Flora and Metabolic Health: Selected Evidence from Animal and Human Studies

Modification of gut flora for health benefits has been an area of research investigation since the beginning of the 20th century after Metchnikoff wrote *The Prolongation of Life* advising people to consume fermented milk containing lactobacilli to prolong their lives (Metchnikoff, 1908). Since that time, products containing various strains of bacteria have been investigated for their beneficial effects on lipid metabolism, cardiovascular health, and most recently in weight reduction (Agerbaek et al., 1995; Schaafsma et al, 1998; de Roos et al., 1999; Bertolami et al., 1999; Kiebling et al., 2002; Kapila et al., 2006; Chang et al., 2011). The bacterial composition of the human GI tract is influenced on a daily basis through diet and can also change as a result of antibiotic use or disease state. Food products that are marketed to boost bacterial-related health benefits (e.g. improved digestive health) are most readily available to consumers in the form of fermented dairy products like yogurt, cultured buttermilk and kefir. These foods serve as a delivery vehicle for live bacteria that are purported to be beneficial, increasing their amount present in the intestinal environment. The bacteria exert their health effects through a variety of mechanisms mediated directly via host-bacteria interactions or indirectly via bioactive substances released from the bacteria in the gut environment which interact with receptors in the host. The bacterial growth substrates and environment determine the type of metabolites generated, so the effects observed in one type of food matrix may not always be seen in another. With respect to the common delivery vehicles of fermented dairy products, it is not completely understood whether their observed effects on metabolism are attributable to bacteria alone or the synergistic action of bacteria and dairy components (Pereira et al., 2002).

The first evidence of the benefits of fermented dairy products on blood lipids was revealed by Shaper et al. (1963) when researchers observed a reduction in serum cholesterol of African tribesmen after they ingested a *Lactobacillus* sp. containing fermented milk. Since then, the consumption of fermented milk products has been shown to reduce total cholesterol (TC) and low-density lipoprotein-cholesterol (LDL-C) in a variety of subjects who are normolipidemic, hyperlipidemic, and those who are overweight or obese (Table 2). The majority of data obtained from these studies varies greatly depending on strain of bacteria used, the amount of product consumed, and the duration of consumption, as well as individual and lifestyle variations among human subjects (Sanders, 2003).

Author	Objective	Subjects	Intervention	Results
Agerbaek et al., 1995	To test the effect of moderate daily intake of FM on lipoprotein levels of middle-aged men	Middle-aged, normocholesterolemic, non-obese, healthy males (n = 58)	<i>E. faecium</i> & <i>S. thermophilus</i>	↓ TC ↓ LDL-C by 10%
Schaafsma et al., 1998	To investigate the effect of a new FM product, fermented by <i>L. acidophilus</i> with FOS added, on blood lipids	Healthy men w/ borderline elevated levels of serum TC (n = 30)	125 ml test probiotic milk with <i>L. acidophilus</i> & FOS	↓ TC by 4.4% ↓ LDL-C by 5.4% ↓ LDL/HDL-ratio by 5.3%
Bertolami et al., 1999	To verify the effects on the lipid profile of FM product	Patients with mild to moderate hypercholesterolemia (n = 21, women; n = 11, men)	200 g daily of <i>E. faecium</i> + <i>S. thermophilus</i> for 8 weeks	↓ TC by 5.3% ↓ ↓ LDL-C by 6.2%
Kiebling et al., 2002	Assessment of hypocholesterolemic effect of yogurt supplementation	Healthy women: Hypercholesterolemic (n = 14) normocholesterolemic (n = 15)	300 g yogurt containing <i>L. acidophilus</i> and <i>B. longum</i> for 21 weeks	↑ HDL-C by 0.32 mmol/L

FM, fermented milk; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; FOS, fructooligosaccharide

With respect to obesity and weight management, *Bifidobacterium* and *Lactobacillus* species are the two most researched groups of beneficial bacteria (Table 3).

Studies investigating the impact of *Bifidobacterium* sp. supplementation on mice fed high fat diets revealed a beneficial effect; with a lowering of body weight and fat weight, and a reduction in the levels of serum triglycerides, cholesterol, and glucose (Kondo et al., 2010; Yin et al., 2010; An et al., 2011). In mice fed high-fat diets and supplemented with *Lactobacillus paracasei*, researchers saw a reduction in body fat accompanied by an increase in circulating levels of the protein fasting-induced adipocyte factor (FIAF) (Aronsson et al., 2010). This increase in circulating levels of FIAF is capable of reducing fat storage by inhibiting lipoprotein lipase (LPL) (Aronsson et al., 2010). Other *Lactobacillus* sp., including *L. acidophilus*, have been investigated for the effect of supplementation on adiposity and metabolic biomarkers in human subjects (Table 3).

Table 3. Summary of Studies Involving the Use of Strains of *Bifidobacterium* sp. and *Lactobacillus* sp. on Adiposity and Metabolic Biomarkers.

<u>Author</u>	<u>Objective</u>	<u>Experimental Model</u>	<u>Intervention</u>	<u>Results</u>
Kondo et al., 2010	To evaluate the antiobesity activity when paired with a HF diet	Male C57BL/6J mice	<i>B. breve</i> B-3	Dose dependent decrease in body weight & epididymal fat; improved serum cholesterol, fasting glucose & insulin levels
Yin et al., 2010	Compare effects of Bifidobacterium on weight change with HF diet	Sprague-Dawley rats	<i>Bifidobacterium</i> sp: L66-5, L75-4, M13-4, FS31-12	Effects on fat distribution are strain specific
An et al., 2011	Assess antiobesity & lipid lowering effects with fat induced diet	Sprague-Dawley rats	HF Chow + LAB supplement (<i>B. pseudocatenulatum</i> , <i>B. longum</i>)	↓ body and fat weight, ↓ Serum TC, LDL-C & HDL-C
Aronsson et al., 2010	To determine the mechanism of altered fat storage	Male C57B/6J mice	HF chow + <i>L. paracasei</i> F19	↓ body fat ↑ circulating levels of FIAF LPL inhibited due to FIAF = ↓ fat storage
de Roos et al., 1999	To investigate whether <i>L. acidophilus</i> L-1 intake lowers serum cholesterol	Healthy men & women	<i>L. acidophilus</i> L-1 containing yogurt for 6 weeks	↓ TC by 0.04 mmol/L (NS)
Woodard et al., 2009	To investigate the effects of daily probiotic use on GI outcomes after RNYGB	Patients undergoing RNYGB	Long-term <i>Lactobacillus</i> supplementation	Greater ↓ in weight loss at 3 months
Chang et al., 2011	To assess the beneficial effects on metabolic syndrome	Healthy adults	Yogurt containing <i>S. thermophilus</i> , <i>L. acidophilus</i> , <i>B. infantis</i> , <i>B. breve</i> , & <i>E. faecalis</i> for 8 weeks	↓ Body weight by 0.24 kg ↓ BMI by 0.10 kg/m ² ↓ LDL-C by 7.71 mg/dL

HF, high-fat; LAB, lactic acid bacteria; FIAF, fasting-induced adipocyte factor; LPL, lipoprotein lipase; TC, total cholesterol; NS, not significant; RNYGB, Roux-en-Y gastric bypass; BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol

Possible Mechanisms for Gut Flora's Role in Obesity

Recent research suggests that the predisposition for excess fat storage of some individuals may be the result of specific gut bacteria that are capable of breaking down dietary compounds ingested by the host that cannot otherwise be digested, thus harvesting extra energy for the host (Cani et al., 2009). The release of lipogenic substrates, which will be stored as adipose tissue, during the breakdown of non-digestible polysaccharides by intestinal bacteria may also contribute to the growth of adipose tissue (Cani et al., 2009). The mechanism by which bacteria can influence fat storage was first investigated using mice (Table 4).

Backhed et al. (2004) tested the hypothesis that gut microbiota act through an integrated host signaling pathway to regulate energy storage in the host. Researchers found that mice conventionalized with normal gut microbiota from conventionally raised mice exhibited suppressed expression of a protein that is responsible for inhibiting the incorporation of lipogenic substrates into adipose tissue (Backhed et al., 2004). Turnbaugh et al. (2006) investigated the importance of the effect of gut bacteria on energy harvest by transplanting normal gut microbiota obtained from obese or lean mice into mice with sterile intestinal environments (germ-free mice), a process known as conventionalization. The result of conventionalization of germ-free mice with the obese microbiota was an increase in body fat, compared to those conventionalized with lean microbiota, despite no concurrent increase in food consumption or decrease in energy expenditure (Turnbaugh et al., 2006). Researchers also found that mice conventionalized with microbiota from obese mice had a higher gain in body fat than mice conventionalized with microbiota from lean mice, suggesting that the differences in gut

microbiota composition have the potential to contribute to or ameliorate obesity (Turnbaugh et al., 2006).

Table 4. Studies Involving Germ-Free Mice Before and After Conventionalization with Gut Microbiota and the Proposed Mechanisms by which Energy Storage is Regulated.			
Author	Objective	Intervention	Results
Backhed et al., 2004	Hypothesis: microbiota act through an integrated host signaling pathway to regulate energy storage in the host.	Colonized GF mice	Fiaf (LPL inhibitor) is suppressed in conventionalized mice
Backhed et al., 2007	Determine the mechanism by which GF mice are protected against obesity produced by consumption of a high-fat, high-sugar Western diet	N/A	GF animals are protected from diet-induced obesity by 1) Elevated FIAF and 2) increased AMPK activity both resulting in increased fatty acid metabolism
Turnbaugh et al., 2006	Demonstrate that changes in gut microbiota affect the metabolic potential of the mouse gut microbiota	Colonized GF mice with obese & lean microbiota	<i>ob/ob</i> donor microbiota > Firmicutes than lean donor; <i>ob/ob</i> colonized mice = greater % increase in body fat without difference in chow consumption = 2% increase of energy extracted from calories consumed.

GF, germ-free; *ob/ob*, obese phenotype

The influence of differences in composition of gut bacteria in humans has also been investigated. In a study designed to examine the extent of gut microbiota's contribution to nutrient absorption or energy extraction in human subjects, Jumpertz et al (2011) investigated the effects of nutrient load on gut microbiota composition and nutrient absorption. Lean and obese male subjects in this randomized crossover study consumed a weight-maintaining diet for 3 days, and were then randomized to either a 2400- or 3400-kcal/d diet for 3 days, which was followed by a 3 day washout period on the weight-maintaining diet. Results revealed that a drastic change in the nutrient load of lean subjects resulted in a rapid change in gut microbiota. Specifically, there was a 20% increase in Firmicutes and corresponding decrease in Bacteroidetes in the gut. This change in gut microbiota composition in lean subjects was significantly associated with an increased energy harvest of approximately 150 kcal per day (Jumpertz et al., 2011).

Conversely, increased nutrient load did not produce observable changes in microbiota in obese subjects. This evidence along with the previous discovery of lean and obese phenotypic differences in bacterial composition suggest that the gut microbiome may play a role in the amount of energy harvested from the diet, resulting in a direct impact on the body weight and the deposition of body fat in an individual.

Backhed et al. (2004) found that the presence of gut bacteria in mice can suppress the expression of the protein Fasting Induced Adipocyte Factor (FIAF) when compared to germ-free mice. A follow up study found that germ-free mice were protected from diet induced obesity in part via elevated FIAF (Backhed et al., 2007). The suppression of FIAF contributes to increased triglyceride storage via increased activity of lipoprotein lipase (LPL) (Cani et al., 2009). FIAF is an endocrine factor that plays a role in energy regulation, nutrient response, and lipoprotein metabolism and has the ability to inhibit LPL, which regulates the release of fatty acids from triglyceride-rich proteins (Backhed et al., 2004). Thus, when FIAF is suppressed, there is a concurrent increase in TG storage. Conventionalization of germ-free mice with gut microbiota from conventionally raised mice resulted in suppression of FIAF and a concurrent increase in LPL activity, which caused an increase in the release of fatty acids from triglycerides and subsequent uptake and storage by adipocytes and an increase in fat mass (Backhed et al., 2004). Figure 2 illustrates the mechanisms by which gut microbiota help to harvest energy from the diet and increase lipogenesis while simultaneously suppressing FIAF expression (Cani et al., 2009).

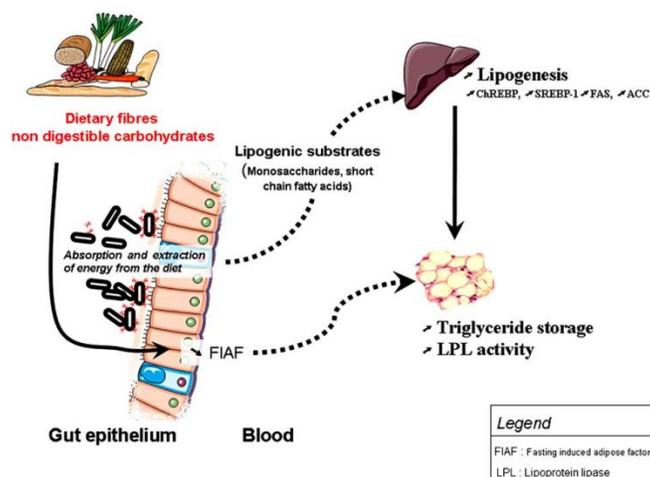


Figure 2. Mechanism by which Gut Microbiota may Increase Fat Storage via FIAF and Excess Energy Extraction from the Diet (Cani et al., 2009).

Diet also has an impact on the composition of the gut microbiome and related changes in gene expression. The types of substrates available in food utilized by bacteria for their growth and colonization plays an integral role in the types and amounts of bacterial species present in the gut. This environmental factor was first illustrated in studies comparing the microbiomes of children from different parts of the world who consumed considerably different diets. Investigations of fecal bacteria composition between children consuming a Western diet and of children in rural Africa revealed a distinct difference between relative amounts of Firmicutes and Bacteroidetes. The microbiota of children ingesting a Western diet included a high number of Firmicutes, which have been previously associated with an obese phenotype, while that of children from rural Africa included an abundance of Bacteroidetes and fewer numbers of Firmicutes (de Filippo et al., 2010). Researchers in this study suggested that throughout history, gut microbiota have been evolving with the changes in diet in order to extract enough calories to survive and thrive (de Filippo et al., 2010). Bacteroidetes are known to preferentially utilize plant fibers, starches and oligosaccharides to produce short chain

fatty acids (SFCAAs) that support colonic epithelial function and also serve as energy substrates (de Filippo et al., 2010). For children in rural Africa, this means that their gut microbial composition supports their survival by efficiently extracting nutrients from their diet. Children and adults consuming a Western diet are most likely not consuming enough plant fibers, starches and oligosaccharides to sustain the Bacteroidetes bacterial colonies and thus exhibit a shift in relative abundance of Firmicutes in these populations.

The Western diet, characterized by a high fat intake, has been associated with a reduction in the amount of *Bifidobacterium* sp. in the feces of diet-induced obese mice on high fat diets (HFD), as compared to mice on a normal-chow regimen (Cani et al., 2007). The relative proportions of *Bifidobacterium* sp. were restored in mice fed HFD when chow was supplemented with the prebiotic, oligofructose (Cani et al., 2007). Results from this study also indicate that the increase in *Bifidobacterium* sp. was positively correlated with improved glucose tolerance and normalized inflammatory tone, which is often associated with metabolic effects resulting from obesity (Cani et al., 2007). In this study, prebiotic supplementation in addition to a high fat diet reduced energy intake and reduced fat mass development compared with mice fed a high fat diet alone (Cani et al., 2007). These findings suggest that dietary approaches to increase the amount of bifidobacteria in the gut may assist in offsetting the adverse metabolic effects associated with diet induced obesity.

Manipulation of Gut Flora using Probiotics, Prebiotics and Synbiotics

Probiotics are defined as live microorganisms that, when ingested in adequate amounts, can provide health benefits to the host (FAO/WHO Working Group Report, 2002). Prebiotics are selectively fermented ingredients that allow specific changes, both

in the composition and/or activity of the GI microbiota that confers benefits upon host well-being and health (Saulnier et al., 2009). Synbiotics are a combination of probiotics and prebiotics, which synergistically affect the host in a beneficial way by improving the survival and colonization of live supplemental microbes in the GI tract (Larsen et al., 2006).

With sufficient evidence to support a role for the gut microflora in obesity, its potential as a novel therapy to treat obesity has gained traction in recent years. Recent observations that *Bifidobacterium* sp. numbers are depleted in obese mice (Ley et al., 2005) as well as obese humans (Ley et al., 2006), suggest that an enhancement in the numbers of bifidobacteria in the gut via dietary means may result in a reduction in fat storage via increased expression of FIAF and reduction of LPL. Probiotics, prebiotics and synbiotics are established and effective approaches to modulating the gut microbiota composition, with an ability to enhance populations of beneficial bacteria in the gut, including *Bifidobacterium* sp. Their potential as a means to prevent or treat obesity thus merits investigation.

Probiotics are typically consumed either in the form of fermented dairy products or as a supplement, and primarily include various strains of lactobacilli and bifidobacteria. Although probiotics' usage has long been suggested for GI disorders, their potential as a tool for weight management is at an exploratory stage. As probiotic effects are often strain-specific and dose-specific, extensive pre-clinical and clinical research efforts may be warranted to identify effective candidates to aid in weight management techniques and/or reduce adiposity (Quigley, 2010).

Although not all probiotics strains colonize the gut and many exert their effects transiently while passing through the gut, many investigators look at colonization as an indicator of potential health benefits. One study by Larsen et al. (2006) investigated the dose-response of *Bifidobacterium animalis* subsp. *lactis* BB-12 (*B. lactis* BB-12) and *Lactobacillus paracasei* subsp. *paracasei* CRL-431 (*L. paracasei* CRL-431) in a probiotic supplement for gut colonization in healthy young adults. The study found that the amount of *B. lactis* BB-12 increased significantly with increasing dosage amounts, whereas *L. paracasei* CRL-431 did not show a dose-dependent increase (Larsen et al., 2006). These results led researchers to conclude that *B. lactis* BB-12, but not *L. paracasei* CRL-431, can be increased in the human intestines through supplementation. Another study conducted by Savard et al. (2011) confirmed these findings using a fermented dairy product containing *B. lactis* BB-12 and *Lactobacillus acidophilus* LA-5 (*L. acidophilus* LA-5). Supplementation with these strains resulted in an increase in bifidobacteria and lactobacilli and a decrease in pathogenic bacteria after consumption. The results from these studies indicate that gut bacteria can be positively influenced and modified with the use of products containing probiotics. However, these studies only reported on the increase in bacterial counts during the consumption period and did not investigate the length of time these bacterial numbers remained increased after consumption had ceased.

Prebiotics are non-digestible carbohydrates that include such substances as inulin-type fructans, galactooligosaccharides, and fructooligosaccharides. Certain gut bacteria, such as lactobacilli and bifidobacteria, can utilize prebiotic substances from the diet in order to enhance their own survival and colonization rates. Bacterial fermentation of prebiotic components produces lactic acid and short-chain carboxylic acids that can alter

the intestinal pH and be absorbed in the human GI tract, thus affecting the GI environment and nutrient absorption from the intestines (Quigley, 2010). Prebiotic substances that can be found in some foods and are often included in dietary supplement formulations include fructooligosaccharides (FOS), inulin, and galactooligosaccharides (GOS). Although prebiotics can provide a beneficial effect, ingestion of large doses of prebiotics aimed at increasing the amount of substrates available to beneficial bacteria is often accompanied by GI discomfort, including bloating and abdominal cramping, that may discourage long-term use.

The rationale behind the synbiotic approach is that beneficial bacteria are more likely to survive and proliferate when dietary substrates needed for their growth and survival are immediately available to them for fermentation (Collins et al., 1999). Currently, research regarding the use and efficacy of synbiotic supplements for the treatment and management of obesity is extremely limited and will be addressed in the following section.

Impact of Pro-, Pre-, and Synbiotics on Metabolic Health and Obesity

The goal of probiotics, prebiotics and synbiotics in foods and supplements is to significantly increase the population of beneficial bacteria in order to reduce the incidence of diseases involving pathogens, the immune system, tumors and abnormal serum lipid concentrations (Ooi et al., 2010).

As mentioned earlier, fermented dairy products are the most common mode of intake of probiotics in the diet. Common strains therein are members of *Bifidobacterium* sp. and *Lactobacillus* sp., including *L. acidophilus*, *L. casei*, *B. bifidum*, *B. infantis*, and *B. longum* (Cruz et al., 2010). *In vitro* and *in vivo* studies involving human subjects have

demonstrated that supplementation with yogurt containing probiotic Bifidobacteria effectively raised high-density lipoprotein cholesterol (HDL-C) levels and led to an improvement in the LDL/HDL cholesterol ratio (Kiebling et al., 2002).

El-Gawad et al. (2005) investigated the effects of *B. lactis* BB-12 on the serum cholesterol in rats fed a cholesterol enriched diet. This study showed that rats supplemented with *B. lactis* BB-12 had lower levels of plasma total cholesterol, VLDL and LDL than rats that were not supplemented, and an inverse relationship was observed between bile acid excretion and total plasma cholesterol (El Gawad et al., 2005). When a yogurt product containing *L. acidophilus* and *B. lactis* was given to hypercholesterolemic human subjects, researchers saw a significant decrease in serum cholesterol when compared to subjects who consumed a control yogurt without the probiotic components (Ataie-Jafari et al., 2009).

L. acidophilus is frequently used as a probiotic and is commonly found in yogurts because it is used as the starter culture. One strain of *L. acidophilus*, LA-L1, has been shown to reduce cholesterol *in vitro* and *in vivo* in hypercholesterolemic humans (Lewis et al., 2005; Anderson et al., 1999). Anderson et al. (1999) saw a 2.9% reduction in serum cholesterol concentration after 4-week double-blind, placebo-controlled, cross-over study with LA-L1. Another strain of *L. acidophilus*, LA-5, has been shown to have the capacity to produce conjugated linoleic acid (CLA), which has been associated with a lowering of body fat and reduction of atherosclerotic lesions (Macouzet et al., 2009).

To date, the effect of probiotics on adiposity has mainly been studied in mouse models in which mice were fed a high-fat, Western style diet supplemented with various beneficial bacteria, including *Bifidobacterium* and *Lactobacillus* strains. Lee et al. (2007)

observed reductions in weights of epididymal, inguinal, mesenteric, and perirenal white adipose tissues with corresponding reduction in total body weight. Aronsson et al. (2010) and An et al. (2011) also saw similar reductions in body weights and fat mass of animals consuming diets supplemented with *Bifidobacterium* and *Lactobacillus* sp.

Besides their ability to stimulate the growth and colonization of beneficial bacteria, prebiotics also possess functional and nutritional properties that confer a physiological benefit (DiBaise et al., 2008). Two studies conducted by Cani et al. (2004 & 2006) investigated the effects of FOS consumption in rats fed standard and high-fat diets and found that food consumption and subsequent energy intake were reduced in FOS supplemented groups providing protection from excess weight gain and fat deposition. In a follow-up study, these effects were shown to be the result of modulation of endogenous gut peptides, namely GLP-1 amide and its precursor proglucagon mRNA, that induce satiety, thereby regulating appetite and body weight (Cani et al., 2006). Inulin was shown to reduce the fat and energy intake when used as a component for a fat replacement formula in a sausage patty, thus supporting the role of prebiotics as a means to reduce body weight (Archer et al., 2004). To determine a mechanism by which probiotics can decrease energy intake in humans, Cani et al. (2006) supplemented subjects with FOS for 2 weeks. In this study researchers saw an increase in satiety following breakfast and dinner that corresponded to a 5% reduction in energy intake per day when compared to the placebo group.

Though the mechanisms have not yet been clearly defined, it has been established that there is a strong relationship between alterations in the gut microbiome and host metabolism (Musso et al., 2010). This has resulted in a recent increase in research efforts

aimed at alleviating the incidence of obesity and metabolic abnormalities through modulation of the gut microbiome using probiotics, prebiotics and synbiotics.

An association between changes in the gut microbiome and body weight has been shown in various studies, however, whether they are a cause or consequence of changes in body weight is still under investigation. The mechanisms by which various strains of bacteria, both beneficial and harmful, exert their metabolic effects on the host are also being explored to enable targeted modulation for health benefits. It has been suggested that some strains of bacteria can increase the risk of becoming obese by extracting extra energy from the diet via hydrolyzing normally indigestible carbohydrates and/or by suppressing the expression of FIAF, thus increasing LPL activity and fat storage.

Previous research from our lab has shown that bioactive substances secreted during active growth of a resident species of Bifidobacterium from the adult human gut are able to enhance secretion of FIAF *in vitro*, along with reducing LPL activity and triglyceride accumulation in adipocytes; (Cotten et al., 2011; Pham et al., 2012). Studies involving the consumption of fermented milk products containing probiotics and prebiotics has also indicated beneficial effects on host metabolic health, possibly resulting from a positive impact on gut flora composition (Tables 2 and 3). These studies suggest that increasing the numbers of Bifidobacteria in the human gut may help manage diet-induced adiposity *in vivo*.

Objectives

The *objective* of this study is to determine whether the regular consumption of a synbiotic formulation containing the probiotic strains *L. acidophilus* LA-5, *L. paracasei* CRL-431, *B. lactis* BB-12 and *Saccharomyces boulardii* in combination with the

prebiotics, inulin and galactooligosaccharides (GOS), will favorably impact adiposity and associated biomarkers of metabolic health in overweight and obese subjects. We investigated these objectives via a pilot-scale randomized, double-blind, placebo-controlled trial with these specific aims: (1) to assess the influence of the synbiotic supplement on body composition (body fat percentage); and (2) to investigate the effect of the synbiotic supplement on obesity-related cardiometabolic biomarkers, specifically total cholesterol, low-density lipoprotein cholesterol (LDL-C), HDL-C, triglycerides and glucose levels in fasting blood samples from the study subjects.

The overall aim of this study is to investigate the potential impact of gut flora manipulation via synbiotic supplement consumption on obesity and dyslipidemia. We hypothesize that the mixture of probiotics and prebiotics in this supplement will exert favorable changes in body compositions and related biomarkers by an increase in the numbers of *Bifidobacterium* sp. in the gut and related increases in FIAF levels. Given that majority of the American population is overweight or obese and potentially ‘at risk’ for chronic metabolic diseases, data from this study could help support the use of synbiotics as a supplementary strategy to manage body weight and associated disease risk. This study is one of the first to explore a role for synbiotics in obesity.

CHAPTER II: MATERIALS AND METHODS

Approval for this trial was granted by the Institutional Review Board (IRB) at Texas State University. (Appendices A and B) All researchers and student volunteers obtained Collaborative Institutional Training Initiative (CITI) certifications to comply with ethics when working with human subjects.

Study Design

This study was conducted as a parallel, double-blind, randomized, placebo-controlled trial. The total duration of the study was 18 weeks. This timeframe included a 2-week run-in period before randomization of subjects into either the treatment or placebo group, a 12-week intervention period, and a 4-week follow-up after supplementation had ceased (Figure 3). This study design and duration was in line with the objective of assessing the effects of a synbiotic supplement on adiposity and lipid biomarkers. The target study population was overweight or obese, but otherwise healthy sedentary or low-active individuals.



Figure 3. Timeline of Assessments for Study Subjects.

Timeline of Assessments

Subjects were required to report to the nutrition laboratory on the Texas State University campus a total of five times over the study period. These visits were scheduled at weeks -2, 0, 6, 12 and 16. Figure 4 shows the timeline of assessments and measurements/samples obtained at each visit.

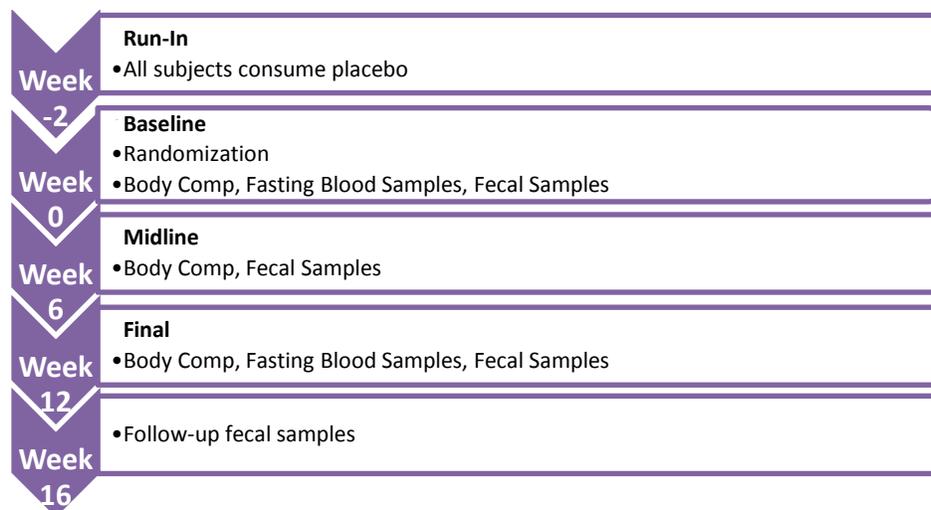


Figure 4. Timeline of Subject Assessment and Measurements Obtained at Each Visit.

All subjects unknowingly completed a two week run-in period (weeks -2 to 0), during which they consumed Product H (placebo capsules), before being allocated to either Group F or Group L for the 12-week intervention period (weeks 0 to 12). The run-in time period was intended as an adjustment period to enable subjects to become accustomed to taking a daily supplement and also allowed researchers to address any issues concerning bowel diary completion with subjects during the second visit. During the second visit at week 0, designated as baseline, anthropometric and body composition measurements were taken, GPAQ was completed, a fasting blood sample and a fecal sample were collected and each subject was given six weeks' worth of supplement, either Product F or Product L. Subject allocation into their respective groups was accomplished

using adaptive randomization software that matched subjects for age, BMI, and gender (University of Reading, Reading, Berkshire). The third visit at week 6 included anthropometric measurements, body composition, GPAQ, and fecal sample collection to obtain midline reference values for each parameter. Additional supplement was provided at this time-point for the following six weeks of supplementation. Visit number four during week 12 included anthropometric measurements, body composition, GPAQ, fasting blood sample, and fecal sample for final measurement values. At this time point, supplementation was discontinued and the following four weeks were designed as follow-up period to monitor any effects after supplementation had ceased. Visit 5 at week 16 included only a fecal sample to determine the rate of washout of gut bacteria following cessation of supplementation.

Synbiotic and Placebo Composition

The synbiotic supplement or ‘treatment’ used in this investigation was a commercially available product, called ProSynbiotic, manufactured by Standard Process (Palmyra, WI). A daily serving of the product (3 capsules) contains 340 mg of a probiotic blend of *Bifidobacterium lactis* (BB-12[®]), *Lactobacillus acidophilus* (LA-5[®]), *Lactobacillus paracasei* (L. casei 431[®]), and *Saccharomyces boulardii*, providing 4 billion colony forming units (CFUs) of probiotic bacteria; along with the prebiotic components inulin (1 g), and galactooligosaccharide (GOS) obtained from milk (100 mg). Maltodextrin capsules of identical form were used as the placebo. Both the treatment and placebo capsules were supplied in a ‘blinded’ manner by Standard Process, in the amounts of 75 capsules per glass bottle labeled as ‘Product L’ or ‘Product F’. In addition,

they also supplied the run-in capsules ‘Product H’, the composition of which was the same as placebo. The inactive ingredients used for formulation were identical in all cases.

Recruitment of Study Subjects

Flyers advertising the study and recruiting participants were posted to information boards on the Texas State University campus, as well as in the local San Marcos, TX and Austin, TX communities (Appendix C). The flyers called for participation from overweight but otherwise healthy individuals between the ages of 18 to 64. Electronic versions of the same flyer were posted to the Texas State University Nutrition program’s website and to www.Craigslist.org under the community: volunteer sections for San Antonio, San Marcos, and Austin. Potential participants were encouraged to call or email researchers for more information and details regarding participation in the study. Community Impact, a local community newspaper, was also utilized to reach out to potential subjects in the Buda, Kyle, and San Marcos communities. The necessary a priori sample size was determined to be 26 subjects per intervention group; using an online sample size calculator (Daniel Soper, California State University, Fullerton, Statistics Calculators version 2.0*). The recruitment goal was thus set as 30 subjects per group. Subjects chosen for this study were given a two-hundred dollar incentive in the form of fifty dollar gift cards to HEB. These incentives were distributed during site visits at weeks 0, 6, 12, and 18.

Screening of Study Subjects

Eligibility of potential subjects for participation was initially assessed using an online screening questionnaire (Appendix D), which was self-administered by each

prospective subject. Potential subjects who were unable to complete the online versions were either sent a paper copy of the questionnaire or were administered the questionnaire over the phone by a trained researcher. Individuals were considered for inclusion if they were between the ages of 18 to 64 years and considered overweight or obese, as determined by a BMI between 25 and 34.9 kg/m², and/or had a waist circumference of > 35 inches for women and > 40 inches for men. Exclusion from the study was determined by a 'yes' answer to any of the following criteria: (a) currently on a weight loss regimen, (b) antibiotic use within the previous three months, (c) regular probiotic, prebiotic, or synbiotic use within the previous month, (d) regular use of anti-inflammatory drugs, weight-loss medication, or supplement within the previous month, (e) personal history of cardiovascular disease, hypertension, cancer, type 1 or type 2 diabetes, or inflammatory GI disorders such as Crohn's disease or colitis, (f) smoking, (g) consumption of more than 2 servings of alcohol per day, (h) pregnancy or lactation, (i) irregular periods, menopause, or hormone replacement therapy, and (j) more than three hundred minutes of exercise per week. Regular use of a medication or supplement was defined as more than three times per week. Eligible individuals were then asked to provide a fasting blood sample, obtained by a trained phlebotomist at the Texas State University's Student Health Center, to be analyzed for blood count, liver function, lipid levels, and fasting glucose in order to ensure that all values were within the normal reference range and the individual could be considered clinically healthy.

Progression of Subjects through the Study

Individuals interested in participating in this trial were first asked to review and sign the study consent form (Appendix E) outlining the details of the study as well as the risks and benefits associated with participation. Using an online screening questionnaire (Appendix D), 404 individuals were pre-screened for inclusion into the study. Of these, 325 were excluded based on the presence of one or more exclusion criteria ($n = 171$), declined participation by the individual ($n = 41$), or lack of response to contact by emails or follow-up phone calls ($n = 113$). Seventy-nine individuals were accepted as study participants, of which three declined participation before randomization. Seventy-six subjects were randomized and assigned to either Group F ($n = 40$) or Group L ($n = 36$). In Group F, five subjects opted out of the study before the intervention began and eight more decided to discontinue the intervention before the intervention period was complete, leaving twenty-seven subjects in that group at the time of completion. In Group L, two subjects opted out of the study before beginning the intervention and four more decided to discontinue the intervention before the intervention period concluded, leaving thirty subjects in Group L at the time of completion. One subject in the Group F and two in Group L were excluded from the analysis after trial completion due to antibiotic use and non-compliance. The progression of participants through each stage of the randomized trial is shown in the consort diagram (Fig. 5).

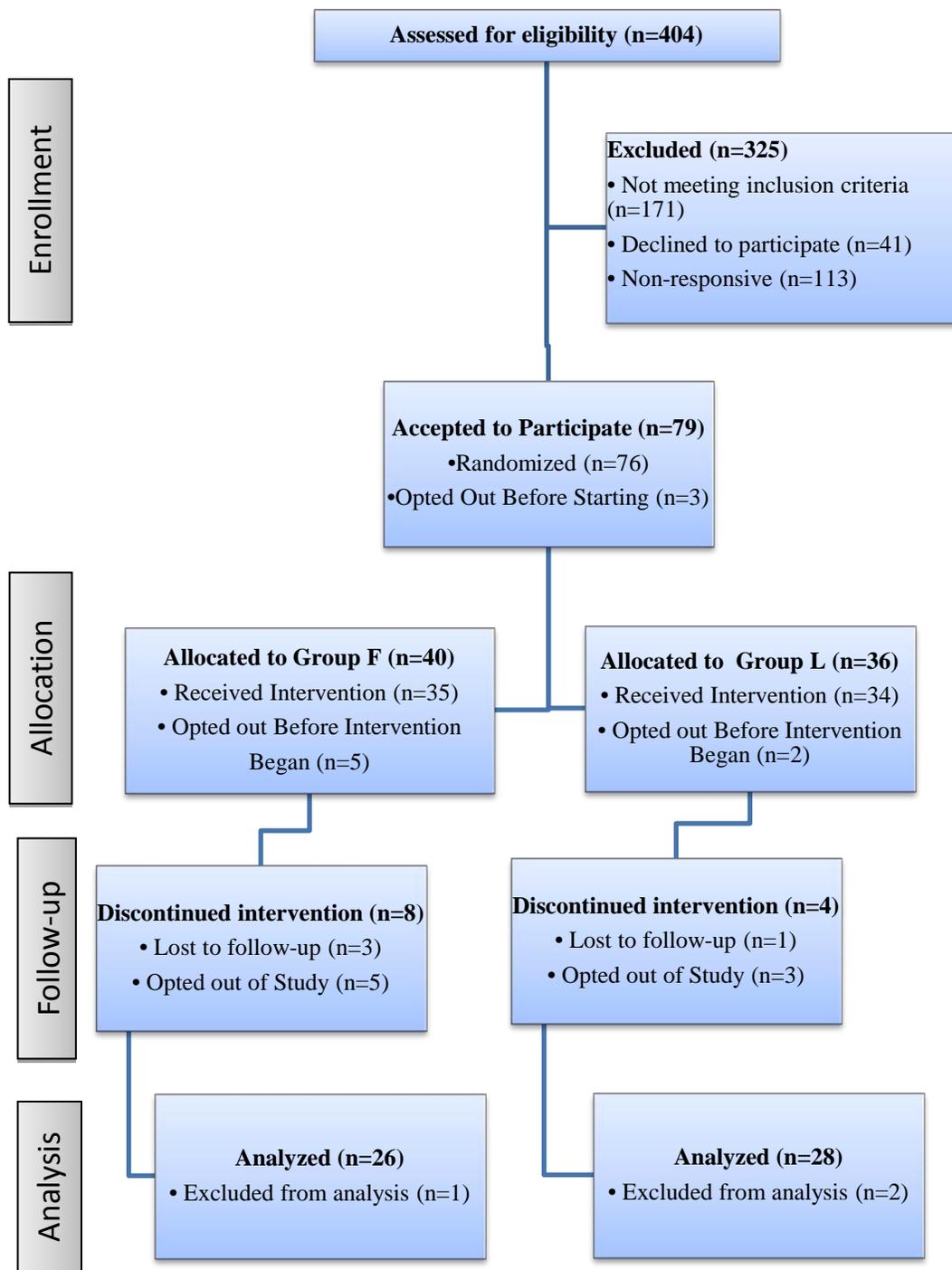


Fig. 5. Consort Flow Diagram Depicting the Progression of Subjects Through the ProSynbiotic Trial.

Pre-intervention Counseling of Subjects

At their first visit, each subject was supplied with an information binder containing a schedule of assessments and all required instructions regarding supplementation, diet, fecal sample collection, fasting blood draw, and dietary recalls. This binder also included the forms for a Bowel diary to be completed daily by each subject and a Global Physical Activity Questionnaire (GPAQ) to be completed with the help of a researcher during each subsequent visit. Each subject visited the site of the study (Nutrition wing of the School of Family and Consumer Sciences at Texas State University) a total of five times throughout the study period, at weeks -2 (begin run-in), 0 (baseline), 6 (midline), 12 (final) and 16 (follow-up).

Supplementation/Intervention

Subjects were instructed to take 3 capsules (340 mg total) of their assigned product and an eight ounce glass of water with breakfast every day for the 14-week period. Product H was consumed by all subjects for the first two weeks (week -2 to week 0), after which time each subject was allocated to either Group F or Group L and instructed to consume the corresponding product for the remaining supplementation period (week 0 to week 12). Subjects were supplied with sealed bottles of the respective product at weeks -2, 0 and 6 and were asked to return those bottles at the following visit (weeks 0, 6 and 12). This protocol was used to monitor subject compliance by allowing researchers to count the amount of supplement left in the bottles and determine the number of capsules consumed between visits. Compliance was also monitored with the use of a 24-hour dietary recall system for dietary analysis.

Subjects were permitted to continue the use of multivitamin supplements during the intervention period, but asked to refrain from consuming any other dietary or weight loss supplements and exclude food products or supplements containing prebiotics, probiotics or synbiotics for the duration of the study. A comprehensive list of such food products (Appendix F) was reviewed with them by research staff and was also provided to each subject in their information binder.

Diet

All subjects were instructed to maintain their routine dietary habits and avoid making any intentional or drastic changes to their habitual diet with respect to food choices and caloric intake, while enrolled in the study. Dietary intake was monitored by researchers by performing dietary recalls at weeks -2, 0, 6, and 12.

Physical Activity

Subjects were screened prior to inclusion in the study to ensure that they did not engage in more than 300 minutes of physical activity per week. To monitor any changes in physical activity, the Global Physical Activity Questionnaire (GPAQ) (Appendix G), developed by the World Health Organization's Department of Chronic Diseases and Health Promotion, was administered during the first four visits (week -2, 0, 6, and 12) by a trained researcher. This 16-question tool was designed to collect information on physical activity participation in three settings (Activity at work, Travel to and from places, and Recreational activities) as well as sedentary behavior. A guide to administration and analysis of the GPAQ was obtained from the World Health Organization's website (www.who.int/chp/steps/GPAQ/en/).

Clinical and Anthropometric Assessments

Blood pressure and anthropometric data, including height, weight, body mass index (BMI), and waist circumference were obtained during each of the first four subject visits (weeks -2, 0, 6 and 12). Before blood pressure was taken, subjects were instructed to sit upright and rest for 15 minutes. Blood pressure measurements were taken using a digital Beurer wrist blood pressure monitor (Win Health Ltd, Jedburgh, Scotland). Three measurements of systolic and diastolic blood pressure were recorded and the average was calculated for data entry. Three measurements of height were taken using a digital, wall-mounted stadiometer (Accurate Technology, Inc., Fletcher, NC). Heights were recorded and averaged for data entry. Body weight was measured during the body composition assessment using the Bod Pod scale as described in the next section. Average height and weight were used to calculate BMI ($\text{weight (kg)} \div \text{height (m)}^2$). An average of three measurements of waist circumference was obtained by a trained researcher who followed the guidelines for measuring tape position as outlined by the National Heart Lung and Blood Institute (www.nhlbi.nih.gov/guidelines/obesity/e_txtbk/txgd/4142.htm).

Body Composition Assessment via Air Displacement Plethysmography

Principle of Air Displacement Plethysmography

During subject site visits at week -2, 0, 6 and 12, body composition was measured using the BOD POD[®] Gold Standard Body Composition Tracking System developed by Life Measurement, Inc. (Chicago, IL). The BOD POD is a two-chambered unit designed to accommodate the subject in the front, or test chamber, and monitor changes in pressure during testing using the instrumentation housed in the back, or reference chamber.

Detailed operating principles of the BOD POD and measurement calculation are described in the operator's manual distributed by COSMED USA, Inc. Briefly, the body volume of the subject is determined by pressure changes that occur between the test chamber and the reference chamber when the subject is seated inside the test chamber. This technique for determining body volume is known as air displacement plethysmography (ADP).

ADP uses the principles of Boyle's Law to show the relationship between pressure ratios in each chamber and can be defined as:

$$\frac{P_1}{P_2} = \frac{V_2}{V_1}$$

where P_1 and V_1 are initial pressure and volume of the test chamber before the subject enters, and P_2 and V_2 are final pressure and volume measured while the subject is in the test chamber. Using this concept, the amount of air displaced by the subject in the test chamber will equal the body volume of the subject. However, since this equation is based on isothermal conditions that are difficult to maintain in the test chamber environment, the BOD POD functions under adiabatic conditions (i.e. air temperature is gaining/losing heat). For this scenario, Poisson's Law more accurately describes the relationship of pressure and volume changes in the BOD POD and can thus be defined as:

$$\left(\frac{P_1}{P_2} \right) = \left(\frac{V_2}{V_1} \right)^\gamma$$

where P_1 and V_1 are initial pressure and volume of the test chamber before the subject enters, and P_2 and V_2 are final pressure and volume measured while the subject is in the

test chamber, and γ is the ratio of specific heat of the gas at constant pressure to that at constant volume ($\gamma = 1.4$ for air). Isothermal air, like air in the lungs, air trapped within fabric of clothing, and air trapped within hair on the head and body, must also be taken into account for body volume measurements. To account for influence of isothermal air, thoracic gas volume is measured, and subjects wear tight fitting synthetic shorts and, for women, bras, as well as a latex cap to compress hair. Calculations for all of these factors are automatically computed by the BOD POD system to determine subject body volume.

To estimate the amount of fat and lean (fat free) tissue in the body, the BOD POD system utilizes the principles of whole body densitometry. Whole body densitometry determines body density (D_B) by measuring body mass (M_B) and body volume (V_B). The formula for calculating body density can be defined as:

$$D_B = \frac{M_B}{V_B}$$

Body mass is measured by weighing the subject using the BOD POD electronic scale, which is linked to the BOD POD computer system. Body weight is automatically saved by the system and used in the calculation of body density after body volume has been measured.

Once body density is calculated, the subject's percent fat mass and absolute amounts of fat mass and fat free mass are automatically calculated by the BOD POD software using the principles of whole body densitometry, as described within the operator's manual provided by COSMED USA, Inc. This system uses previous research from cadaver studies, performed to determine the densities of fat and fat free mass based on age, race and gender to determine the weights and percentages of fat and fat free mass

of study subjects. Fat free mass includes protein, water, mineral and glycogen composition and uses the known densities of each component for this calculation. Body fat and fat free mass percentages and absolute values can be calculated from this information and by using several different equations that have been developed to account for population differences.

BOD POD Assessment of Body Composition

All body composition assessments were conducted in the morning between 7:00 am and 12:00 am. Subjects were instructed refrain from eating, drinking or engaging in any physical activity for 2 hours prior to body composition assessments as these variables would affect body composition measurements. All subjects were given spandex shorts and a swim cap to wear for testing in order to minimize the influence of isothermal air. For this same reason, female subjects were asked to wear a sports bra for use during the body composition analysis. Before testing, subjects were instructed to change into the clothing required for testing and remove all jewelry in order to obtain an accurate reading of body density.

Once the subject was dressed appropriately, a researcher instructed the subject regarding procedures to obtain thoracic gas volume, weight, and body density using the BOD POD system. Thoracic gas volume was obtained during each subject's first site visit at week -2, after which this measurement was used for all consecutive visits. If thoracic gas volume could not be obtained at the first site visit due to any reason, this measurement was estimated by the system, and the measurement was attempted again at the second site visit at week 0. The program estimates thoracic gas volume using a standard prediction equation, explained in detail in the BOD POD Operator's Manual. If

this measurement was still not attainable (due to an inability of some subjects to breathe as per the required pattern), the estimated thoracic gas volume of the subject was used for all body composition analyses. To obtain the measurement of body density, subjects were instructed to enter the seating chamber, sit still during the measurement, and breathe normally for the duration of the assessment. Two measurements lasting approximately 1 minute each were conducted as per BOD POD protocol and a third measurement was sometimes necessary to obtain an accurate measurement. Upon conclusion of the body composition assessment by the BOD POD system, values for body mass (weight), percentage of fat mass and fat free mass, and absolute amounts of fat and fat free mass were recorded as outcome measures for later analysis.

Blood Sample Collection

Fasting blood samples were collected during subject visits at week -2 (Baseline) and week 14 (Final) for analysis of lipid and glucose levels. Subjects reported to the Student Health Center on the Texas State University campus where they were met by a student researcher for assistance. All subjects were instructed to fast for 10 hours prior to blood sample collection and avoid consuming large meals high in fat and calories the night before the fasting blood sample was to be collected. Subjects were also instructed and drink sufficient amounts of water throughout the 10-hour fast to prevent dehydration and ensure easy sample collection. We collaborated with Dr. Emilio Carranco, Director of the Student Health Center at Texas State University in San Marcos, TX to perform standard laboratory analysis of fasting blood samples. A trained phlebotomist collected 16 mL of fasting blood into two BD Vacutainer Cell Preparation tubes containing sodium heparin for isolation of PBMC and plasma for gene expression and immunological assays

not reported in this thesis. An additional 2 mL of fasting blood was drawn into a serum-separating tube (SST) for analysis of triglycerides, total cholesterol, HDL, LDL, total cholesterol to HDL ratio, and fasting glucose. Samples were analyzed by a clinical laboratory contracted by the Student Health Center.

Dietary Recall

To monitor intentional or unintentional changes to diet during subject inclusion in the study, dietary recalls were conducted using the Nutrition Data System for Research (NDSR) developed by the University of Minnesota (Minneapolis, MN). Dietary intake data was gathered by a trained research assistant using a multiple-pass approach interview methodology to obtain dietary intake information over the previous 24-hour period. Dietary recalls were conducted either over the phone prior to the subject's upcoming visit or in-person during subject visits. The interviewer collected detailed information from each subject about each reported food and beverage, including the amount consumed and the method of preparation, and information regarding the subject's use of supplements. Information regarding supplement use was used to monitor subject adherence to protocol and supplementation instructions. Upon completion, the information was reviewed for completeness and correctness and nutrient content of all food and beverage items consumed was calculated by the software.

Data Analysis

Statistical analysis was performed using SPSS® software, Version 20.0 - GradPak (SPSS, Inc., Chicago, IL). Differences within groups were assessed by using Wilcoxon-Sign Rank test. Analysis of covariance was used to compare differences between groups

over time, while controlling for confounding variables of age, gender, weight, and measurements obtained at baseline assessments. Values are stated as means \pm standard deviation unless otherwise stated. All tests were considered to be significant at p -value < 0.05 .

CHAPTER III: RESULTS

Baseline Characteristics of Subjects

Subject characteristics at baseline are shown in Table 5. The subject characteristics were matched at baseline, with no significant differences in any characteristic except total cholesterol. The number of males and females in each group was similar, with more females than males in both groups. The average BMI for each group fell within the overweight classification of 25 to 30 kg/m².

There were no significant differences between the two groups with regards to body composition measurements (body mass, fat mass, % fat mass, fat free mass and % fat free mass), BMI, waist circumference or blood pressure. Blood pressure and fasting blood lipid values were within normal reference ranges as indicated in Table 5. The measured values for fasting blood lipids and glucose were also similar between the two groups, with the exception of total cholesterol levels. Mean total cholesterol levels in Group F (162.92 ± 34.31 mg/dL) were significantly lower than in Group L (180.71 ± 31.13) at baseline as indicated by $p < 0.05$.

Table 5. Baseline Characteristics of Study Subjects†			
Characteristic [normal values]	Group F (n=26)	Group L (n=28)	P Value*
Men	7	9	
Women	19	19	
Age (yr)	28.46 ± 8.39	30.54 ± 9.51	0.394
Body Mass (kg)	83.316 ± 11.13	82.62 ± 14.71	0.782
Fat Mass (%)	37.75 ± 7.56	35.34 ± 8.96	0.382
Fat Free Mass (%)	62.25 ± 7.56	64.66 ± 8.96	0.382
Fat Mass (kg)	31.53 ± 7.83	30.06 ± 11.54	0.377
Fat Free Mass (kg)	51.78 ± 8.88	53.32 ± 11.57	0.653
BMI (kg/m ²)	29.39 ± 3.36	28.99 ± 4.04	0.634
Waist Circumference (cm)	38.82 ± 3.14	39.13 ± 4.21	0.910
Females	38.90 ± 2.55	38.62 ± 3.80	0.729
Males	38.61 ± 4.64	40.21 ± 5.02	0.536
Systolic BP (mmHg)	119.12 ± 9.53	117.21 ± 10.70	0.410
[< 120 mmHg]			
Diastolic BP (mmHg)	73.04 ± 7.73	72.57 ± 8.43	0.567
[< 80 mmHg]			
Total Cholesterol (mg/dL)	162.92 ± 34.31	180.71 ± 31.13	0.039
[125-200 mg/dL]			
HDL-Cholesterol (mg/dL)	51.00 ± 9.39	52.71 ± 11.25	0.567
[> 40 mg/dL, men]			
[> 46 mg/dL, women]			
LDL-Cholesterol (mg/dL)	93.42 ± 27.44	105.64 ± 25.20	0.096
[< 130 mg/dL]			
Triglycerides (mg/dL)	92.15 ± 44.75	112.21 ± 51.43	0.141
[< 150 mg/dL]			
Glucose (mg/dL)	88.58 ± 8.68	88.68 ± 5.91	0.493
[65-99 mg/dL]			
† values are means ± standard deviation			
* p-value < 0.05 indicates significant difference between treatment and control.			

Anthropometric Measurements

Table 6 displays the values of measured anthropometric parameters for each group before and after the 12-week intervention, i.e., body mass, fat mass, % fat mass, fat-free mass, % fat-free mass, BMI and waist circumference. Measured blood pressure values pre- and post-intervention are also indicated. Fat mass decreased in Group L group

by 0.79 kg, with a corresponding increase in fat free mass of 0.38 kg, although these changes did not reach significance. No changes in these values were seen in Group F nor were they significantly different between the two groups. Other body composition measurements, as well as BMI and waist circumference values also remained unchanged in response to intervention in either group with no significant difference between groups. Unexpectedly, there was a reduction seen in systolic blood pressure ($119.12 \text{ mmHg} \pm 9.53$ vs. $113.92 \text{ mmHg} \pm 8.55$) and diastolic blood pressure ($73.04 \text{ mmHg} \pm 7.73$ vs. $69.85 \text{ mmHg} \pm 7.10$) in Group F between baseline and final measurements, respectively. There was no difference in blood pressure between the groups however.

	Group F			Group L			<i>p</i> -value [‡]
	<u>Baseline</u>	<u>Final</u>	<u><i>p</i>-value[†]</u>	<u>Baseline</u>	<u>Final</u>	<u><i>p</i>-value[†]</u>	
Body Mass (kg)	83.32 ± 11.13	83.62 ± 11.97	0.523	82.62 ± 14.71	82.97 ± 15.12	0.332	0.737
Fat Mass (kg)	31.53 ± 7.83	31.77 ± 9.01	0.660	30.06 ± 11.54	29.27 ± 9.87	0.385	0.214
Percent Fat Mass	37.75 ± 7.56	37.72 ± 8.22	0.956	35.34 ± 8.96	35.12 ± 9.18	0.540	0.633
Fat Free Mass (kg)	51.78 ± 8.88	51.86 ± 8.86	0.784	53.32 ± 11.57	53.70 ± 11.86	0.112	0.189
Percent Fat Free Mass	62.25 ± 7.56	62.28 ± 8.22	0.956	64.66 ± 8.96	64.90 ± 9.18	0.540	0.633
BMI (kg/m ²)	29.39 ± 3.36	29.42 ± 3.56	0.819	28.99 ± 4.04	29.11 ± 4.10	0.242	0.634
Waist Circumference (cm)	38.82 ± 3.14	39.02 ± 3.53	0.585	39.13 ± 4.21	39.26 ± 4.36	0.639	0.910
Systolic Blood Pressure (mmHg)	119.12 ± 9.53	113.92 ± 8.55	0.018	117.21 ± 10.70	117.29 ± 9.05	0.909	0.410
Diastolic Blood Pressure (mmHg)	73.04 ± 7.73	69.85 ± 7.10	0.037	72.57 ± 8.43	71.86 ± 6.55	0.562	0.567
* mean values ± standard deviation							
† <i>p</i> -value < 0.05 indicates significant difference between baseline and week 12							
‡ <i>p</i> -value < 0.05 indicates significant difference between treatment and control at week 12							

Metabolic Biomarkers

Table 7 shows concentrations of total cholesterol, HDL-C, LDL-C, triglycerides and glucose at baseline and final time points for each group. There were no significant differences seen in serum lipid levels or serum glucose concentrations within either group over the treatment period or between groups at final measurements. Analysis of differences between groups at the final time point were completed using analysis of covariance (ANCOVA) with baseline measurements as covariates to account for any differences between groups at baseline. Within Group L, total cholesterol levels did not differ before and after supplementation (180.71 mg/dL \pm 31.13 vs. 179.46 mg/dL \pm 22.80), nor did LDL-C levels (105.64 mg/dL \pm 25.20 vs. 102.43 mg/dL \pm 21.61). Similar results were observed in Group F. Before supplementation total cholesterol and LDL-C levels were 162.92 \pm 34.31 and 93.42 \pm 27.44, respectively. After supplementation, total cholesterol and LDL-C levels were not significantly different (162.04 \pm 33.30 and 92.27 \pm 23.71, respectively).

	Group F			Group L			P value ^{‡,a}
	Baseline	Week 12	P value [†]	Baseline	Week 12	P value [†]	
Total Cholesterol (mg/dL)	162.92 \pm 34.31	162.04 \pm 33.30	0.732	180.71 \pm 31.13	179.46 \pm 22.80	0.882	0.305
HDL-Cholesterol (mg/dL)	51.00 \pm 9.39	50.77 \pm 12.30	0.799	52.41 \pm 11.25	52.39 \pm 13.26	0.979	0.980
LDL-Cholesterol (mg/dL)	93.42 \pm 27.44	92.27 \pm 23.71	0.602	105.64 \pm 25.20	102.43 \pm 21.61	0.311	0.700
Triglycerides (mg/dL)	92.15 \pm 44.75	94.81 \pm 53.58	0.684	112.21 \pm 51.43	122.68 \pm 73.73	0.446	0.509
Glucose (mg/dL)	88.58 \pm 8.68	89.96 \pm 4.94	0.100	88.68 \pm 5.91	89.00 \pm 6.50	0.720	0.493

* mean values \pm standard deviation
[†] p -value < 0.05 indicates significant difference between baseline and week 12
[‡] p -value < 0.05 indicates significant difference between Group F and Group L at week 12
^a Values obtained using ANCOVA with baseline values as covariate to adjust for differences between groups at baseline

Physical Activity Assessment

Data regarding regular physical activity was collected using the Global Physical Activity Questionnaire, as described in Methods, to monitor changes within each group. No differences were seen within groups or between groups and the level of physical activity stayed the same over time (Table 8).

Table 8. Results of Global Physical Activity Questionnaire				
	0 wk ^{\$}	6 wk ^{\$}	12 wk ^{\$}	<i>p</i> value between wk0, wk6 and wk12
Group L (n=28)	37.5±7.8	33.3±7.0	30.4±7.1	0.496
Group F (n=25)	30.6±7.3	34.4±7.4	31.6±8.2	0.930
<i>p</i> value between groups	0.773			
\$ Data present as mean ± S.E.M, Met-h/ week: Metabolic Equivalent hour per week. Comparison between time points in each group was performed by <i>Kruskal-Wallis test (One-Way ANOVA for nonparametric data)</i> . Comparison between groups over the whole study period was performed by <i>Mixed-Design 'Split plot' ANOVA with baseline as the covariate (General Linear model Repeated Measures)</i> .				

Dietary Assessment

Table 9 shows the average nutrient composition of the diet in both groups as recorded at the baseline and the final dietary recalls. There was a significant increase (*p*-value = 0.018) in carbohydrate consumption from the baseline dietary recall (214.55g ± 84.61) to the final dietary recall (257.03g ± 102.79,) in Group F. No significant changes from baseline were observed within Group L. No significant differences were observed in energy or nutrient intake between the two groups over the duration of the study.

Table 9. Analysis of Nutrient Intake by NDSR Method*

	Group F			Group L		
	<u>Baseline</u>	<u>Final</u>	<u>p-value</u> †	<u>Baseline</u>	<u>Final</u>	<u>p-value</u> †
Energy (kcal)	1976.62 ± 732.31	2091.04 ± 749.30	0.292	1908.18 ± 769.36	1889.03 ± 738.84	0.733
Fat (kcal)	767.15 ± 354.46	712.19 ± 364.52	0.424	732.00 ± 423.48	730.29 ± 425.44	0.868
Total Fat (g)	87.05 ± 40.48	80.95 ± 41.41	0.439	83.25 ± 47.92	82.89 ± 47.86	0.820
Saturated Fat (g)	29.51 ± 14.47	25.89 ± 15.26	0.151	27.07 ± 20.75	29.77 ± 18.46	0.350
Trans Fat (g)	3.60 ± 2.57	2.53 ± 1.55	0.069	2.28 ± 2.12	2.69 ± 3.51	0.316
Cholesterol (mg)	354.88 ± 434.31	288.35 ± 164.41	0.694	261.96 ± 276.10	316.64 ± 288.32	0.158
Sodium (mg)	3727.23 ± 1809.44	3829.00 ± 2235.53	0.909	3267.00 ± 1543.33	3439.11 ± 1241.62	0.509
Carbohydrate (g)	214.55 ± 84.61	257.03 ± 102.79	0.018	217.21 ± 83.10	207.94 ± 86.19	0.716
Fiber (g)	18.61 ± 7.70	20.05 ± 10.31	0.949	21.55 ± 13.26	18.63 ± 11.62	0.927
Sugar (g)	79.60 ± 57.25	87.32 ± 53.04	0.409	81.41 ± 33.77	81.35 ± 53.82	0.891
Protein (g)	83.47 ± 37.66	85.59 ± 35.57	0.657	75.88 ± 30.39	81.48 ± 32.93	0.316
Vitamins & Minerals						
Vitamin A (IU)	7607.89 ± 9303.77	5030.50 ± 4675.14	0.909	4756.46 ± 5695.08	7120.07 ± 11871.96	0.151
Vitamin C (mg)	50.13 ± 35.63	58.62 ± 50.39	0.657	77.27 ± 55.46	74.18 ± 77.35	0.633
Calcium (mg)	810.65 ± 364.55	963.27 ± 631.43	0.341	822.04 ± 436.41	899.54 ± 429.44	0.699
Iron (mg)	13.53 ± 6.51	15.32 ± 8.99	0.238	14.69 ± 8.28	14.31 ± 8.18	0.855

* mean values ± standard deviation
† p-value < 0.05 indicates significant difference between baseline and week 12
‡ p-value < 0.05 indicates significant difference between Group F and Group L at week 12

CHAPTER IV: DISCUSSION AND FUTURE DIRECTIONS

Based on previous research closely linking the incidence of obesity to variations within the gut microbiome, a synbiotic supplement containing several strains of *Lactobacillus* sp. and *Bifidobacterium* sp. in a formulation with prebiotics, inulin and GOS, was evaluated for its potential effects on adiposity and related metabolic biomarkers. Although the intricate relationship between the human microbiome and obesity is still being investigated, it seems plausible that manipulating the microbial composition of the intestinal tract with the use of prebiotics, probiotics and synbiotics to promote the growth and survival of beneficial bacteria can afford protection against obesity and metabolic syndrome. While several clinical studies have explored the role of probiotics or prebiotics in metabolic health (Agerholm-Larsen et al., 2000; Lu et al., 2004; Parnell et al., 2009; Kadooka et al., 2010; de Luis et al., 2011; Ejtahed et al., 2012; Vulevic et al., 2013), not many have looked at synbiotics. One of the reasons for this may be the less than spectacular results obtained in many studies with probiotics, with only one trial reporting a significant decrease in anthropometric measures (Kadooka et al., 2010) and modest impact on blood lipids in others. Also in clinical studies, probiotics, as well as prebiotics, tend to be more effective in improving lipid profiles in individuals with dyslipidemia, impaired glucose tolerance and type 2 diabetes rather than in clinically healthy subjects. Lastly, with probiotics, strain specificity is a challenge, and with

prebiotics, significant effects are usually obtained at doses unachievable through diet, and high supplemental doses are associated with abdominal discomfort. Bearing all of the above in mind, exploration of synbiotics in this context seems a logical way forward as synbiotics often contain multiple strains of probiotics combined with prebiotics. Presence of prebiotics in theory may increase the efficacy of probiotics by enhancing both survival and growth in the gut and the synergistic effects may potentiate the otherwise modest effects observed. Secondly, by combining both approaches, more realistic quantities of prebiotics may be used in the formulation, enhancing the chances of long-term use by the consumer due to lack of adverse GI symptoms. While a food, rather than supplement-based approach is preferable for health promoting effects of many dietary bioactives, in the case of probiotics, a supplement approach may be preferable. This is because the bacteria lose their viability and efficacy very quickly in a food matrix and many studies report dramatically lower actual numbers of bacteria in food products than those reported on labels. This is especially true of many *Bifidobacterium* sp., which are not as aerotolerant as *Lactobacillus* sp., and thus best consumed in a freeze-dried form as a supplement. As mentioned earlier, a synbiotic approach may enhance the individual benefits of probiotics or prebiotics; in both food and supplement forms.

Based on the above facts, the probiotic strains in the supplement tested were combined with prebiotic carbohydrates, inulin and GOS. These two dietary compounds are widely used in synbiotic formulations because of their safety, stability, resistance to digestion in the upper GI tract and their fermentability in the colon (Macfarlane et al., 2008). *Bifidobacterium* sp. and, to a lesser extent, *Lactobacillus* sp. have been shown to have the ability to grow on these carbohydrate sources (Macfarlane et al., 2008). A study

conducted by Gibson and Beatty et al. (1995) revealed that male volunteers who consumed 15 g/day of inulin had a significant increase in bifidobacteria concentrations in fecal samples. Some *Lactobacillus* sp. have been shown to possess the ability to hydrolyze inulin (de Souza Oliveira et al., 2012). Inulin has been shown to aid in the growth of *Lactobacillus paracasei* subsp. *paracasei* 8700:2 (Makras et al., 2005). However, due to the diversity of the gut microbiome and individual differences in subjects, diets and lifestyles, designing a specific combination of probiotic and prebiotic components for formulation into a synbiotic supplement that will have a beneficial impact on adiposity and metabolic biomarkers in all individuals is extremely challenging.

Differences in adiposity have been associated with variations in gut microbial composition at the phylum level, with a higher proportion of Firmicutes present in obese subjects and a higher proportion of Bacteroidetes seen in lean subjects. Bacterial gene sequencing conducted by Turnbaugh et al. (2009) found that obesity was associated with a significant reduction in the level of bacterial diversity, which may be the result of abnormal energy input associated with our present obesogenic environment. When a reduction in body weight was achieved, there was a corresponding decrease seen in levels of bacteria from the phylum Firmicutes and an increase in the amount of bacteria from the phylum Bacteroidetes (Ley et al., 2005). Considering that the majority of research regarding adiposity and gut microbiota focuses on the change in gut microbiota after weight reduction, this study is one of the first of its kind to investigate whether a change in gut microbiota can induce a positive effect on body weight and adiposity.

One of the key strengths of our study is the design and methods of data collection, giving us a high degree of confidence in the results obtained. These include: a

randomized, double-blinded, placebo-controlled design, considered the gold standard of clinical trials, power analysis to assess the number of subjects required in each arm of the study, data collection on background diet and physical activity, often omitted in trials, and detailed analysis of gut flora changes, often ignored in many trials as well.

We sought to minimize confounding variables by recruiting individuals who had similar BMIs, participated in similar exercise duration and intensity, were not on an active weight loss regimen and who agreed to monitor diet throughout the study, such that any changes in body composition and blood lipids obtained in response to the intervention would likely be the result of the synbiotic supplement being investigated. Our hypothesis was that a synbiotic intervention will produce favorable changes in body composition and associated markers (fasting blood lipids and glucose) as a function of increases in *Bifidobacterium* sp. in the gut, and potential ability to increase FIAF levels based on prior *in vitro* data from our laboratory. While we did note both of these alterations in our study subjects (Group L), i.e. increase in Bifidobacteria numbers in fecal samples and increased gene expression of FIAF (not part of this thesis), the changes did not translate to significant alterations in body composition and blood lipid levels or glucose levels.

However, the results from our study did reveal a slight reduction in body fat mass in Group L (-0.79 kg), while Group F exhibited a slight, but also insignificant, increase in fat mass (+0.24 kg). While studies from our lab were conducted on *B. longum*, a strain not present in ProSynbiotic, in another study by Aronsson et al. (2010), *Lactobacillus paracasei* and *Bifidobacterium lactis* BB12, part of ProSynbiotic formulation, were also shown to upregulate the transcription of FIAF mRNA in colonic cell lines. As mentioned

previously, FIAF is an inhibitor of LPL activity with the potential to reduce triglyceride storage in adipocytes and have a positive impact on body weight and fat accumulation. Given that our subjects in Group L showed a significant change in *Bifidobacterium* sp. and FIAF levels, significant difference in fat deposition as a result of this mechanism might be observed given a larger sample population. Obesity-related interventions are notorious for requiring a largest sample size (Patel et al., 2006), from several hundred subjects to over a thousand subjects for minor treatment effects to become detectable, due to the complex etiology of this disease. The studies typically may also need to be carried out for a longer time period, but that generates the problem of high drop-out rate (typically between 30-80% in weight loss studies) (Huisman et al., 2009), as subjects may lose interest if they do not lose weight while enrolled in the study. As this study was intended as a pilot-scale study, the goal of which is to determine how and whether to launch a full scale study, the data lays sufficient grounds to conduct a full-scale trial of a similar nature.

Dietary intake of macronutrients (energy, carbohydrates, fats, etc.) was not significantly different between groups. However, changes within groups could have affected results within groups even if the changes were not significant. Results from previous studies have shown that various strains of *Bifidobacterium* sp. and *Lactobacillus* sp. can lower cholesterol concentrations *in vitro* (Tahri et al. 1996; Brashears et al., 1998; Pereira et al., 2002). *Bifidobacterium* BB-12 and *Lactobacillus acidophilus*, two of the strains included in our formulation, have specifically been shown to have hypocholesterolemic effects in animal studies and human studies (El-Gawad et al., 2005; Lewis et al., 2005). Although, a comparable study conducted by Larsen et al. (2006),

which used a probiotic supplement containing *Bifidobacterium* BB-12 and *Lactobacillus acidophilus* and was similar in population size (n=71) to our study, and saw no significant change in blood lipids.

While the study has not yet been ‘unblinded’, based on the findings and trends observed in this study, and also data from gut bacterial changes not reported in this thesis, we predict that Group L was the intervention group consuming the ProSynbiotic supplement. Although this pilot study did not reveal any changes in blood lipids, specifically TC and LDL-C, perhaps a larger study population would minimize the effects of individual variation that often accompany human studies in an effort to determine potential benefits, if any, associated with synbiotic supplementation.

In order to minimize any effects from non-compliance or change in dietary behaviors, participants were regularly monitored using dietary recall software throughout the study. Overall compliance with respect to supplement/placebo consumption was 88%, with a compliance rate of 89% and 86% in Group L and Group F, respectively. In terms of diet, Group F exhibited a significant reduction in consumption of *trans* fats (-1.07 g) and a significant increase in carbohydrates (+ 42.48 g) over the course of the study. Group L slightly increased consumption of *trans*-fats (+0.41 g) and reduced consumption of carbohydrates (-9.27 g), though neither change was significant when compared to baseline. The change in carbohydrate consumption of Group F may have impacted our results by unintentionally influencing the proportions of intestinal bacteria through strain specific preference for available carbohydrate substances leading to a blunting effect on differences in some outcomes within groups. Any specific effects of carbohydrate sources

are difficult to pin point in this instance, because dietary recalls were not conducted every day and fiber consumption was not significantly increased in this group.

Regular physical activity was also a potential confounding factor that was controlled for, with the administration of the Global Physical Activity Questionnaire (GPAQ) developed by the World Health Organization at each subject assessment. This questionnaire has been validated and designed to assess physical activity in three domains: activity at work, travel to and from places and recreational activities, as well as collect information on sedentary behavior. Based on our analysis, physical activity was unchanged in either group over the period of the study. Groups were also similar in amounts of physical activity participation before and after the study. Therefore, we have determined that physical activity was not a significant factor in any changes seen in body composition.

Surprisingly, a significant reduction in systolic and diastolic blood pressure was observed in Group F, with over 65% of subjects reducing their systolic and diastolic blood pressure after the 12-week supplementation period. We have no explanation for this reduction except that it could have been the result of some unknown confounding factor that was not addressed in this study.

While well-designed, this study has some limitations. First, samples sizes of both groups was relatively small and included individuals of varying ethnicities, though we did previously determine that an $n=26$ would give statistical power to our data. Secondly, our study subjects were normolipidemic at baseline measurements and considered clinically healthy. Much of the previous research regarding the effects of probiotics on blood lipids is conducted using individuals with hypercholesterolemia or in animal

models with induced hypercholesterolemia due to a high-fat diet. The synbiotic supplement investigated here may prove to have a stronger effect in subjects with elevated cholesterol and triglyceride levels. Third, diets and physical activity were controlled for as much as possible, but were not monitored on a daily basis over the 12-week supplementation period. Future research should include investigations aimed at maximizing the formulation of synbiotic supplements by determining which prebiotic substances are best suited to increase the numbers and growth of specific probiotic organisms. Improved synbiotic supplement formulations will need to be examined for their effectiveness in reducing energy extraction from the diet, increasing satiety of subjects, and their impact on fat accumulation in adipocytes. To accomplish this task, more research needs to be conducted to establish mechanisms by which modulation of gut flora via prebiotics, probiotics, and synbiotics can positively impact health and contribute to a reduction in the obesity epidemic and associated metabolic syndrome seen in today's society.

To summarize, synbiotic supplementation did not significantly affect adiposity or metabolic biomarkers in healthy, overweight individuals in this study. However more research is required to determine if synbiotic supplementation will prove to be a beneficial addition to current weight management strategies.

APPENDIX A

Institutional Review Board Certificate of Approval



Institutional Review Board Application

Certificate of Approval

Applicant: Vatsala Maitin

Application Number : 2011F7611

Project Title: Pilot study on the influence of a synbiotic formulation (ProSynbiotic) on gut microflora composition, adiposity and selected markers of cardiometabolic health in overweight or moderately obese individuals

Date of Approval: 06/24/11 14:31:01

Expiration Date: 06/23/12

Handwritten signature of M. Blunde in black ink.

Assistant Vice President for Research
and Federal Relations

Handwritten signature of Jon Lane in black ink.

Chair, Institutional Review Board

APPENDIX B

Institutional Review Board Certificate of Continuation/Change



Institutional Review Board

Continuation/Change

Certificate of Approval

Applicant: Vatsala Maitin

Original IRB Application Number : 2011F7611

Date of Approval: 06/16/12

Expiration Date: 06/16/13

A handwritten signature in black ink, appearing to read "M. Blonds".

Assistant Vice President for Research
and Federal Relations

A handwritten signature in black ink, appearing to read "Jon Lane".

Chair, Institutional Review Board

Appendix C

Study Advertisement Flyer

PARTICIPANTS NEEDED FOR NUTRITION RESEARCH STUDY



\$200 Incentive!!!

Research Topic: Can increasing 'good bacteria' in the gut through diet help with weight control?

Recruiting: Now!

Study Begins: September 2011



Do You Qualify?

- ✓ Are you overweight or obese but otherwise healthy?
- ✓ Are you between the ages of 18 – 64?

What Will You Need To Do?

- Take a dietary supplement (capsule) daily for 14 weeks.
- Visit the Nutrition Lab at Texas State University for 5 scheduled visits over a 4-month period (mornings).
- Provide 3 blood & 4 stool samples.
- Have body muscle and fat measured in a BodPod (gold standard!).

How Will You Benefit?

- Learn about your health for free! (over \$500 value)
 - Free blood test (blood sugar, lipids, liver enzymes)
 - Free body composition test
 - Free diet analysis
 - Free gut bacteria test
- **\$200 incentive**

Interested? Please email maitinlab@gmail.com or call (512) 245-7054.

Texas State Nutrition Study 512-245-7054 maitinlab@gmail.com								
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APPENDIX D

Study Screening Questionnaire

Screening questionnaire for Texas State ProSynbiotic Study

This questionnaire is an initial assessment of your eligibility to participate in a clinical study designed to explore the effect of a synbiotic* formulation called ProSynbiotic, on the make-up of your gut bacteria, your body composition (body fat percentage) and some markers of health status related to overweight or obesity. [*Contains probiotics ('good' bugs) and prebiotics (food for the 'good' bugs), more information about ProSynbiotic can be obtained by e-mailing maitinlab@gmail.com]

Please answer all questions. If unsure about a question, please contact us at 512-938-2552 or at the e-mail address above for clarification.

1. Name (Last, First):
2. Telephone number (include area code):
3. E-mail address:
4. Preferred means of contact (Phone/e-mail/either):
5. Date of Birth (MM/DD/YYYY):
6. Gender: Male/Female
7. Height:
8. Weight:
9. Waist circumference:
10. Are you actively trying to lose weight (e.g. a weight-loss program, dieting or intense regular exercise of more than 5 hours per week)? *Yes/No*
11. When was the last time you took antibiotics? *Less than 3 months ago/More than 3 months ago/More than 6 months ago*
12. Do you currently consume foods or supplements that contain probiotic bacteria, prebiotics or synbiotics *regularly* (more than 3 times a week)? *Yes/No*

Examples of such products include *DanActive, Biosalud, Activia, Lifeway Probiotic Kefir, Stonyfield Probiotic Yoghurt, Good Belly, Phillips Colon Health, Align, Yakult probiotic drink, Culturelle, Prebiotin, Syntol, VSL #3 and Vitalizer*, please see list on page 4&5 for more examples.

If yes, please list product name(s) _____, and skip to Q-14.
If no, go to Q-13.

13. When was the last time you *regularly* (more than 3 times a week) consumed products containing probiotic bacteria, prebiotics or synbiotics? *Less than 1 month ago/More than 1 month ago/Never*

14. Are you currently taking anti-inflammatory drugs (Steroids or NSAIDs), such as cortisone, Aspirin, Ibuprofen, Motrin, Aleve or Celebrex *regularly* (more than three times a week)? *Yes/No* If yes, please name the drug(s)_____ and skip to Q-16. If no, go to Q-15.

15. When was the last time you *regularly* used anti-inflammatory drugs (more than 3 times a week)? *Less than 1 month ago/More than 1 month ago/Never*

16. Are you currently taking weight-loss medication or a dietary supplement for weight-loss or have done so within the last one month? *Yes/No* If yes, please name the medication(s) or supplement(s)_____.

17. Are you taking any other supplements (including multivitamins)? *Yes/No* If yes, please name the supplements_____.

18. Are you taking fiber supplements such as Metamusil or Benefiber? *Yes/No*

19. Have you undergone weight-loss surgery? *Yes/No*

20. Have you been diagnosed with any of the following?

a. Cardiovascular/Heart disease: *Yes/No*

b. High Blood Pressure: *Yes/No*

c. Cancer: *Yes/No*

d. Type 1 diabetes: *Yes/No*

e. Type 2 diabetes: *Yes/No*

f. Crohn's disease: *Yes/No*

g. Colitis: *Yes/No*

h. Inflammatory Bowel Disease (IBD)? *Yes/No*

i. Antibiotic-associated diarrhoea (Clostridium difficile)? *Yes/No*

j. Gluten allergy: *Yes/No*

k. Lactose intolerance: *Yes/No*

l. Stomach ulcers: *Yes/No*

m. Diverticulitis: *Yes/No*

21. Are you immunocompromised or on immunosuppressant drugs? **Yes/No**
22. Are you a smoker? **Yes/No**
23. Do you consume more than 2 units of alcohol per day? **Yes/No**
24. Do you exercise for more than 300 minutes (5 hours) per week? **Yes/No**
25. If short-listed for the study, will you be willing to provide a fasting blood sample for a blood test (measurement of blood count, liver function, lipid levels and fasting glucose)? **Yes/No**
26. If enrolled in the study, would you be willing to consume a synbiotic supplement called ProSynbiotic from Standard Process or a placebo capsule containing Maltodextrin daily for 14-weeks? **Yes/No**
27. The study capsules contain gelatin. Would this be acceptable to you in case of any dietary restrictions such as vegetarianism? **Yes/No**
28. If enrolled in the study, would you be willing to visit the Department of Family and Consumer Sciences at Texas State University-San Marcos for height, weight, waist circumference and body composition measurements and provide a stool and blood sample (5-6 visits each lasting approximately 2 hours over a period of 4-5 months)? **Yes/No**
29. Do you have regular bowel movements and do you feel reasonably confident that you will be able to produce a stool sample for the study during your scheduled visit? **Yes/No** (If not, alternative arrangements can be made for stool collection)

Questions 30- 35 are to be answered by females only:

30. Are you pregnant or planning for it in the next 6 months? **Yes/No**
31. Are you currently breast-feeding? **Yes/No**
32. Do you have irregular periods? **Yes/No**
33. Are you post-menopausal? **Yes/No**
34. Are you on hormone-replacement therapy (HRT)? **Yes/No**
35. Have you undergone hysterectomy? **Yes/No**

I declare that I have answered the above questions honestly and truthfully to the best of my knowledge.

Signature: _____

Date: _____

APPENDIX E

Study Consent Form

Consent form for Texas State ProSynbiotic Study

IRB Approval #: 2011F7611

Principal Investigator: Dr. Vatsala Malin, Nutrition and Foods Program,
Department of Family and Consumer Sciences, Texas State University, San
Marcos, TX. E-mail: v.malin@txstate.edu

You are invited to participate in this IRB-approved research study exploring the effect of consuming a dietary supplement called ProSynbiotic containing 'good' bacteria (probiotics) and food for the 'good' bacteria (prebiotics) on the make-up of bacteria in your gut, your body composition (amount of body fat and muscle) and some markers of overall health status related to overweight or obesity. The study is sponsored by Standard Process Inc., Palmyra, WI.

What the study is about:

Summary: Type of bacteria in the gut of obese and lean people have been shown to be different, with lower numbers of 'good' bacteria called Bifidobacteria in obese people. In this study, we will investigate if increasing the numbers of these good bacteria via a probiotic and prebiotic-containing dietary supplement (ProSynbiotic) in the gut of people who are overweight or obese but otherwise healthy can help with weight management and related health conditions. The study will monitor changes in the make-up of your gut bacteria, your body-composition, plasma lipids, glucose, markers of inflammation as well the levels of certain genes that affect these changes.

Details:

Scientists have shown that the type of bacteria present in our gut is related to our tendency to be lean or obese by affecting calories extracted from the diet and storage of this energy as fat in the body. The numbers of a type of "good" bacteria in the gut called Bifidobacteria has been reported to be low in obese people. The numbers of these "good" Bifidobacteria can be boosted in your gut by foods or supplements that contain probiotic bacteria or fiber-like indigestible carbohydrates called 'prebiotics'. Products that combine probiotic bacteria and prebiotics together are called synbiotics. In this study, we will explore whether regular consumption of a synbiotic formulation called ProSynbiotic containing probiotic bacteria *Lactobacillus acidophilus* LA-5, *Lactobacillus paracasei* CRL-431, *Bifidobacterium lactis* BB-12 and *Saccharomyces boulardii* in combination with prebiotics inulin and Galactooligosaccharide (GOS) will increase the numbers of Bifidobacteria and Lactobacilli and reduce the numbers of Clostridia (a class of 'bad' bacteria) in the gut of healthy overweight or obese subjects. We suggest that these changes in gut bacterial make-up will favourably impact body composition (reduce fat mass) in these subjects leading to reduced inflammation. The study will involve measurement of

changes in the numbers of Bifidobacteria, Lactobacilli and Clostridia in the stools of study volunteers before and after ProSynbiotic or placebo supplementation, along with alterations in body composition, plasma inflammatory biomarkers, blood lipid and glucose levels. To understand the mechanism behind the changes, levels of expression of relevant genes will also be measured in cells collected from feces and blood.

What you will be asked to do: You will be requested to complete a screening-questionnaire to assess your initial eligibility for the study. If you meet the eligibility criteria and volunteer to participate, you will be requested to provide a blood sample for a blood-test (blood count, liver function, lipid levels, fasting glucose) to confirm that the test values are normal. If your blood-work is normal and you are selected to participate in the study, you will be requested to do the following:

- Consume ProSynbiotic capsules or placebo capsules (maltodextrin) daily for the duration of the study (12 weeks), at the recommended dose, while maintaining your normal diet and physical activity level.
- Report after an overnight fast (In the morning) to the Nutrition wing of the Family and Consumer Science department of Texas State University-San Marcos for 5 scheduled visits (Weeks -2, 0, 6, 12 and 16) for data and sample collection.
 - You should not eat or drink anything aside from water before your visit. You should also not exercise for at least two hours before your visit.
 - Data collection will include measurement of your height, weight, waist circumference, blood pressure and body composition (body fat percentage). Body composition will be measured using an instrument called a Bod-Pod, where you will be asked to sit inside an egg-shaped enclosed chamber for 2 minutes, to measure the amount of air displaced by your body for calculation of your body fat percentage. For this test, women will need to wear a swimsuit or lycra shorts and a sports bra and men a speedo or tight lycra shorts. A swim cap will also need to be worn. If you do not own these items of clothing, these will be provided to you by the investigator upon prior notice. If you feel claustrophobic during the 2-minute test period, the measurement can be stopped at any time.
 - Sample collection will include 4 stool samples (weeks 0, 6, 12 and 16) and 2 blood samples (weeks 0 and 12)
 - You will also be requested to complete a physical activity questionnaire (maximum of 16 questions).
 - Each visit is expected to last approximately 2 hours.
 - You will be provided breakfast at the end of each visit.

- Participate in a 24-hour dietary recall over the telephone on 4 occasions during the course of the study where you will be asked about what you ate over the past 24 hours. Each call is expected to take 20-30 minutes.
- Stick to the diet and physical activity guidelines provided to you as a participant in the study.

Risks and benefits: The study carries minimal risks as the probiotics and prebiotics present in ProSynbiotic, and maltodextrin present in the placebo capsules have a long history of use in foods and supplements without any reported ill-effects in healthy subjects aside from occasional abdominal cramps or flatulence. The yeast strain present in ProSynbiotic (*Saccharomyces boulardii*) has been reported to cause fungemia at high doses in patients which are immunosuppressed, those with intravenous catheters, diabetes or antibiotic-associated diarrhoea and those on parenteral nutrition. Risks associated with study procedures include discomfort during drawing of blood in individuals with difficult veins or during Bod-Pod measurement if the subject is claustrophobic. Some participants may feel disappointed or angry if participation in the study does not result in a perceivable benefit such as weight-loss. Based on literature and preliminary experimental data, potential benefits may include improved gut bacteria make-up, enriched in *Bifidobacteria*, reduced adiposity, reduced inflammation, plasma lipid levels and fasting glucose, thus potentially reducing the risk of overweight-related health conditions. Participants may also benefit from improved gut-health, although this will not be tested in the current study. Participants in the study will help assess whether addition of probiotics and prebiotics to one's diet can be a supplementary strategy to calorie-counting and exercise in managing the worsening problem of obesity and related chronic diseases in the United States.

Taking part is voluntary: Taking part in this study is completely voluntary. Even if you are already enrolled in the study, you are free to withdraw at any time without prejudice or jeopardy to your standing with the University. Participation in the study does not imply giving up any of your legal rights.

Compensation: For participation in the study, you will receive a nominal compensation in a pro-rated manner: \$50 gift card each at week 0, week 6, week 12 and week 16, for a total value of \$200.

Confidentiality: Any personal identifying data will be kept confidential in the Principal Investigator's office and accessible only to authorized investigators. Scientific data generated will be de-identified (coded) for dissemination to the sponsor, as well as for publication. Electronic files will be password-protected and hard copies will be stored under lock and key. All identifying data will be destroyed two years from the publication of the results of the study, unless a secondary investigation is planned upon appropriate consent and IRB-protocols.

Biological materials: Biological samples collected will only be used for the purposes stated in this study. Unless specifically permitted otherwise, blood samples and faecal samples collected will be anonymised and de-identified at the end of the study such that they cannot be traced to any personal information about the participant they were collected from. If any secondary investigation is planned with the samples leftover from the study, this will be conducted in accordance with the IRB regulations at Texas State University-San Marcos. If no further investigation is planned, the samples will be destroyed two years after the study results have been published.

Contact information: Any pertinent questions about this research, your rights as a study participant and/or any research-related injuries should be directed to the chair of the IRB committee at Texas State University Dr. Jon Lasser (512-245-3413, lasser@txstate.edu), or to Ms. Becky Northcut, Compliance Specialist (512-245-2102).

Study results: Upon the completion of the study, you may request a summary of the findings if interested, by contacting the Principal Investigator via e-mail (v.mallin@txstate.edu).

Statement of consent: I have read the above information and have received satisfactory answers to any questions. I understand the risks and benefits associated with participation in this study. I consent voluntarily, without coercion in any form, to be a participant in this research and understand that I have the right to withdraw from the research at any time without any consequence. I have received a copy of this consent form for my personal records.

Participant Name: _____

Participant's signature: _____

Researchers' names: _____

Researchers' signatures: _____

Date (Month/Date/Year): _____

APPENDIX F

List of Probiotic, Prebiotic, and Synbiotic Products for Subjects to Avoid Consuming

Please use this list to check if you are regularly taking probiotics or prebiotics in your diet.

List of products containing probiotics and prebiotics available in local area supermarkets

- Accuflora – Probiotic Acidophilus capsule supplement
- Align – Digestive Care probiotic supplement
- American Health – Acidophilus & Bifidum chewable wafers
 - Probiotic with Citrus Pectin capsule supplement
 - Probiotic Acidophilus capsule supplement
- Bio Salud – Probiotic yogurt drink
- Buddy Bear – chewable probiotic supplement
- Central Market – Quattro Probiotic supplement
 - Dual action Acidophilus & Bifidus capsule supplement
- Culturelle – probiotic digestive health capsule supplement
 - probiotic for kids supplement
- Dannon - DanActive Probiotic drink
 - Activia yogurt
 - Activia probiotic yogurt drink
 - Activia Light yogurt
 - Activia Light probiotic yogurt drink
- DDS – Acidophilus tablet supplement
 - Acidophilus capsule supplement
 - Acidophilus PLUS+ capsule supplement
- Digestive Advantage - Intensive Bowel Support Probiotic capsule supplement
- Enzymatic Therapy – PearlsIC Probiotic supplement
- GanedenBC30 – Digestive Advantage capsule supplement
- Garden of Primal Defense – probiotic formula capsule supplement
- Gerber - DHA & Probiotic single grain rice
 - DHA & Probiotic single grain oatmeal
- Good Belly - Probiotic drink
- GT's - Kombucha Tea

- H.E.B. – Active Cultures Cereal
 - Active yogurt
 - Active yogurt drink
 - Active Light yogurt
 - Active Light yogurt drink
- Jarrow Formulas – Baby's jarro-dophilus powder supplement
 - jarro-dophilus EPS capsule supplement
 - jarro-dophilus +FOS capsule supplement
 - jarro-dophilus +FOS powder supplement
- JELL-O - LiveActive dark chocolate puddings
- Lifeway - Biokefir probiotic yogurt drink
 - Kefir Probiotic yogurt drink
- MegaFood – Megaflora optimal potency probiotic supplement
- Monkey Bar – prebiotic granola bars
- Monkey Brains - prebiotic oatmeal
- NATROL – Acidophilus capsule supplement
- Nature's Bounty – Acidophilus chewable probiotic supplement
- Nature's Life – Lactobacillus drink
 - Pro-96 Acidophilus drink
- Nature's Way – Primadophilus Reuteri Probiotic capsule supplement
 - Acidophilus & Rhamnus capsule supplement
 - Bifidus capsule supplement
 - Children capsule supplement
 - Kids capsule supplement
- Now Naturals – 4x6 Acidophilus capsule supplement
 - 8 blend Acidophilus & Bifidus capsule supplement
 - Acidophilus Powder supplement
- Nutrition Now - PB8 capsule supplement
- ORIGIN – Prebiotic Formula Acidophilus caplet supplement
- PHD – To go Probiotic water
- Philips – Colon Health daily probiotic capsule supplement
 - Colon Health probiotic and fiber supplement
- Rainbow Light – Probiolicious Gummies
- Spring Valley – Probiotic Multi-Enzyme supplement
 - Super Strength Probiotic Acidophilus capsule supplement
 - Probiotic Acidophilus capsule supplement
- Sustenex – Probiotic Gummies
- Vobranz – Kumbucha tea
- White Mountain – Bulgarian yogurt
- Yakult - probiotic drink

APPENDIX G

Global Physical Activity Questionnaire (GPAQ)

Visit: _____

Volunteer number: _____

Global Physical Activity Questionnaire (GPAQ)

Department of Chronic Diseases and Health Promotion
Surveillance and Population-Based Prevention
World Health Organization
20 Avenue Appia, 1211 Geneva 27, Switzerland
For further information: www.who.int/chp/steps

Physical Activity		
Activity at work		
Think first about the time you spend doing work. Think of work as the things that you have to do such as paid or unpaid work, <u>study/training, household chores, harvesting food/crops, fishing or hunting for food, seeking employment</u> . [Insert other examples if needed]. In answering the following questions 'vigorous-intensity activities' are activities that require hard physical effort and cause large increases in breathing or heart rate, 'moderate-intensity activities' are activities that require moderate physical effort		
Questions	Response	Code
1 Does your work involve vigorous-intensity activity that causes large increases in breathing or heart rate like [carrying or lifting heavy loads, digging or construction work] for at least 10 minutes continuously?	Yes 1 No 2 If No, go to P4	P1
2 In a typical week, on how many days do you do vigorous- intensity activities	Number of days <input type="text"/>	P2
3 How much time do you spend doing vigorous-intensity activities at work	Hours : minutes <input type="text"/> : <input type="text"/> hrs mins	P3 (a-b)
4 Does your work involve moderate-intensity activity that causes small increases in breathing or heart rate such as brisk walking [or carrying light loads] for at least 10 minutes continuously?	Yes 1 No 2 If No, go to P7	P4
5 In a typical week, on how many days do you do moderate- intensity activities	Number of days <input type="text"/>	P5
6 How much time do you spend doing moderate-intensity activities at work	Hours : minutes <input type="text"/> : <input type="text"/> hrs mins	P6 (a-b)
Travel to and from places		
The next questions exclude the physical activities at work that you have already mentioned. Now I would like to ask you about the usual way you travel to and from places. For example <u>to work, for shopping, to market, to place of worship</u> . [insert other examples if needed]		
Questions	Response	Code
7 Do you walk or use a bicycle (pedal cycle) for at least 10 minutes continuously to get to and from places?	Yes 1 No 2 If No, go to P10	P7
8 In a typical week, on how many days do you walk or bicycle for at least 10 minutes continuously to	Number of days <input type="text"/>	P8
9 How much time do you spend walking or bicycling for travel on a typical day?	Hours : minutes <input type="text"/> : <input type="text"/> hrs mins	P9 (a-b)

Recreational activities		
The next questions exclude the work and transport activities that you have already mentioned. Now I would like to ask you about <u>sports</u> , <u>fitness</u> and <u>recreational activities (leisure)</u> .		
Questions	Response	Code
10 Do you do any vigorous-intensity sports, fitness or recreational (leisure) activities that cause large increases in breathing or heart rate like <u>running or football</u> , for at least 10	Yes 1 No 2 If No, go to P13	P10
11 In a typical week, on how many days do you do vigorous- intensity	Number of days <input type="text"/>	P11
12 How much time do you spend doing vigorous-intensity sports, fitness or recreational activities	Hours : minutes <input type="text"/> : <input type="text"/> hrs mins	P1 2 (a- b)
13 Do you do any moderate-intensity sports, fitness or recreational (leisure) activities that cause a small increase in breathing or heart rate such as <u>brisk walking, (cycling, swimming, volleyball)</u> for at least	Yes 1 No 2 If No, go to P16	P13
14 In a typical week, on how many days do you do moderate-intensity sports, fitness or recreational	Number of days <input type="text"/>	P14
15 How much time do you spend doing moderate-intensity sports, fitness or recreational (leisure) activities on a typical	Hours : minutes <input type="text"/> : <input type="text"/> hrs mins	P1 5 (a- b)
Sedentary behavior		
The following question is about sitting or reclining at work, at home, getting to and from places, or with friends including time spent [<u>sitting at a desk, sitting with friends, travelling in car, bus, train, reading, playing cards</u> or <u>watching television</u>], but do not include time spent sleeping.		
Questions	Response	Code
16 How much time do you usually spend sitting or reclining on a typical day?	Hours : minutes <input type="text"/> : <input type="text"/> hrs mins	P1 6 (a-

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