PROBIOTIC MODULATION OF DIGESTIVE ENZYMES AND FASTING-INDUCED ADIPOCYTE FACTOR (FIAF) AS MEDIATORS OF DIET-RELATED OBESITY

Presented to the Graduate Council of
Texas State University-San Marcos
in Partial Fulfillment
of the Requirements

for the Degree

Master of SCIENCE

by

Reese Cotten, B.S., R.D.

San Marcos, Texas
December 2010

ACKNOWLEDGMENTS

I would like to thank my friends and family for their support throughout this endeavor, for their patience and understanding, and for their enthusiasm. I would also like to thank my advisor Dr. Vatsala Maitin for her constant guidance and dedication to my overall success with my thesis and my personal growth. I would like to thank Dr. Dhiraj A. Vattem for allowing me to train in his lab as well as for providing direction and input throughout my college experience. This thesis was submitted on June 29, 2010.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure **Page**

I. GENERAL INTRODUCTION

OBESITY

Obesity has become a significant health concern within the United States and is commonly viewed as one of the most serious public health problems of the 21st century. It has been understood that the increase in obesity throughout the western world is directly linked to the abundance of highly palatable foods and the rise in sedentary lifestyles (Zandbergen et al. 2006). Obesity can be briefly defined as a condition characterized by an excessive or atypical amount of body fat (NIH). A consensus within the healthcare community classifies an individual as obese if their body mass index (BMI) is >30 kg/m^2. Current statistics indicate that almost 65% of the US population is overweight which correlates to approximately 500 million people (Roault 2008; Backhed et al. 2004). Additionally, obesity is a risk factor for several diseases including, but not limited to, diabetes, hypertension, hypercholesterolemia, sleep apnea, as well as various forms of cancer (Kondo et al. 2010).

CAUSES OF OBESITY: DIETARY/ENVIRONMENTAL, GENETIC, GUT FLORA

There are various causes of obesity and normally the onset of obesity stems from a multitude of interrelated factors. Commonly accepted factors include environmental

factors such as a poor diet philosophy including foods high in saturated fat, cholesterol and refined sugars; a sedentary lifestyle lacking in physical activity; and genetic factors such as hereditary or genetic predisposition and genetic disorders. The rise in obesity rates has been particularly steep over the past three decades, with NHANES statistics (Raoult 2008) showing that the number of obese people in the USA doubled from 15% to over 30% within a 20 year period (1980-2000). This dramatic rate of increase is inconsistent with genetic factors and suggests environmental factors as the primary cause of obesity. Examples include influence of diet, exercise, socio-economic status, education and environmental chemicals such as endocrine disruptors (Tilg et al. 2009; Farooqi and O'Rahilly 2006). From a nutritional standpoint, caloric excess and poor dietary choices have been significant contributors. While the primary treatment regimen for obesity includes dieting and exercise, most people are unable to make long-term dietary/physical activity changes to support weight management. Non-compliance to an exercise and dietary intervention can lead to subtle weight gains even if energy intake surpasses expenditure by less than 1% (Hill 2006). It is thus important and generally accepted that novel approaches are needed to combat the obesity epidemic.

A novel environmental factor that has been identified to contribute to an individual's obesity-risk is the composition of one's gut microflora (Martinez et al. 2009). It was shown that lean and obese individuals differ in the relative proportions of the two major groups of bacteria in the human gut: the Firmicutes and the Bacteroidetes (Ley et al. 2005). While obese individuals possessed a higher relative proportion of Firmicutes, lean individuals possessed a higher proportion of Bacteroidetes. The authors

suggested that the 'obese' phenotype exerted by certain groups of gut bacteria is mediated by their ability to impact caloric extraction from the diet (Ley et al. 2005).

HUMAN GUT MICRBIOTA

The human GI tract is one of the first sites of exposure to the external environment including diet and also the main portal to the rest of the organs in the human body. Through its interactions with environmental stimuli, the gastrointestinal tract plays an important role in the preservation of health and the etiology of disease. A significant component of gut-mediated influences on human health is the gut microflora (Tilg et al. 2009).The abundance of microbes that colonize the human intestines is estimated to be in the trillions. The residing microbiota, dominated by 2000 species of anaerobic bacteria, represents a huge bacterial community mainly localized in the colon (Tilg 2009). The distribution of microbiota varies among anatomical site but can be divided into three main sections: the stomach, small intestine (SI), and large intestine (colon). Bacterial concentrations within the stomach and small intestine are relatively low compared to that of the large intestine and colon (Fig. 1.1). The microbial inhabitants within the stomach are facultative anaerobes and predominately belong to *Lactobacillus* sp., *Streptococci* sp., *and Enterococcus* (Xiao-Xing Li 2009). The distribution and diversity of the human microbiota is a result of numerous factors such as the influx of new species, physiochemical dynamics (pH, oxygen content, and motility) of the gut, and substrate availability (Quigley 2010). The residing microbes within the small intestines of healthy individuals are predominately facultative anaerobes (*Lactobacillus* sp., *Bacteroidetes* sp., *Clostridium* sp., *Bifidobacterium* sp., *and Streptococci*). Upon reaching the terminal ileum of the intestines, bacterial concentrations dramatically increase to levels nearly

reaching 10^9 CFU/ml (Quigley 2010). The human large intestines and colon is the section of the GI tract that contains the largest and most diverse microbial habitat than any other region of the human body (Fig.1.1). The bacterial concentrations, although not all species have been identified, can reach levels as high as 10^12 cfu/ml (Hooper et al. 2002). Although *ex-vivo* cultivation of microbes from the gut is limited, gene-based sequencing has led to the discovery that microbial densities are as high as 10^14 cfu/ml (Gil et al. 2006; Hooper et al. 2002).

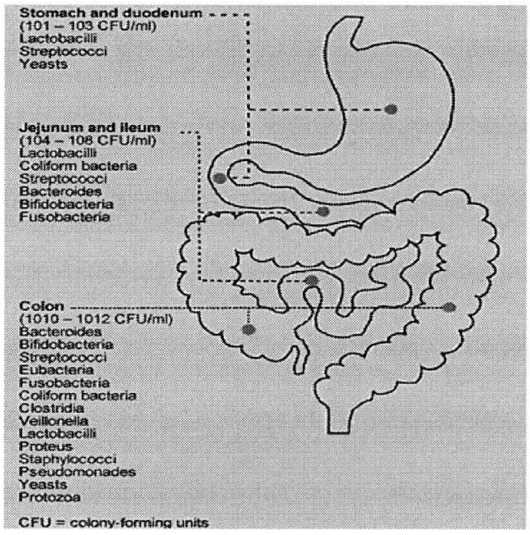


Figure 1.1: Schematic overview of microflora within the human gut (Wilson 2005)

Based on the understanding of the impact of gut bacteria on health, they have been broadly placed in three categories (Yang et al. 2005; Hooper et al. 2002): a) those with harmful or pathogenic influences, b) those that have beneficial effects, and c) those that may have both. This classification was primarily based on the ability of certain gut-bacterial species to impart localized benefits such as pathogen-resistance and immune-modulation (Saulnier et al. 2009; Tilg et al. 2009). However, we now know (Haemer et

al. 2009; Wolf and Phil 2006; Freitas et al. 2003) that the multifaceted bacterial-host interactions arising from the presence of these gut bacteria promote a symbiotic environment that influences host metabolism 'beyond the gut'. The collective genomes of the residing bacteria contain approximately 100 times more genes than the human genome (Backhed et al. 2004). The bacterial genome or the microbiome present in the human gut can thus be considered a separate metabolic organ that explicitly adapts to an individual's physiology (Dumas et al. 2006). The resulting symbiotic relationships have been shown to substantially influence host physiology, gene expression, as well as metabolic capacities that have evolved primarily due to the residing microbiota (Wolf and Phil 2006).

GUT MICROFLORA AND DIETARY ENERGY HARVEST

By virtue of their location in the human digestive tract, gut microbes are effectively situated at the interface of diet and gut to potentially influence nutrient host interactions. As mentioned earlier, an association has been established between the abundance of certain bacterial divisions in the human gut and the incidence of obesity (Vrieze et al. 2010; Musso et al. 2010; Cani and Delzenne 2009). Pioneering studies conducted by Jeffery Gordon and colleagues indicated that mice with a normal gut microbiota (conventionally-raised mice) had approximately 40% more total body fat than their germ-free littermates consuming the same diet. Moreover, conventionally-raised mice required 30% less caloric intake to maintain their body weight than their germ-free counterparts. Transplantation of gut microbes from conventionally-raised mice to the recipient germ-free mice resulted in a 60% increase in body fat content and insulin resistance within two weeks (Kondo et al 2010; Buck et al. 2008). The experiments

suggest that the presence of gut microflora contributes to obesity by influencing caloric extraction from the diet or dietary 'energy harvest'. Conventionalization of germ-free mice resulted in alterations in transcription of various intestinal mediators that are vital in nutrient absorption, mucosal barrier and metabolic functions (Tilg et al. 2010; Hooper et al. 2002).

In a human study, analysis of distal gut microflora unveiled that an obese phenotype is related to the altered ratio of Firmicutes to Bacteroidetes, the two predominant bacterial divisions within the gut. The study determined that obese individuals exhibited a 50% reduction in beneficial Bacteroidetes and equivalent increase in the less beneficial Firmicutes (Ley et al. 2005). Relative abundance of the phylum Firmicutes is associated with an obese phenotype; resulting from increased 'energy-harvest' or caloric extraction from the diet via modulation of carbohydrate and lipid metabolism (Backhed et al. 2007; Turnbaugh et al. 2006). The human gut microbiome has a vast array of genes that encoding for enzymes necessary for the degradation and metabolism of numerous sugars, starch and glycans as well as methanogenesis (Raoult 2008). For example, gut microbes possess a wide array of glucoside hydrolases; processing complex dietary carbohydrates into monosaccharides and short-chain fatty acids (SCFAs) (Fig. 1.2).

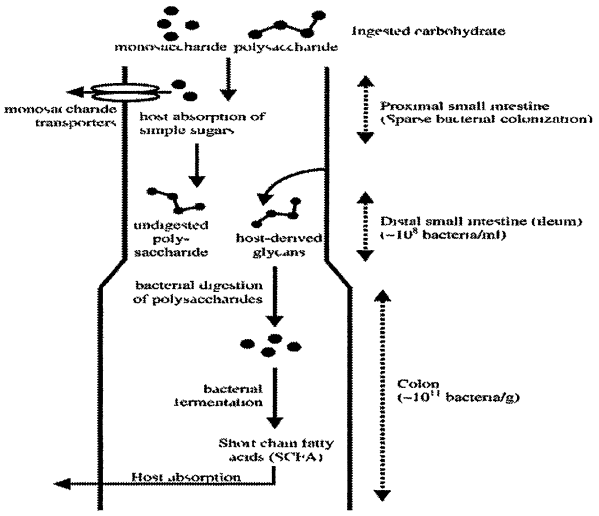


Figure 1.2. Schematic view of microbial carbohydrate digestion throughout the intestines (Hooper et al. 2002)

Relevant studies have indicated that *Bacteroides thetaiotaomicron (B. thetaiotaomicron)*, a highly abundant anaerobe found in the human colon, contains approximately 64 such enzymes (Sonnenburg et al. 2005). The increased processing of these complex carbohydrates in the colon is followed by increased absorption of the monosaccharides and SCFAs, by modulation of the relevant host receptors by the gut microbiota. For instance, it has been reported that *B. thetaiotaomicron* colonization promoted the increased expression of the sodium/glucose transporter (SGLT-1) within the human mucosal epithelium (Sonnenburg et al. 2005). Similarly, SCFAs produced by bacterial fermentation of complex carbohydrates were absorbed by regulating the levels of GPR41 (Samuel et al. 2008).

This increased intestinal absorption and utilization of liberated monosaccharides and SCFAs has led to the understanding of how the gut microbiome can contribute to serum glucose and insulin levels, as well as lipogenesis. Conventionalization of germ-

free mice resulted in the elevated expression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (Fas) both which are necessary for de novo hepatic lipogenesis (Fava et al. 2006). In addition, it is known that insulin and glucose impact lipogenesis by increasing the expression of ACC and Fas (Musso et al. 2010).

Recent advances (Backhed et al. 2007) have elucidated the exact mechanism by which gut microbes modulate host adiposity. Microbiomes associated with obese individuals resulted in a general increase in lipoprotein lipase (LPL) activity; an enzyme responsible for triglycerides hydrolysis and storage from circulating lipoproteins (Fava et al. 2006). The 'obese' microbiota resulted in this increased LPL activity by suppressing the expression levels of intestinal fasting-induced adipocyte factor (FIAF), an LPL inhibitor (Backhed et al. 2007). Differential effects on carbohydrate and lipid metabolism by gut microbiota are thus partly responsible for their varying contributions to dietary 'energy harvest', ultimately resulting in increased fat storage and adiposity.

FIAF/ANGPTL-4

Fasting-induced adipose factor (FIAF) also known as angiopoietin-like 4 (ANGPTL4), is a circulating lipoprotein lipase inhibitor and is expressed in several metabolically active tissues including the adipose tissue, heart, liver, skeletal muscle, pancreas, lung, kidney, and brain (Yin et al. 2009; Mandard et al. 2005). This secreted protein plays an important role in glucose and lipid metabolism, angiogenesis and strongly up-regulated by fasting in white adipose tissue and liver, as well as during adipogenesis (Mandard et al. 2005; Kersten et al. 2000).

Mechanistically, the circulating FIAF protein tightly binds LPL and converts the enzyme from dimers (active) to monomers (inactive), thus rendering the activity of LPL (Yin et al. 2009). Upon dislocation of the dimer molecule, ANGPTL4 is then released. The newly formed monomers of LPL remain stable but are incapable of reforming their active dimer configuration (Yin et al. 2009). The expression of FIAF is directly regulated by certain transcription factors called peroxisome proliferator-activated receptors (PPARs) (Zandbergen et al. 2006). More specifically, it was discovered that FIAF was a target gene of PPARα and PPARγ. It was also realized that, although PPARα does induce FIAF expression, PPARγ is the prime controller of adipogenesis (Mandard et al. 2004). Studies conducted to better characterize this adipocytokine discovered that plasma FIAF existed in two main forms; native protein (~45-50kDa) and a truncated form (~32kDa), but the amount of expression and processing varies by tissue (Kersten 2009; Dutton et al. 2007; Zandbergen et al. 2006).

As mentioned earlier, FIAF protein can be detected in various tissues and in blood plasma, suggesting that it has an endocrine function. FIAF is a human gene encoding for a secreted protein that is directly involve in regulating glucose homeostasis, lipid metabolism, and insulin sensitivity (Mandard et al. 2005; Zandbergen et al. 2005). Its plasma abundance is increased by fasting and decreased by chronic high-fat feeding. In addition, FIAF also plays an important role in the determination of adipose tissue size and plasma lipid levels (Kersten et al. 2000). Decreased expression of intestinal FIAF has been linked to chronic diseases including type-2 diabetes and CVD, as well as altered LDL, HDL, and serum TG (Martinez et al. 2009).

Previous studies by Backhed et al. (2004) have reported that FIAF suppression is essential for the gut microbe-induced deposition of triglycerides in adipocytes, underlining the role of gut microbes as an important environmental factor influencing energy harvest and energy storage in the host. Recent advances (Backhed et al. 2007) have elucidated the exact mechanism by which gut microbes modulate host adiposity. It has been determined the gut microbiota of conventionally-raised mice differentially suppress the expression of fasting-induced adipocyte factor (FIAF), a circulating inhibitory protein of lipoprotein lipase (LPL); the enzyme responsible for serum triglyceride hydrolysis and storage in adipocytes (Backhed et al. 2007). Lipoprotein lipase is an enzyme that hydrolyzes lipids found in lipoproteins such as chylomicrons and VLDL. Hydrolysis of lipids results in the liberation of fatty acids from triglycerides and ultimately affects their uptake by adipose tissue. (Resta 2009; Mead et al. 2002). It has now been established that one of the key modulators for microbiota-induced fat deposition is due to their innate ability to influence triglyceride storage within adipocytes (Tilg et al. 2009).

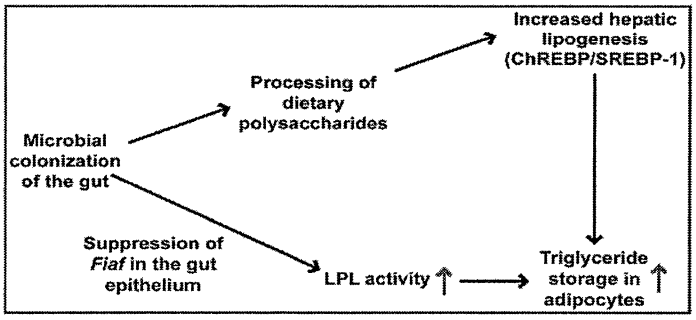


Figure 1.3. Schematic view of how the gut microbiota effects host fat storage (Backhed et al. 2004)

To further understand the contribution of FIAF to the relation between gut microbiota and adiposity, comparisons were made using germ-free mice and germ-free knockout (FIAF -/-) mice. Results from the experiment showed that unlike regular germ-free mice, FIAF (-/-) knockout mice were not protected from diet-induced obesity and exhibit the same degree of adiposity as their conventionally-raised siblings (Backhed et al. 2004). Therefore, it was concluded that FIAF is the key modulator in the microbiota-induced increase in fat storage.

An addtional recent study (Buck et al. 2009) discussed that gut microbes possess a wide array of glucoside hydrolases; processing complex dietary carbohydrates into SCFAs. Interestingly, microbiomes associated with obese individuals have a greater capacity to produce short-chain fatty acids (SCFAs) from complex polysaccharides. On top of SCFAs being the main energy source for enterocytes, it is understood that SCFAs are ligands for specific receptors, including G protein-coupled receptor 41 (Tazoe et al. 2009; Resta 2009). Gpr41-bound SCFAs will stimulate the expression of leptin. Leptin is a polypeptide hormone that affects both appetite and energy metabolism in adipocytes. This study revealed the importance of Gpr41 in the metabolic cycles directly related to the gut microbiome (i.e. regulation of the flow of calories between host and diet).

Results from experimental studies provide enormous insight into the ways that the gut microflora interacts with host-metabolism to influence obesity. The symbiosis, composition, and biological importance of the resident microbes provide the rational basis for developing methods to beneficially alter the make-up of our microbiomes. The most widely-used dietary approach for favourable modulation of gut microflora is the

consumption of probiotics, prebiotics and synbiotics (Sanders and Marco 2009; Yang et al. 2005).

PROBIOTICS, PREBIOTICS AND SYNBIOTICS

Probiotics are defined as live microbial feed supplements that beneficially affect the host by improving its intestinal microbial balance (Sanders and Marco 2010; Resta 2009; Sauleir et al. 2009). Prebiotics are selectively fermented ingredients that allow specific changes, both in the composition and/or activity of the gastrointestinal microbiota that confers benefits upon host well-being and health (Sanders and Marco 2010; Roberfroid et. al 2010; Yang et al. 2005; Gibson et al. 2004). Synbiotics are mixtures of pro- and prebiotics, which beneficially affect the host, by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract (Cruz et al. 2010; Panesar et al. 2009).

Most of the bacteria used as probiotics belong to the *Bifidobacterium* and *Lactobacillus* genera and are administered via fermented dairy products (Sanders and Marco 2010). Within the *Lactobacillus* and *Bifidobacterium* genera, the most often utilized species are L. *acidophilus*, *L. casei*, *B. bifidum*, *B. infantis*, and *B. longum* (Cruz et al. 2010; Nagpal and Sachdeva 2009). Foods that contain selected probiotic strains are normally active within the human gut, but other sites of action have been observed. To date, many efforts are now being focused in the direction of the gut microbiome and probiotics for manipulation of host metabolism and involvement in numerous conditions including obesity, insulin resistance, hypertension, and cancer. For many years, probiotics and prebiotics have been associated with numerous health-promoting activities

ranging from immune-modulation and lipid metabolism to lowering blood pressure as well as beneficially altering the microbial balance within the gut (Sauleir et al. 2009). Although insights regarding the mechanisms responsible for the beneficial effects of probiotic supplementation remain inadequately defined, the effects are attributed to intricate microbe-microbe and microbe-host interactions (Sanz et al. 2010). While the influence that various gut-bacterial species including 'probiotics' have towards the gastrointestinal tract alone is well-established, documentation of their implications throughout the human body are growing. With the understanding that probiotic consumption shifts the composition of the intestinal microbiota as well as impacting bacterial-host metabolic communication, the implications that probiotics and their consumption may have on human health are rapidly expanding.

On the molecular level, little was known of the interactions that commercial preparations of probiotics had within the human body. For example, commercial preparations of probiotics have been used as a means of promoting weight gain in farm animals (Armougom et al. 2009). More importantly, probiotic products used in the farming industry belong to the Firmicutes, in particular *Lactobacillus* and *Enterococcus* spp. The author urges that given that the growth promoting ability of species such as *Lactobacillus* sp., their use as probiotic supplements for humans should be a cause of concern. To reinforce this notion, studies have indicated that obese individuals have a higher concentration of *Lactobacillus* sp. in their gut than that of lean or anorexic subjects (Armougom et al. 2009). Considering the newly discovered significance of gut microflora in metabolic health, it is critical to re-visit our understanding of the

manipulation of gut flora by probiotics and prebiotics in this context, by focusing on their impact on underlying molecular mechanisms.

LITERATURE RELEVANT TO THE APPLICATION OF PROBIOTICS IN OBESITY

As mentioned above, obesity is a result of an imbalance between energy expenditure and energy intake. This disease is regulated by multiple pathways that are driven by various metabolites and hormones (Sanz et al. 2010). Regardless of years of intensive research, the processes implicated in the progression of metabolic disorders (i.e. type-2 diabetes, CVD etc.) remain poorly defined. Recently, it has been established that imbalance in the intestinal microbiome directly influences host metabolic processes, whether the aberrations are seen at the phyla, genus, or species level (Cani and Delzenne 2009). The gut microbial composition, more often than not, correlates with the degree of low-grade inflammation closely linked with the onset of obesity and diabetes (Cani et al. 2007). Interestingly, it was observed that the proportion of *Bifidobacterium* sp., a commonly used probiotic bacteria, was inversely associated with the insulin resistance, glucose tolerance, and metabolic disorders linked to obesity (Cani and Delzenne 2009). As can be expected, the use of probiotics towards metabolic diseases such as diabetes and obesity is a concept that is growing in validity.

There are numerous pharmaceutical and therapeutic approaches geared towards the treatment of obesity and the accompanying conditions, but combating these co-morbidities with the least offensive side effects has proven to be difficult. A novel approach to combat obesity and metabolic disorders has been introduced in which probiotic ingestion is coupled with pharmaceutical drugs. One such study revealed that

Bifidobacterium, given its beneficial probiotic nature, was a practical candidate to become the delivery vector for anti-obesity medications. In brief, recombinant *B. longum* was transformed with plasmids containing the human gene for oxyntomodulin (OXM), a potent regulatory molecule of body weight and appetite (Long et al. 2010). The OXM-transformed *B. longum* exhibited the ability to reduce food intake, body weight and lower blood triglyceride levels in mice while maintaining its normal beneficial attributes. Further investigations will be conducted to ensure that this delivery method of probiotics and anti-obesity compounds compliments the residing microbiome.

A new study conducted in rats with diet-induced obesity rats provided preliminary data for the beneficial use of selected probiotics as anti-obesity agents. Rats maintained on a high-fat diet supplemented with the probiotic *Bifidobacterium breve* exhibited lower total body fat, improved insulin sensitivity, and an improvement in serum triglycerides, cholesterol, and glucose. Significantly, it has been established that a high-fat diet diminishes the levels of *Bifidobacterium* sp. within the intestine. The study indicated that the anti-obesity effects were due to the bacterial-mediated increase in the expression of FIAF and pro-glucagons within the intestines (Kondo et al. 2010). It is known that the suppression of FIAF is pivotal in microbial-induced fat deposition within adipocytes (Fava et al. 2006, Stappenbeck et al. 2002). Therefore, these results further support the understanding of how probiotic supplementation and the composition of the human microbiome impart benefits to the host.

Recently it was revealed that a specific probiotic strain of Lactobacillus, L. paracasei F19, was able to induce FIAF expression in HCT-16 cells. The study further determined that the metabolite responsible for the induction was, in fact, secreted as well

as heat-stable (Aronsson et al. 2010). These results reinforce the idea that specific probiotic strains can be utilized for the targeted treatment of obesity via FIAF/LPL.

A study conducted in rats demonstrated that LAB, specifically *L gasseri*, decreased adipose tissue weight, growth, and size in a manner related to increased fecal fatty acid excretion, lower triacylglyceride transportation, absorption, and lower serum leptin levels (E. M. Hamad et al. 2009). Although the exact mechanism by which this LAB contributes to decreased adipocyte size and adipose tissue weight in rats is not known; probably candidates include reduction in serum leptin levels, alterations of adipocyte hypertrophy and circulating lipoprotein lipase (LPL) activity. These effects on adipocytes could not however be replicated in mice which obesity was already present prior to LAB supplementation. A study conducted using synbiotics revealed another possible approach in weight management and the treatment of obesity. A synbiotic powder containing inulin, *Bifidobacterium* sp. and *Lactobacillus* sp. improved host digestion and weight by beneficially altering the ecology and digestive enzyme activities of the GI tract (Yang et al. 2005). The results of the study indicated that rats maintained on a high dose of synbiotics for 8 weeks showed a significantly lower body weight than the low-dose and control groups.

Interestingly, it was also observed that, although a lower body weight was achieved in the high-dose group, digestive enzyme activities (sucrase, lipase, isomaltase) increased. This observation is possibly due to the fact that the microbial metabolic reaction upon administration is dependent upon dosage, subjects, duration of administration, interactions with commensal microbes and the specific strain used (Yin et al. 2010). In a study comparing the effects of four different strains of Bifidobacteria on

high fat diet-induced obesity in rats, it was determined that the degree of fat distribution or weight gain is subject to manipulation. For example, when weight gain is medically needed, a particular Bifidobacterium strain (B. M13-4) can be administered to achieve more effective fat absorption. In contrast, in the incidence where intervention is carried out for weight loss, another Bifidobacterium strain (B. L66-5) could be utilized as an effective candidate for controlling adiposity (Yin et al. 2010). The improvement of the intestinal enzyme activity, mucosal health, microbial ecology and body weight provided by the ingestion of selected probiotics and prebiotics reveals an alternative means to combat obesity.

OBJECTIVES

The long-term goal of our research is to further investigate the role of the human gut microflora in human energy metabolism and explore the mechanisms involved, particularly their impact on host adiposity. An interest in the role of gut microflora in metabolic health has undergone a huge revival in the past couple of years since *a link was reported between gut flora and obesity* (Turnbaugh et al. 2006; Ley et al. 2005; Backhed et al. 2004). As mentioned earlier, it was shown that lean and obese individuals differ in the relative proportions of the two major groups of bacteria in the human gut: the Firmicutes and the Bacteroidetes (Musso et al. 2010; Turnbaugh et al. 2006; Backhed et al. 2004). While obese individuals possessed a higher relative proportion of Firmicutes, lean individuals possessed a higher proportion of Bacteroidetes. The authors suggested that the 'obese' phenotype exerted by certain groups of gut bacteria is a consequence of their capacity for 'energy-harvest' or extraction of extra calories from food in the gastrointestinal tract.

A demonstrated role gut flora in obesity has made gut flora the latest therapeutic target for obesity (Musso et al. 2010; Cani and Delzenne 2009; Turnbaugh et al. 2006). The best understood dietary approach towards modulation of gut flora is the consumption of probiotics, prebiotics or synbiotics. Previous studies have shown that host microflora as well as probiotic supplementation can beneficially affect metabolic parameters including; cholesterol, triglycerides, diabetes, overall energy utilization and fat storage (Pan and Zhang 2008; Wolf and Phil 2006). Consumption of foods and supplements containing pro-, pre- or synbiotics generally result in an increase in the gut population of Bifidobacteria and Lactobacilli. While these bacterial genera are associated with a multitude of reported health benefits, the status of Lactobacillus has been thrown into controversy as it belongs to the obesity-related bacterial phylum Firmicute. Majority of probiotic-containing foods preferably use Lactobacillus as it is more aerotolerant than Bifidobacterium and thus easier to use as a 'live bacterium' in foods that are aerobically processed. Considering the widespread use of Lactobacillus as a probiotic and conflicting reports about its role in promoting adiposity, this investigation is aimed at comparing the influence of commonly used probiotic Lactobacillus and Bifidobacterium species against a commensal gut bacterium from the phylum Bactereoidetes; on microbe-mediated mechanisms of obesity. The representative bacteria chosen for this investigation are *Lactobacillus casei* (probiotic species from the obesity-related phylum Firmicutes), *Bifidobacterium longum* (probiotic species from the phylum Actinobacter, with no reported link to obesity) and *Bacteroides thetaiotaomicron* (a commensal bacterium from the phylum *Bacteroidetes,* correlated with leanness).

The objectives of the study are to investigate and compare the ability of metabolites in the cell-free supernatants (CFS) from the selected bacteria in this study to: (1) modulate the activity of host digestive enzymes, namely disaccharidases present at the intestinal brush-border (sucrase/maltase) and the main lipid-digesting enzyme in the intestine pancreatic lipase and (2) determine the contribution of selected species of gut bacteria to fat storage and adiposity via modulation of FIAF/LPL.

Previous investigations have established the ability of gut microflora to influence carbohydrate and lipid metabolism. In the context of carbohydrate metabolism, these studies have mainly focused on the contribution of *bacterial* enzymes to the digestion of indigestible polysaccharides in the colon. Our goal was to investigate if gut microflora can have a direct influence on digestion and absorption of carbohydrates and lipids in the small intestine, by potentially influencing *host* digestive enzymes, namely disaccharidases present at the intestinal brush-border (sucrase/maltase) and the main lipid-digesting enzyme in the intestine, pancreatic lipase. This could serve as an alternate mechanism via which gut bacteria may influence host adiposity. We will compare the ability of cell free supernatants (CFS) from each bacterial strain to modulate sucrase, maltase and pancreatic lipase using Caco-2 cells capable of enterocytic-like differentiation and expression of brush-border enzymes.

Other key mediators of microbial effects on diet-induced obesity include intestinal levels of FIAF and related influences on the activity of lipoprotein lipase (LPL) and triglyceride storage in the adipocytes (Kondo et al. 2010; Ley et al. 2005), hence these were chosen as representative biomarkers for investigating the comparative effects of CFS from Lactobacillus and Bifidobacterium. Effects of bacterial CFS on intracellular

and secreted FIAF will be studied using the human colonic cell line HT-29 and murine adipocytic cell line 3T3-L1 will be used to study their effects on LPL activity and triglyceride deposition.

An advanced comprehension of the effect bacteria have regarding the etiology of human disease will provide practical tools for the adoption of progressive dietary strategies to enhance health outcomes in humans. Furthermore, a thorough understanding of how the gut microbiome responds to changes in resource availability, as well as alter host metabolism could lead to revolutionizing nutrition by matching one's diet to their gut microbiota (Sonnenburg et al. 2006).

II. CELL-FREE SUPERNATANTS FROM LACTOBACILLUS SP., BIFIDOBACTERIUM SP., AND BACTEROIDETES SP. ALTER HOST DIGESTIVE ENZYME ACTIVITY

ABSTRACT

Composition of an individual's gut microflora is a recently recognized factor in diet-related obesity. An obese phenotype has been associated with a relative abundance of the bacterial phylum Firmicutes, resulting in increased 'energy-harvest' or caloric extraction from the diet. Firmicutes are predominantly *Clostridium* sp., but include *Lactobacillus* sp., some of which are probiotics. The objective of this study was to compare 'energy-harvesting' ability of probiotics *Lactobacillus casei* (Firmicute) with *Bifidobacterium longum* (Actinobacterium), in terms of effects on intestinal carbohydrate- and lipid-digesting enzymes. We compared their effects with *Bacteroides thetaiotaomicron* (*Bacteroidetes*) as this strain is a predominant commensal of the human GI tract. Effect of cell-free supernatants (CFS) from pure cultures of *L. casei*, *B. longum,* and *B. thetaiotaomicron* on intestinal disaccharidases maltase and sucrase was investigated in differentiated Caco-2 cells. Pancreatic lipase activity was measured by an *in vitro* turbidimetric assay. Un-inoculated bacterial growth medium was the control. *L. casei* CFS resulted in an increase in maltase and sucrase activity by 38% and 24% respectively. *B. longum* did not significantly alter maltase, and the >5kDa fraction

increased sucrase activity by 17%. *B. thetaiotaomicron* CFS decreased maltase activity by 17% with no effect on sucrase activity. *L. casei* whole CFS did not influence lipase activity but the >5 fraction significantly decreased lipase activity by 44%. *B. longum* increased lipase activity by 17%, and *B. thetaiotaomicron* decreased lipase activity by 22%. Overall, the results indicate a greater capacity for caloric-extraction by *L. casei*, partially offset by a decrease in lipase activity.

INTRODUCTION

Human gut microbiome is a significant component of human health. While it is well-recognized that certain gut-bacterial species including 'probiotics' impart localized benefits such as pathogen-resistance and immune-modulation in the gut, their metabolic implications beyond the gut have only recently been realized. It is now known that relative abundance of a phylum of bacteria called 'Firmicutes' is associated with an obese phenotype; resulting from increased 'energy-harvest' from the diet via modulation of carbohydrate and lipid metabolism (Backhed et al. 2007; Ley et al. 2006). This increased energy-harvest by certain gut flora has been attributed to more efficient breakdown of undigested complex carbohydrates in the human colon by the action of bacterial glycosylhydrolases, and uptake of the released monosaccharides. The aim of this study was to investigate whether gut microbes are also able to influence host digestive enzyme activity in the small intestine and thus impact dietary carbohydrate and lipid digestion and absorption. Along with *Clostridium*, its predominant member, the obesity-related phylum Firmicutes also contains *Lactobacillus* sp. Considering the widespread use of *Lactobacilli* as probiotic microorganisms in dairy products, we sought to determine if a representative member *Lactobacillus casei* contributes to obesity by exploring its effect

on intestinal brush-border disaccharidases sucrase and maltase and the activity of pancreatic lipase. Humans possess six enzyme activities required for the digestion of starches present within the diet. These six enzymes (two luminal endo-glucosidases and four exo-glucosidases/α-glucosidase) work interactively to breakdown larger carbohydrate molecules into useable glucose molecules (Sim 2010, Quezada-Calvillo et al. 2007). The α-glucosidase activities are associated with: maltase-glucoamylase (MGAM) and sucrase-isomaltase (SI) which are small intestinal membrane-bound enzymes. Sucrase-Isomaltase (SI) and maltase-glucoamylase (MGAM) are type II brush border membrane proteins that play an important role in the final stage of carbohydrate digestion. These enzymes work collectively in the hydrolysis of an assortment of linear and branched dietary starches (Sim et al., 2010). Given the role that MGAM and SI play in the breakdown of dietary sugars and starches, they frequently become targets by pharmaceuticals as a method for stabilizing plasma glucose levels in individuals with type 2 diabetes (Sim et al. 2010).

Dietary lipids are hydrolyzed to mono-acylglycerides and free fatty acids by gastric and pancreatic lipases for their subsequent absorption by the body (Mun et al. 2006). Given the extensive prevalence of obesity and its related disorders, newer approaches for the treatment of obesity have targeted dietary triglyceride digestion and absorption via the inhibition of pancreatic lipase (PL).

We studied the effects of *L. casei* relative to another common probiotic *Bifidobacterium longum,* which does not belong to the obesity-associated phylum Firmicutes and also to the commensal gut bacterium *Bacteroides thetaiotaomicron.*

MATERIALS

Bacterial Strains

Bacterial strains *Lactobacillus casei* (ATCC 334), *Bifidobacterium longum* (ATCC 15707), and *Bacteroidetes thetaiotaomicron* (ATCC 29148) were obtained from American Type Culture Collection (ATCC) (Manassas, VA).

Cell-free supernatant (CFS) preparation

Each bacterial strain was grown in their respective medium as follows: *Lactobacillus casei* in LDM-III (Jim Versalovic, Baylor College of Medicine, Houston, TX), *Bacteroides thetaiotaomicron* in BHI TM Brain Heart Infusion (Bacto), *Bifidobacterium longum* in MRS (Oxoid).

CFS were prepared by centrifugation (4000 g, 10 min, 4°C) of log-phase cultures (OD~0.7-0.8) of *L. casei, B longum, B. thetaiotaomicron* in LDM-III, MRS and BHI respectively. This OD corresponds to log phase growth and bacterial numbers of ~10^8 - 10^9 cfu/ml which correlates to the microbial density in the human small intestine (Rambaud et al., 2007). The bacterial numbers were experimentally confirmed by performing bacterial growth curves and viable plate counts for all three bacteria. Vivaspin Columns (Sartorius Stedim Biotech, France) with the MWCO of 5000Da were used for the fractionation of bacterial CFS into a larger molecular weight fraction (>5000 kDa) and a smaller molecular weight fraction (<5000 kDa). Conditions such as temperature, time, sample collection/aliquots, and final volume of CFS after centrifugation were kept constant to further remove any confounding variables. CFS pH was adjusted to 7.0 using 2M NaOH, and filter-sterilized through a 0.2 micron Corning

Sterile Syringe Filter and samples were not subjected to freeze-thaw. Optical density was measured using a BioMate3 Thermo Spectronic at 600nm.

Tissue Culture

The human adenocarcinoma-derived Caco-2 cell line was purchased from American Type Culture Collection (ATCC) (Manassas, VA). Caco-2 cells are a widely used model of the human intestinal epithelium and capable of enterocytic differentiation and tight junction formation, as well as, expression of brush-border enzymes sucrase and maltase (Gull et al. 2009). Cells were revived and maintained in EMEM supplemented with 20% FBS, 1% L-glutamine, 1% NEAA, and 1% Antibiotic/Antimycotic solution and grown in 100mm tissue culture dishes pre-treated with 0.1% collagen.

Brush border Enzyme Assays (Sucrase/Maltase)

Prior to treatment, cells were changed to a medium containing 1% FBS. CaCo-2 cells were treated with the bacterial whole supernatants or fractions for 8-days. Cell treatments (whole CFS, <5kDa CFS, >5kDa CFS) were categorized as follows: 20% CFS (v/v), 20% bacterial growth medium (control)(v/v), and No-treatment. Cells had medium and treatments changed every two days. CaCo-2 cells were collected following the 8-day treatment period and lysed in PBS (with calcium and magnesium) containing 0.5% Triton X-100, 0.35M sodium chloride and EDTA-free protease inhibitors (Roche Diagnostics, Indianapolis, IN). Cells were incubated in lysis buffer for 1 hour on ice, followed by centrifugation for 10 minutes at 12,000xg at 4°C. Cell lysates were stored in aliquots at -80°C and used for enzyme assays without being subjected to freeze-thaw. Enzyme assays were performed in a 96-well microtiter plate utilizing cell lysates as enzyme.

Maltase activity in cell lysates from Caco-2 cells post-treatment was measured at pH 7.0 and 37 °C with an Ultra microplate reader using p-nitrophenyl-β-D-galactopyranoside (PNPG) as substrate. Enzyme activity was reported as the amount of p-nitrophenol product formed after a15 min incubation.

Sucrase activity in cell lysates from Caco-2 cells post-treatment was analyzed at pH 7.0 with an Ultra microplate reader using 4% sucrose as substrate. DNS (3, 5 Dinitrosalicylic acid) reagent was used to halt reaction. Sucrase activity was reported as the amount reducing sugars released from sucrose.

Enzyme activities were expressed as % control and normalized to total cellular protein to yield specific activity.

Pancreatic Lipase Assay

Lipase activity was measured kinetically at pH 8.0 and 37 degrees Celsius with a Bio-Tek ELx808™ Series Ultra Microplate Reader and using an olive oil emulsion as the substrate. The effect of bacterial CFS and fractions was measured using porcine pancreatin (Sigma-Aldrich, St. Louis, MO) as the enzyme. Porcine pancreatin extract was gently suspended in 0.1M phosphate buffer (pH 8.0) and incubated on ice for 15min prior to use. Olive oil emulsions were prepared in 25ml of phosphate buffer (pH 8.0) containing 1% Tween. Olive oil solution was sonicated (3 min) to form the emulsion. Whole CFS as well as fractions (<5kDa and >5kDa) of *L casei*, *B. longum* and *B. thetaiotaomicron* were utilized to determine effect on lipase activity during reaction. Each assay was performed in triplicate and repeated 2-3 times (n=6-9) using LDM-III, MRS and BHI as the control. Appropriate substrate and enzyme controls were used.

After incubation for 30 min at 37 °C, enzyme activity was determined by measuring the reduction in turbidity of the olive oil emulsion due to triglyceride hydrolysis into free fatty acids. Activation or inhibition was calculated with respect to the values obtained with un-inoculated LDM-III, MRS and BHI broth as control.

Protein Determination

Protein concentration was determined according to the Bio Rad DC method using BSA (1-2mg/ml) as reference.

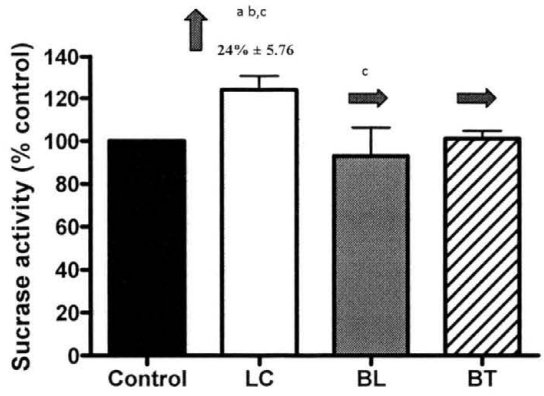
RESULTS

Whole supernatants from L. casei increased sucrase activity whereas those from B. longum and B. thetaiotaomicron had no net effect on sucrase activity.

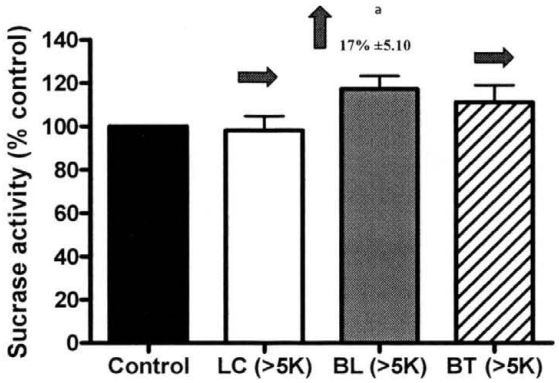
The ability of the selected bacterial strains to affect sucrase activity was determined using Caco-2 cell lysates, known to possess brush-border enzyme activity. Two representative probiotics and one commensal bacterium; *Lactobacillus casei, Bifidobacterium longum*, and *Bacteroides thetaiotaomicron* respectively were examined. All strains were tested for sucrase activity with respect to control (un-inoculated bacterial growth medium). Upon treatment of Caco-2 cells with *L. casei* CFS, an increase in sucrase activity was observed by whole CFS (24% ± 5.76) as well as <5 kDa fraction respectively compared to control (Fig.2.1A, C). *L. casei* >5 kDa fraction did not have a net effect on sucrase activity (Fig.2.1B). Treatment with CFS fraction >5kDa from *B. longum* resulted in an increase in sucrase activity (17% ± 5.10) as compared to control. The whole CFS or <5kDa fraction of *B. longum* had no effect on sucrase activity. *B. thetaiotaomicron* did not shown any net effect on sucrase activity (Fig. 2.1A, B, C). These data indicate that secreted bioactive factors from actively growing *L. casei* exhibit

a significant increase in sucrase activity. Increase in sucrase activity in cells treated with *B. longum* was due to the larger molecular fraction only and not with the whole supernatant.

A



B



C

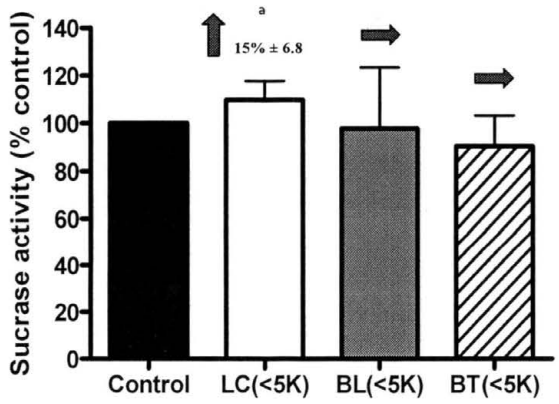
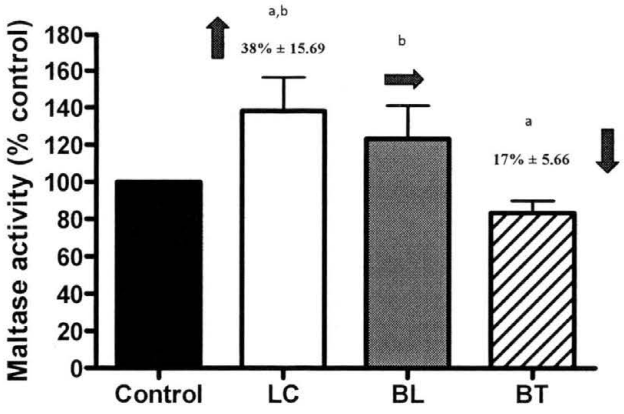


Figure 2.1. : Effect of (A) Whole CFS (B) >5 kDa fraction and (C) <5 kDa fraction from *Lactobacillus casei* (LC), *Bifidobacterium longum* (BL) and *Bacteroides thetaiotaomicron* (BT) on sucrase activity. Data shown are mean ± SEM, n=3. a=significant difference from control; b=significant difference from BT; c=significant difference between LC and BL. Caco-2 cells were treated according to Methods for 8 days and sucrase activity was measured in cell lysates.

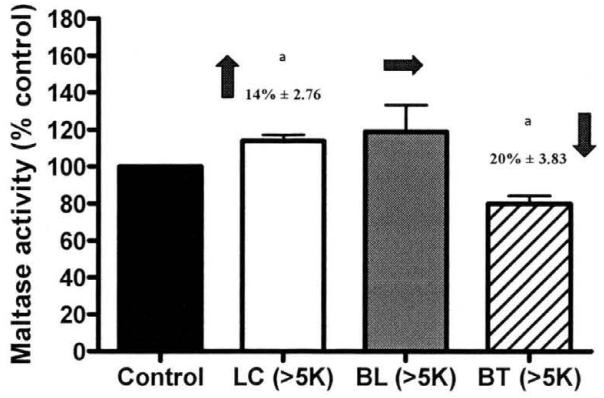
Whole supernatants from L. casei and B. thetaiotaomicron showed an increase and decrease in maltase activity respectively, while those from B. longum did not alter maltase activity.

The ability of the selected bacterial strains to affect maltase activity was determined using Caco-2 cell lysates, known to possess brush-border enzyme activity. Two representative probiotics and one commensal bacterium; *Lactobacillus casei, Bifidobacterium longum*, and *Bacteroides thetaiotaomicron* respectively were examined. All strains were tested for maltase activity with respect to control. Once Caco-2 cells were treated, a significant increase in maltase activity in response to *L. casei* was observed by whole CFS (38% ± 15.69) as well as both >5 kDa (14% ± 2.76) and <5 kDa fractions (21% ± 4.15) compared to control (Fig.2.2A, B, C). CFS from *B longum* did not show any effect on maltase activity. Whole CFS and the >5kDa fraction of *B. thetaiotaomicron* resulted in a decrease in maltase activity of 17% ± 5.66 and 20% ± 3.83 respectively compared to control (Fig.2.2A, B). The <5kDa fraction of *B. thetaiotaomicron* did not shown any net effect on maltase activity. These data indicate that secreted bioactive factors from *L. casei* contribute to an increase in maltase activity and those from *B. thetaiotaomicron* decrease maltase activity. The effects of *L. casei* CFS are mediated both by small metabolites as well as larger molecular weight compounds. In case of *B. thetaiotaomicron,* inhibition of maltase activity is primarily due to larger molecular weight compounds of molecular weight > 5kDa.

A



B



C

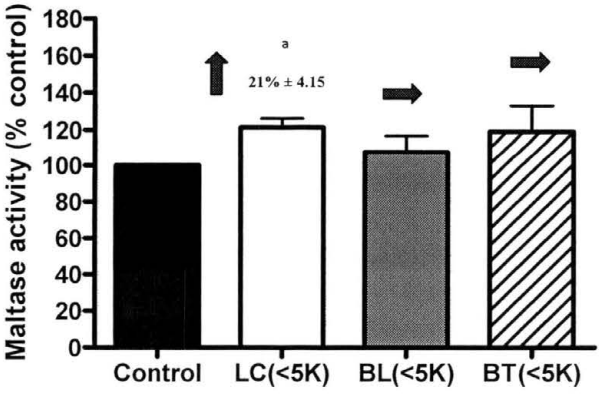
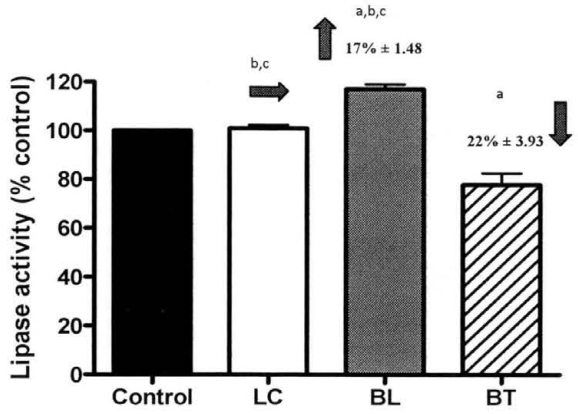


Figure 2.2.: Effect of (A) Whole CFS (B) >5 kDa fraction and (C) <5 kDa fraction from *Lactobacillus casei* (LC), *Bifidobacterium longum* (BL) and *Bacteroides thetaiotaomicron* (BT) on maltase activity. Caco-2 cells were treated according to Methods for 8 days and cell lysates were collected to be used as enzyme. Data shown are mean ± SEM, n=3. a=significant difference from control; b=significant difference from BT; c=significant difference between LC and BL.

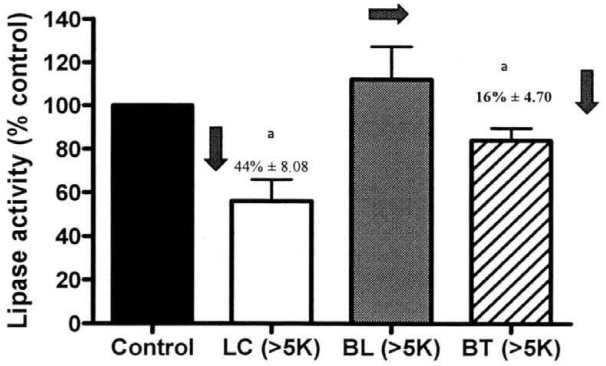
Whole supernatants from B. longum, B. thetaiotaomicron and L. casei increased, decreased and had no effect on pancreatic lipase activity respectively

The ability of the selected bacterial strains to modulate pancreatic lipase activity *in vitro* was determined kinetically using an olive oil emulsion as the substrate. Two representative probiotics and one commensal bacterium; *Lactobacillus casei, Bifidobacterium longum*, and *Bacteroides thetaiotaomicron* respectively were examined. All strains were tested for pancreatic lipase activity with respect to control. Whole CFS from *B. longum* and *B. thetaiotaomicron* resulted in an increase of 17% ± 1.48 and a decrease of 22% ± 3.93 in pancreatic lipase activity respectively (Fig.2.3A). No effect on pancreatic lipase activity was observed by whole *L. casei* CFS. The >5kDa fraction isolated from both *L. casei* and *B. thetaiotaomicron* decreased lipase activity by 44% ± 8.08) and 16% ± 4.70 respectively compared to control (Fig.2.3B). *B. longum* >5kDa fraction did not indicate any effect on lipase activity. Small bacterial metabolites present in <5kDa fractions from all three bacteria did not influence lipase activity (Fig.2.3C). These data indicate that the bioactive compound associated with the observed effects on lipase activity is of a large molecular weight.

A



B



C

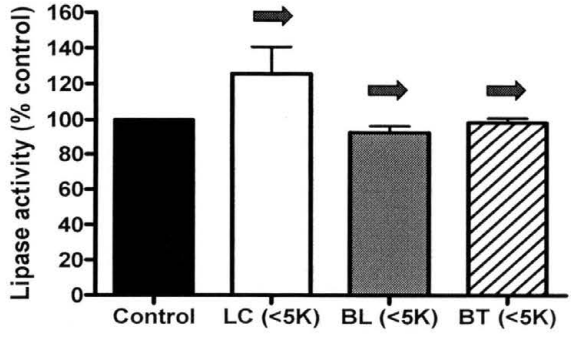


Figure 2.3.: Effect of (A) Whole CFS (B) >5 kDa fraction and (C) <5 kDa fraction from *Lactobacillus casei* (LC), *Bifidobacterium longum* (BL) and *Bacteroides thetaiotaomicron* (BT) on lipase activity. Data shown are mean ± SEM, n=3. a=significant difference from control; b=significant difference from BT; c=significant difference between LC and BL. Whole CFS as well as fractions (<5kDa and >5kDa) of *L. casei*, *B. longum* and *B. thetaiotaomicron* were utilized to determine effect on lipase activity during reaction. Porcine pancreatin and olive oil emulsions were utilized as enzyme and substrate according to Methods.

DISCUSSION

The data presented show that secreted factors from probiotic bacteria *L. casei* and *B. longum* are able to differentially modulate the activity of maltase, sucrase and pancreatic lipase *in vitro* and may exhibit similar *in vivo* activity in the small intestine, of which *L. casei* and *B. longum* are resident members (Booijink et al. 2007).

Whole supernatants from *L. casei* caused an increase in the activity of both disaccharidases investigated in this study, namely maltase and sucrase. Increased maltase activity was contributed by both >5 kDa and <5 kDa fractions of *L. casei*, whereas increased sucrase activity was due to the <5kDa fraction. Small molecular weight compounds contributing to increases in maltase and sucrase activity with *L. casei* may be short-chain fatty acids such as propionate and butyrate (Martin et al. 2009; Resta 2009) present in the CFS as products of bacterial growth and metabolism. Increased maltase and sucrase activity may also be due to bacterial effects on PPARα activation. Lactobacillus sp. has been shown to upregulate PPARα (Aronsson et al. 2010), which in turn has been shown to play a role in enterocytic differentiation and the activity of brush-border enzymes (Yang et al. 2005, Collins and Gibson 1999). Whole supernatants from *L. casei* did not exhibit an influence on pancreatic lipase activity, but a strong inhibitory effect was interestingly observed due to the large molecular weight fraction (>5 kDa) when it was separated from the small molecular weight fraction. This may be due to the separation of the inhibitory compound from a small metabolite that may be preventing its action on lipase activity.

Whole supernatants from *B. longum* had no net effect on maltase or sucrase activity, but increased the activity of pancreatic lipase. Similar to the observed effect of

L. casei on pancreatic lipase, the large molecular weight fraction (> 5k Da) from *B. longum* caused an increase in sucrase activity when separated from the small metabolite fraction. Again, this shows that perhaps a small metabolite may be preventing the required interaction of the activating compound with sucrase. Colonization with Bifidobacterium sp. has previously been shown to increase sucrase activity in mice by promoting enterocyte maturation (Yang et al. 2005). The large-molecular weight compounds activating sucrase and maltase may potentially be polyamines such as spermine or spermidine that have been shown to be produced by gut bacterial fermentation and play a role in small intestine growth and development and thus affect intestinal disaccharidases (Kleesen 2005; Yang et al. 2005).

Activation of pancreatic lipase by *B. longum* whole supernatants but not by its fractions suggests that some synergestic action between a large molecular weight compound and a small metabolite is required to cause pancreatic lipase activation.

CFS from the commensal bacterium *B. thetaiotaomicron* caused a decrease in maltase and lipase activity and no change in sucrase activity; decrease in both cases was mediated by the >5 kDa fraction.

Overall, *Lactobacillus casei* exhibited a greater 'energy-harvesting' ability from carbohydrates compared to *Bifidobacterium longum and B thetaiotaomicron.* This study demonstrates that secreted bioactive metabolites present in the CFS from the chosen bacterial species differentially modulate enzymes that are necessary in carbohydrate and lipid digestion, serving as an additional mechanism for microbe-mediated effects on caloric extraction from the diet.

The human gut microbiome has a vast array of genes encoding for enzymes necessary for the degradation and metabolism of numerous sugars, starch and glycans, dietary lipids, as well as methanogenesis (Resta 2009; Raoult 2008). For example, gut microbes possess a wide array of glucoside hydrolases; processing complex dietary carbohydrates into monosaccharides and short-chain fatty acids (SCFAs) (Fava et al. 2006). Other studies have shown that an abundance of certain probiotics in the gut resulted in the host production of digestive enzymes including; sucrase, maltase, and lipase (Yang et al. 2005; Collins and Gibson 1999). It has also been shown that *B. thetaiotaomicron* colonization promoted the increased expression of the sodium/glucose transporter (SGLT-1) within the human mucosal epithelium (Sonnenburg et al. 2005). This increased expression was also notable in rats fed a diet high in simple sugars (Ogawa 2000). It is well-known that the intestinal flora possesses the capacity to alter the differentiation of the intestinal epithelium (Pai 2008). Additionally, it can be proposed that the observed increases in the small intestinal enzyme activity are due, in part, to bacterial-mediated stabilization and modification in the mucosal morphology (Kleesen and Blaut 2005). Studies have shown that the abundance of certain LAB increased villus height and crypt depth, thus resulting in the enhanced digestive and absorptive capacities of host intestines (Kleesen and Blaut 2005; Yang et al. 2005).

Previous research has shown that colonization and formation of the normal flora within the GI tract results in the augmentation of pancreatic lipase and co-lipase, enzymes essential in the hydrolysis of dietary triglycerides (Hooper et al. 2002, Yang and Lowe 2000). The modulation of the gut microbiota by probiotics is species-dependent and may alter different regulatory mechanisms of involved in host energy metabolism (Martin et

al. 2009). Numerous studies have provided insight into how the microbial composition of the gut is tied to the synthesis and deposition of dietary lipids. Additionally, reports have concluded that the presence of selected probiotic bacteria, namely *Lactobacillus* sp., possess the ability to alter fat metabolism within the small intestine (Martin et al. 2007). The study disclosed that mice administered the probiotic *L. paracasei* exhibited lower quantities of intermediates in the small intestine necessary in lipogenesis, thus implying that LAB mediate intestinal fat metabolism. Ultimately, the gut microbiome differentially modulates the degree in which dietary lipids are synthesized and subsequently metabolized by the intestines (Martin et al. 2007).

The lipase data generated in our study implies that the inhibitory and stimulating bioactive factors are small proteins, large peptides or polysaccharides. Proteins, being amphiphilic, have been shown to inhibit lipase activity by suppressing enzyme adsorption and/or competing for interfacial binding to lipid droplets (Armand 2007; Aloulou 2006). Particular metabolites secreted by probiotics upon ingestion and transit through the GI may possess a proteolytic capacity that could ultimately interfere with the lipolytic activity of host digestive lipases (El-Salam et al. 2010). The basis for this proposed mechanism is reinforced by the fact that probiotic LAB possess a proteolytic activity against proteins in dairy-based functional foods (Ramchandran and Shah 2009; Donkor et al. 2007). Paradoxical to our results, a known pancreatic lipase inhibitor, namely protamine, is of a small molecular weight and is effective at decreasing post-prandial serum triglycerides *in vivo* (Armand 2007; Byun et al. 1999). It is probable that other metabolites present in the CFS of the selected bacteria are temporarily offsetting the observed effect on lipase activity. Additionally, the decrease in lipase activity by *L. casei*

and *B. thetaiotaomicron* may also be acting in a similar fashion to other known lipase inhibitors that bind to the lipid-water interface or act as C-terminal domain/co-lipase lures (Armand 2007; Aloulou et al. 2006). As for the increase in lipase activity by *B. longum* CFS, it is possible that lipolysis was augmented in a mechanistic fashion similar to ALTU-135; a microbial derived lipase and commonly used tool to increase fatty acid bioavailability (Armand 2007).

The results thus suggest that the contribution of an individual's gut-microflora to diet-induced obesity may be attributed in part to their effect on the host's digestive enzymes in the gut. Considering the varying and opposing effects of the probiotics studied, more research is warranted in terms of their effects on metabolic health. This includes understanding synergistic effects of probiotic bacteria, their interaction with commensal gut bacteria, as well as host digestive enzymes. Also, considering that the effects on enzyme activity vary by the molecular weight of the secreted bioactives present in bacterial supernatants, targeted health benefits may then be achieved in future using a 'postbiotic' or 'pharmabiotic' approach where isolated bioactive ingredients from probiotics may be used, rather than whole cells (Shanahan 2009).

III. CELL-FREE SUPERNATANTS FROM PROBIOTIC *BIFIDOBACTERIUM LONGUM* INCREASE INTESTINAL FIAF LEVELS *IN VITRO*

ABSTRACT

Gut bacterial composition has been shown to differentially modulate fat storage in the adipose tissue. The effects of intestinal microbes on fat storage in the adipose are mediated by their influence on intestinal expression of a lipoprotein lipase (LPL) inhibitor called Fasting Induced Adipocyte Factor (FIAF). The levels of FIAF in turn impact LPL activity and LPL-mediated triglyceride hydrolysis, fatty acid uptake and triglyceride storage in the adipose tissue. Modulation of FIAF by gut microbes has thus been reported as an indicator of their potential in promoting or suppressing adiposity.

We evaluated the influence of secreted bioactive compounds from representative probiotic strains from two different bacterial phyla: (a) *Lactobacillus casei* (Phylum: Firmicutes) and (b) *Bifidobacterium longum* (Phylum: Actinobacter) on intestinal FIAF levels and downstream effects on LPL-activity and triglyceride storage. Secreted bioactives from *L. casei* had no significant influence on FIAF levels, LPL activity or triglyceride levels in adipocytes whereas those from *B. longum* demonstrated potential in suppressing adiposity by significantly increasing intracellular and secreted intestinal FIAF levels (by 47.16±20.23 and 83.15±17.51 % respectively), decreasing adipocyte LPL-activity (by 30.68±9.80 %) and triglyceride levels (by 10.01%) *in vitro*.

INTRODUCTION

Initial investigations provided various explanations for the observed contribution of the gut microbiome and the progression of adiposity. The increased energy storage is mediated, in part, by regulating the expression of fasting-induced adipose factor (FIAF) from the intestinal epithelium (Ley 2005) (Fig.3.1). A pioneering study conducted by Jeffery Gordon and colleagues indicated that FIAF, a circulating lipoprotein lipase inhibitor, is a specific target by which the gut microflora is able to influence diet-induced obesity (Backhed 2007). These studies performed in germ-free mice indicated that an influx of certain bacterial species within the gut allows for modulation of host gene expression including FIAF, affecting fat accumulation in host adipocytes and thus promoting obesity (Cani 2009; Backhed 2007). Additionally, germ-free mice lacking the FIAF gene are not protected from diet-induced obesity as compared to their germ-free littermates still possessing the gene (Wolf and Phil 2006), suggesting that FIAF is a key component in gut flora's effects on diet-induced obesity. Selective suppression of FIAF by the intestinal microbiota results in an increased LPL activity, repression of peroxisomal proliferator activated receptor coactivator-1 α (PGC-1α), and the successive deposition of triglycerides in adipocytes (Musso 2010; Ley 2005). FIAF acts as a potent signal that stimulates fat mobilization and prevents its deposition, as well as possibly improve blood glucose levels (Mandard et al. 2006). With the understanding that FIAF is a secreted, circulating protein that is affected by the gut flora, it is probable that FIAF exerts its affects distal to its site of production (Kersten et al. 2000).

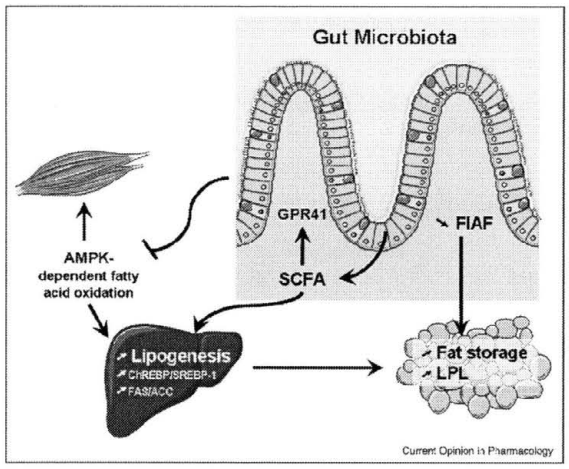


Figure 3.1. Proposed mechanism of gut microbiota-mediated energy storage

The role of lactic acid bacteria in modulating triglyceride storage in adipocytes independent of FIAF has also been conducted. A study performed in rats demonstrated that lactic acid bacterium, specifically *L. gasseri and L. plantarum*, decreased adipose weight, growth, and adipocyte size via increased fecal fatty acid excretion, lower triglyceride transport, absorption, and lower serum leptin levels (Takemura 2010; E. M. Hamad et al. 2009).

Our initial experimental approach was to determine the contribution of selected species of gut bacteria to fat storage and adiposity via modulation of FIAF/LPL utilizing a human colonic epithelial cell line (HT-29). Expression was studied by examining the both intracellular and secreted FIAF protein levels, using Western Blot. Lipoprotein lipase (LPL) activity and triglyceride accumulation was examined in 3T3-L1 adipocytes.3T3-L1 cells are a well-established model for investigating environmental influences on adipocyte biology including differentiation from pre-adipocytes into adipocytes, LPL activity, triglyceride storage and FIAF expression (Dutton and Trayhurn 2007; Mandard et al. 2004; Yoon et al. 2000).

MATERIALS

Bacterial CFS preparation

Bacterial CFS from *L. casei, B. longum*, and *B. thetaiotaomicron* were prepared as described in Ch. II Methods, except that *L. casei* was cultured in MRS broth instead of LDM-III.

CFS Size-Fractionation

Vivaspin Columns (Sartorius Stedim Biotech, France) with the MWCO of 5 kDa were used for the fractionation of bacterial CFS into a larger molecular weight fraction and a small metabolite fraction. ≤5 kDa fractions were specifically used for experiments with 3T3-L1 cells because only small bacterial metabolites are capable of reaching this target tissue through general blood circulation following absorption into the enterocytes.

Cell Culture

Cell lines (HT-29 and 3T3-L1) were purchased from American Type Culture Collection (ATCC) (Manassas, VA).

Human colon adenocarcinoma cell line HT-29 was maintained in HG-DMEM supplemented with 10% FBS, 1% L-glutamine, 1% NEAA, and 1% antibiotic/antimycotic solution and grown in T-25 flasks. HT-29 cells were utilized in this study due to their ability to possess characteristics of mature enterocytes as well as show differential expression levels of PPAR-target genes (Are et al. 2007, Howell 1992).

Once confluent (90%), HT-29 cells were switched to serum-free DMEM and then treated with bacterial cell free supernatants (CFS) for 24 hours. Treatment groups were categorized as follows: 20% CFS (v/v), 20% bacterial growth medium (control) (v/v),

50μM PPAR Agonist (WY14643), and No treatment (Cell culture medium only). Bacterial CFS was incubated with these cells to investigate the effect of bacterial metabolites on FIAF expression.

Pre-adipocytes (3T3-L1) were maintained in DMEM supplemented with 10% newborn calf serum (NCS), 1% L-glutamine, 1% NEAA, and 1% antibiotic/antimycotic. Once cells reached 48-hours post-confluence (Day 0 in terms of experiment), differentiation was induced by the addition of 0.25 μM dexamethasone, 50 mM 3-isobutyl-l-methylxanthine (IBMX), and 5mg/μl (0.174 mM) insulin in DMEM supplemented with 10% FBS, 1% L-glutamine, 1%, NEAA, and 1% antibiotic/antimycotic for 96 hours. After differentiation (Day 4), cells were allowed to mature in DMEM containing 10% FBS, 1% L-glutamine, 1%, NEAA, 1% antibiotic/antimycotic with the addition of 5mg/μl (0.174 mM) insulin for 96 hours (Day 4-Day 8). Cells were then serum-starved prior to treatment. Fully matured adipocytes were subjected to ≤5kDa bacterial supernatant fractions. This fraction (≤5kDa) was utilized due to the fact that metabolites of this molecular weight are capable of being absorbed into enterocytes and interacting with target host cells (Chae 2005). Same treatment groups were used as above, except that the agonist treatment was omitted.

Conditioned medium from HT-29 cells was collected in phenyl-methyl-sulfonyl-fluoride (PMSF)-containing tubes and centrifuged at 5500rpm for 5 mins to remove non-viable cells and debris. Conditioned medium was then transferred to separate tubes and followed by a 12.5% TCA precipitation for 2 hours on ice and centrifuged for 15mins @ 12,000g. Following centrifugation, protein pellets were washed twice with acetone (200μl) and centrifuged for 15mins @ 12,000 g. Pellets were air-dried for 30 min to

remove acetone and re-suspended in 300 µl of re-hydration buffer containing 0.125M Tris and 4% SDS. Protein concentrations in the resulting samples were determined by the Bio-Rad DC protein assay (microplate method).

Cell lysates from HT-29 cells were also collected post treatment. Cells were washed twice with cold PBS and detached from T-25 flasks by scraping. Suspended cells were pelleted by centrifugation for 2500 rpm for 5min. PBS was then removed and cell pellets were lysed on ice for 20 min in 50µl of RIPA buffer containing protease inhibitors. Lysates were then collected following removal of un-lysed cells by centrifugation at 12000 g for 10min.

FIAF Western Blot Analysis

Soluble proteins from supernatant collected via TCA precipitation (12.5%) of conditioned medium from HT-29 cells or from cell lysates were separated by SDS-PAGE on 10% polyacrylamide gels, and transferred to pre-treated PVDF membranes. Membranes were blocked using 5% blotto (Santa Cruz Biotechnology, Santa Cruz, CA) dissolve in 1X TBS with 0.05% Tween for 2 hours at room temperature and then incubated with primary anti-ANGPTL-4 purified goat IgG (1:500) (R&D systems) in 5% blotto/1X TBS with 0.05% Tween overnight (12-15 hours) at 4°C. Secondary antibody HRP-conjugated anti-goat IgG (1:1000) (R&D systems) was conducted in 5% blotto/1X TBS with 0.05% Tween for 1 hour at room temperature. Blocking as well as antibody incubations were followed by washings in 1X TBS with 0.05% Tween (4x10min). Immuno-reactivity was detected using an enhanced chemiluminescent plus ECL substrate kit (PerkinElmer). Secreted FIAF was normalized against Ponceau-S stained total protein. For normalization of intracellular FIAF levels, the corresponding membranes

were stripped and re-probed with anti-beta-actin antibody and intracellular FIAF levels were normalized to the levels of beta-actin. Primary mouse monoclonal anti-β-actin antibody (AC15, Sigma-Aldrich, St. Louis, MO) and its corresponding secondary antibody, IgG-HRP goat-anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Restore PLUS Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL) was used to strip membrane of antibodies. Stripping buffer was applied to the membranes and allowed to incubate for 10 mins at room temperature. The stripping solution was then removed and the membranes washed twice with 1X TBST for 5 mins before re-probing. The intensity of the FIAF bands, beta-actin bands as well as ponceau-stained total protein was quantified using GE ImageQuant© TL software. Protein concentration was determined according to the Bio Rad DC method using BSA (1-2mg/ml) as reference.

Lipoprotein Lipase (LPL) enzymatic activity in 3T3-L1 cells

Lipoprotein lipase enzyme assay was performed according to Gonzales and Orlando 2007 with modifications. Adipocytes were subjected to two different treatment regimens: (1) treatment post- maturation [Day 8-Day 14] (2) treatment beginning at induction of differentiation [Day 0-Day 8]. Pre-adipocyte cells were seeded in 6-well plates, allowed to differentiate and reach maturation. Following treatments as described previously, cells were then washed twice with phosphate buffered saline (PBS), and incubated with 200 μg/ml heparin prepared in PBS for 15 min at 25°C. Following incubation, the solution containing the heparin-released LPL was collected and centrifuged (15,000 × g, 5 min, 4°C) to remove cell debris. The LPL-containing solution was diluted 1:5 and plated in triplicate in 96-well microtiter plates. The substrate PNPB

(p-nitrophenyl butyrate, 10 mM) was diluted, per protocol, 1:10 for a final concentration of 1mM in cold acetone and added to each well. Absorbance at 405 nm was recorded following a 10 minute incubation using a Genesys UV Spectrophotometer. LPL activity was reported as the amount of p-nitrophenol product formed over the 10 min incubation at 37°C, expressed as a percentage of LPL activity following the control treatment (un-inoculated MRS). Assays for LPL activity were conducted on Day(s) 0, 8 and10.

Triglyceride (TG) accumulation in 3T3-L1 cells

TG accumulation in cells was monitored by Oil Red-O staining as described previously (Vankoningsloo 2005) with some modifications. Cells were subjected to similar treatment regimens as for the measurement of LPL activity. At the end of the treatment period, cell monolayers were washed with PBS and then fixed for 1 hour with 3.7% paraformaldehyde (Sigma) in PBS. Oil red-O (Sigma) was added for 1 hour at room temperature and cells were washed with deionized water to remove Oil Red-O debris. Isopropanol elution and quantification was conducted on Day(s) 0, 8, and 14. TGs accumulation was confirmed by visible lipid-droplet accumulation by phase contrast microscopy. Quantitative determination was performed by measuring the absorbance at 490 nm of the eluted oil-red O stain from the lipid-droplets by isopropanol elution.

RESULTS

B. longum increases the levels of secreted intestinal FIAF *in vitro*

An imbalance of the two predominate bacterial phyla within the gut (Firmicutes to *Bacteroidetes*) can result in host adiposity; part of which is due to a suppression of intestinal FIAF, a key modulator in microbial-regulated energy storage (Tilg et al. 2010,

Backhed et al. 2004). Therefore, to determine how certain probiotic CFS could potentially play a role in adiposity, FIAF protein levels were analyzed by western blot. Treatment of HT-29 cells with *L. casei* did not result in a net effect in FIAF protein levels compared to control (Fig 3.2.). Cells treated with *B. longum* resulted in an 83.15%±17.51 increase in FIAF protein levels (P=0.001) compared to control. These data indicate that CFS from the probiotic *B. longum* results in a significant increase in FIAF expression *in vitro*, indicating its anti-obesegenic potential.

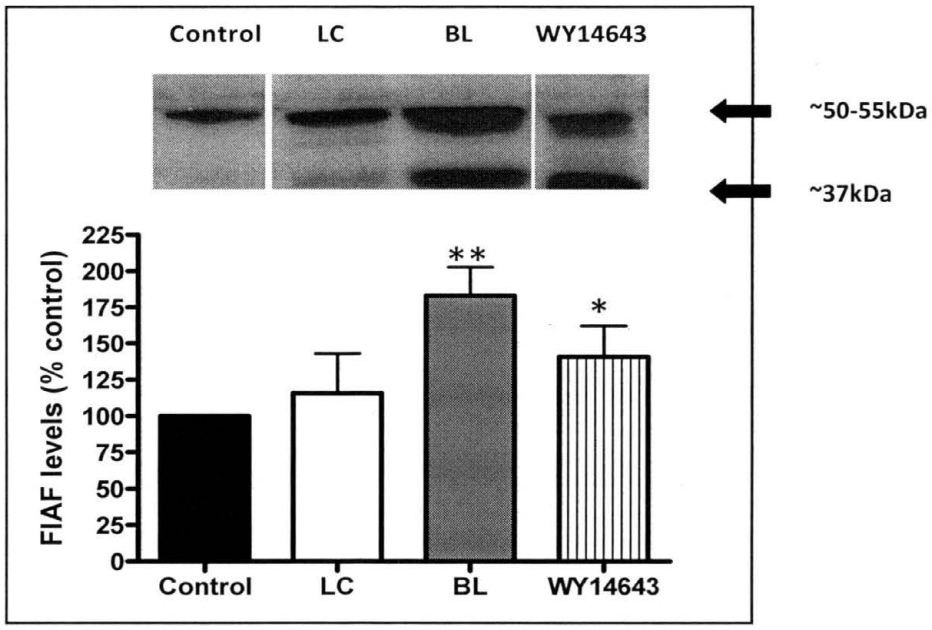


Figure: 3.2 *B. longum* increases the levels of secreted intestinal FIAF *in vitro*. HT-29 cells were treated with bacterial cell-free supernatants from *Lactobacillus casei* (LC) or *Bifidobacterium longum* (BL) for 24 hours and secreted FIAF was detected by immunoblotting. Treatment with a PPAR-α agonist WY14643 was used as a positive control for induction of FIAF expression. Results are presented as % control, relative to treatment with uninoculated bacterial growth medium MRS. * and ** indicate a significant difference from control at $p<0.05$ and $p=0.001$respectively.

Bioactive compounds of a molecular weight >5 kDa in CFS from B. longum result in the greatest increase in secreted FIAF levels

For further investigation into the nature of the secreted bioactive factor or metabolite responsible for a increase in secreted FIAF protein levels, *L. casei* and *B. longum* CFS were fractionated into a large molecular weight fraction > 5 kDa and a small molecular weight fraction < 5 kDa. HT-29 cells treated with either the >5kDa or <5kDa fraction of *L. casei* CFS resulted in no significant increase in FIAF protein levels compared to control (Fig 3.3). Cells treated with either the >5kDa or <5kDa fraction of *B. longum* CFS resulted in a significant increase ($P<0.05$) in FIAF protein levels by 137.96%±52.00 and 49.51%±15.87 respectively compared to control (Fig 3.3). These data indicate that the >5kDa fraction from *B. longum* resulted in the greatest increase in FIAF expression *in vitro*. Additionally, these results signify that the bacterial bioactive factor is of a large molecular weight.

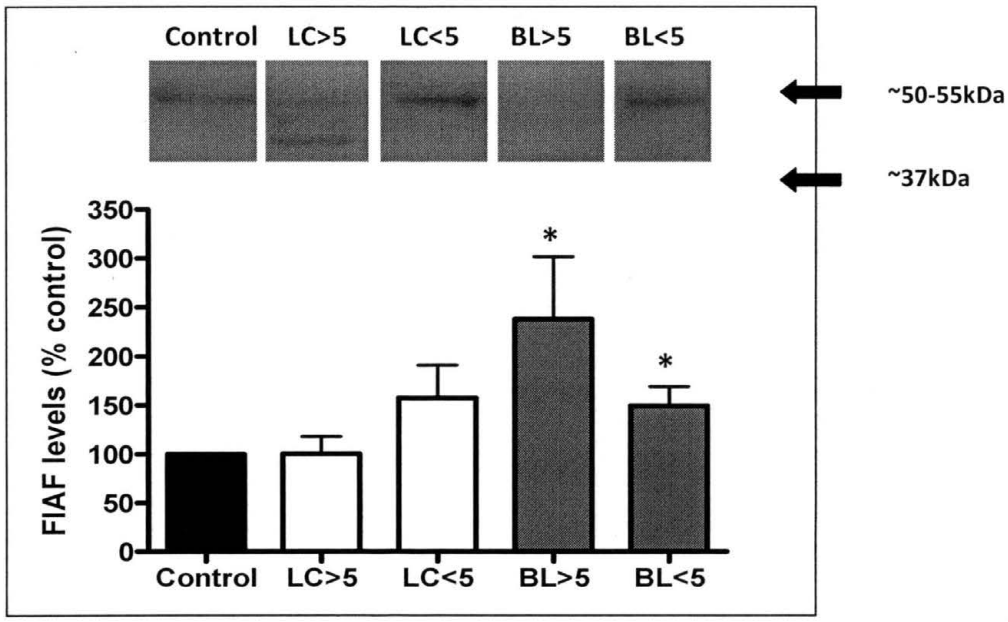


Figure: 3.3. Bioactive compounds of a molecular weight >5 kDa in CFS from *B. longum* result in the greatest increase in secreted FIAF levels. HT-29 cells were treated with >5 kDa or <5 kDa fractions of CFS from *Lactobacillus casei* (LC) or *Bifidobacterium longum* (BL) for 24 hours and secreted FIAF was detected by immunoblotting. Results are presented as % control, relative to treatment with similarly fractionated uninoculated bacterial growth medium MRS. * indicates a significant difference from control at $p<0.05$.

B. longum **increases the intracellular levels of intestinal FIAF** ***in vitro***

To investigate whether observed increases in secreted FIAF levels in response to *B. longum* CFS were related to increased intracellular FIAF levels; intracellular FIAF levels were also analysed by Western Blot. HT-29 cells treated with *L. casei* CFS had no significant effect on intracellular FIAF protein levels compared to control (Fig 3.4.). Cells treated with *B. longum* CFS resulted in a 47.16%±20.23 increase ($P<0.05$) in intracellular FIAF protein levels compared to control (Fig 3.4.). These data show that increased levels of secreted intestinal FIAF are partly due to increased intracellular FIAF levels in response to *B. longum* CFS.

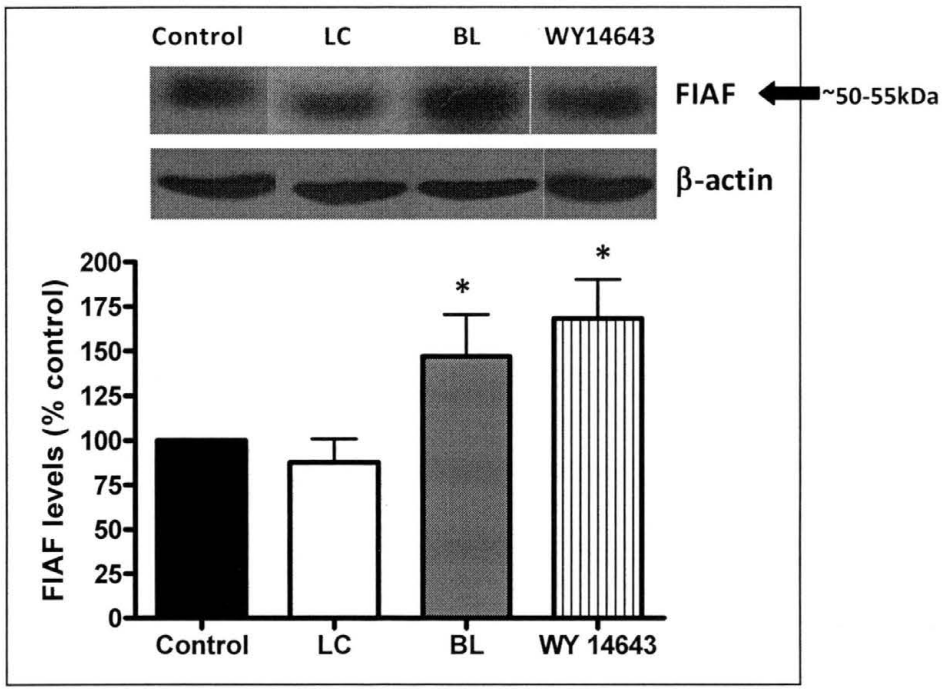


Figure: 3.4. *B. longum* increases the intracellular levels of intestinal FIAF *in vitro*. HT-29 cells were treated with bacterial cell-free supernatants from *Lactobacillus casei* (LC) or *Bifidobacterium longum* (BL) for 24 hours and intracellular FIAF in cell lysates was detected by immunoblotting. Treatment with a PPAR-α agonist WY14643 was used as a positive control for induction of FIAF expression. Results are presented as % control, relative to treatment with uninoculated bacterial growth medium MRS. * indicates a significant difference from control at $p<0.05$.

***Bifidobacterium longum* decreases lipoprotein lipase (LPL) activity in 3T3-L1 adipocytes**

The adipose tissue is an integral player in the regulation of metabolism due to its contribution to energy utilization and fat storage (Gonzales and Orlando 2007). Adipocyte hypertrophy is, in part, maintained by the hydrolysis of circulating lipoproteins and chylomicrons resulting in the subsequent uptake of extracellular fatty acids (Gonzales and Orlando 2007; Zandbergen et al. 2006). Recent research has designated that the gut microbial environment possesses the capacity to influence adiposity via FIAF; a potent inhibitory protein of LPL (Kersten et al. 2000). In our experiments, mature 3T3-L1 adipocytes treated with CFS from the probiotic *B. longum* significantly decreased LPL activity ($P<0.05$) to 69.32%±9.80 of control (3.5A). Pre-adipocytes subjected to co-treatment with *B. longum* CFS during differentiation and maturation to adipocytes also significantly decreased LPL activity ($P<0.05$) to 90.80%±4.72 of control (3.5B). CFS from *L. casei* did not result in any significant alteration in LPL activity in either fully matured or pre-adipocytes as compared to control. Our data indicate that bioactive factors present in the CFS of the probiotic *B. longum* decrease adipocyte LPL activity.

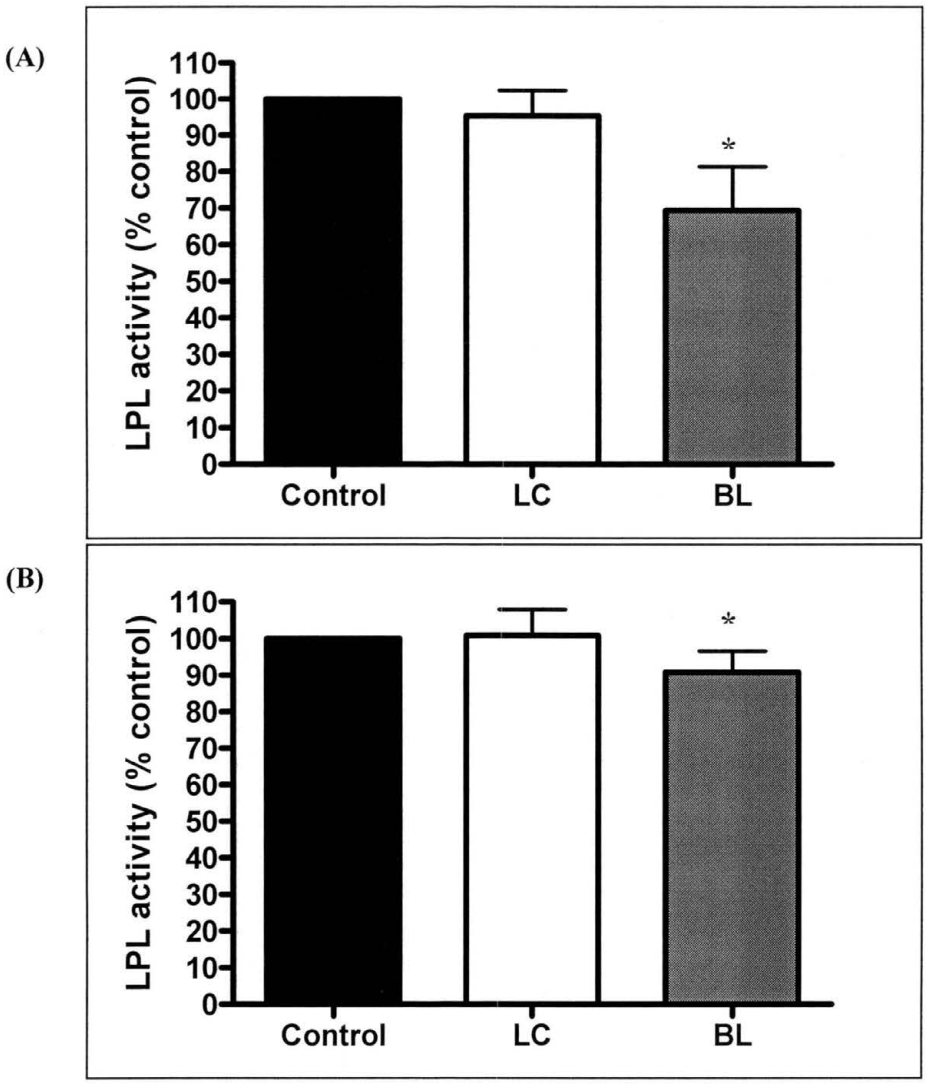


Figure: **3.5.** *Bifidobacterium longum* decreases lipoprotein lipase (LPL) activity in 3T3-L1 adipocytes. (A) Differentiated 3T3-L1 cells were treated with cell-free supernatants from *Lactobacillus casei* (LC) or *Bifidobacterium longum* (BL) for 48 hours, LPL was released from the cell-surface by heparin treatment and LPL activity was assayed by measuring p-nitrophenol released from p-nitrophenyl butyrate substrate. (B) 3T3-L1 preadipocytes were treated with CFS from LC or BL during cell differentiation and maturation as described in Methods and LPL activity was measured as in set (A). Results are presented as % control, relative to treatment with uninoculated bacterial growth medium MRS. * indicates a significant difference with respect to control, at $p<0.05$.

***Bifidobacterium longum* decreases triglyceride accumulation in 3T3-L1 adipocytes**

Triglyceride deposition and accumulation within various lipid-metabolizing tissues is related to different disease states and pathologies (Vankoningsloo et al. 2005). Studies have discussed possible microbial related mechanisms responsible for TG accumulation within host adipose tissue, in particular, modulation of lipid metabolism via FIAF/LPL (Backhed et al. 2004; Vankoningsloo et al. 2005).

3T3-L1 cells treated with CFS from *B. longum* resulted in a significant decrease ($P<0.05$) in TG deposition to 89.99%±5.14 of control (3.6A). Treatment of pre-adipocytes with CFS from *B. longum* during adipocyte differentiation and maturation also indicated a significant decrease ($P<0.001$) in TG deposition to 84.95%±0.70 of control (3.6B). The observed effects on TG accumulation were not observed upon treatment with CFS from the probiotic *L. casei*. These data indicate that secreted bioactive factors present in the CFS from *B. longum* results in a noteworthy decrease in TG deposition within pre-adipocytes as well as fully matured adipocytes.

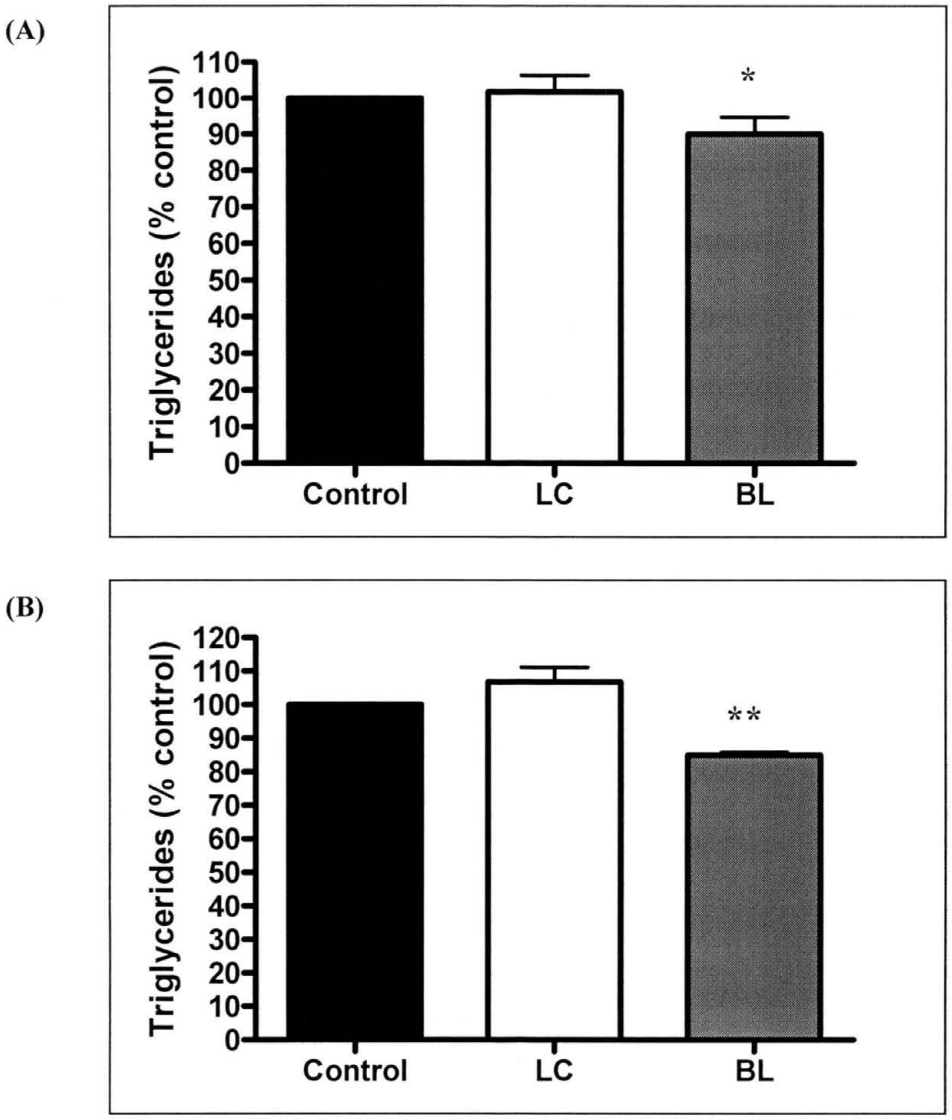


Figure 3.6: *Bifidobacterium longum* decreases triglyceride accumulation in 3T3-L1 adipocytes. (A) Differentiated 3T3-L1 cells were treated with cell-free supernatants from *Lactobacillus casei* (LC) or *Bifidobacterium longum* (BL) for 6 days and triglyceride levels were quantified by Oil-Red O staining of the intracellular lipids. (B) 3T3-L1 preadipocytes were treated with CFS from LC or BL during cell differentiation and maturation as described in Methods and triglyceride levels were measured as in set (A). Results are presented as % control, relative to treatment with uninoculated bacterial growth medium MRS. * indicates a significant difference with respect to control, at $p<0.05$ and ** at $p<0.001$.

DISCUSSION

This study was prompted by a several landmark studies from Prof. Jeff Gordon's group (University of Washington, St. Louis, MO) showing that (a) mice with gut flora are obese in comparison to germ-free mice partly due to suppression of intestinal levels of an LPL-inhibitor FIAF by gut microbes; resulting in increased LPL activity and increased triglyceride hydrolysis from circulating lipoproteins and increased fatty acid uptake and fat storage in the adipose tissue and (b) obese humans have a greater proportion of bacteria belonging to the phylum Firmicutes, compared to lean individuals. This led to two nutritionally significant questions pertaining to the role of probiotic bacteria in human health. Commonly used probiotic bacteria in foods and nutritional supplements belong to the genus Lactobacillus or Bifidobacterium. While studies have been conducted for several decades on the health benefits of probiotic bacteria in digestive and inflammatory disorders, their potential role in metabolic health has only received interest in the past few years. We chose to study a representative species from both genera Lactobacillus and Bifidobacterium, namely *L. casei* and *B. longum* based on their usage as probiotic strains in foods and also their presence in the human intestinal tract (Gill et al. 2006; Dumas et al. 2006). Interestingly, Lactobacillus sp. belongs to the obesity-related phylum Firmicutes whereas Bifidobacterium sp. belongs to the phylum Actinobacter, with no reported link to obesity. Additionally, a 'bifidogenic' diet in infants, promoted by breastfeeding has been related to decreased risk of long-term obesity as an adult. Our goal was to therefore conduct a systematic *in vitro* evaluation of the influence of *L. casei* and *B. longum* on the novel biomarker of microbe-mediated

effects on obesity, namely FIAF, as well as downstream effects on LPL activity and triglyceride storage.

Our results show that although Lactobacillus sp. belongs to the phylum Firmicutes, it did not significantly influence FIAF levels, LPL activity or triglyceride storage *in vitro*, suggesting that its use as a probiotic in foods does not pose an increased risk of obesity mediated via FIAF. In fact, a recent report has shown that another closely related strain of Lactobacillus (*Lactobacillus paracasei*) in fact increased FIAF levels and decreased fat storage in mice (Aronsson et al. 2010). It is thus likely that other bacterial species such as Clostridium sp. which is the predominant member of Firmicutes may be contributing to an obese phenotype rather than Lactobacillus sp.

Although Bifidobacterium sp. has been correlated with low risk of obesity, such as in breast-fed infants, the mechanisms behind this observation have not been very well understood. In light of the role of FIAF in microbe mediated obesity and our results showing an increase in both intracellular as well as secreted FIAF upon treatment with CFS from *B longum*, this could be a contributing mechanism to the anti-obesity effects of Bifidobacterium. Another recent study showed an ability of *Bifidobacterium breve* Strain B-3 (Kondo et al. 2010) in suppressing high-fat diet induced obesity in mice by increasing intestinal FIAF gene-expression.

We have indicated that the increase in intestinal FIAF protein levels by *B. longum* metabolites is primarily contributed to a large molecular weight molecule (>5kDa). These results suggest that the active metabolite is possibly a large peptide, protein, nucleic acid, and/or polysaccharide. Our study also examined intracellular FIAF and it was discovered

that active factors present in the CFS from the probiotic *B. longum* significantly increased intracellular FIAF protein levels. This suggests that increased levels of secreted FIAF are due to increased transcription and translation of the FIAF gene in HT-29 cells. However, the observed increase in secreted FIAF levels is much greater than increase in intracellular FIAF levels, suggesting additional mechanisms may be involved such as decreased post-translational degradation or increased trafficking to the cell-surface.

Secreted FIAF was visible as two distinct bands of approximately 50 kDa and 37 kDa, the lower molecular weight truncated form has primarily been shown to be present in secreted FIAF from various tissues (Koster et al. 2005; Dutton et al. 2007; Zandbergen et al. 2006) and generated by post-translational proteolytic cleavage. The 37 kDa band was not observed in intracellular FIAF, exhibiting a single band of roughly 50 kDa. Although the levels of both the 50 kDa band and the cleaved 37 kDa band were increased in response to PPAR-agonist WY14643 and *B. longum* CFS, the cleavage itself is not required for LPL-inhibitory activity. Therefore, total FIAF levels including both bands were considered when evaluating the influence of bacterial CFS on FIAF. Interestingly, the >5 kDa fraction of B. longum CFS primarily increased the levels of the 37 kDa band whereas the < 5 kDa fraction primarily increased the levels of the 50 kDa band with respect to control.

To confirm the specificity of the bands observed on western blots, we also tested TCA precipitates from bacterial cell-free supernatants as such as well as TCA precipitates from the cell culture medium used for treatments and neither resulted in immunoreactivity with the FIAF-antibody (data not shown).

The < 5kDa fraction of *B. longum* CFS was tested for effects on LPL activity and triglyceride deposition using two separate experimental designs: treatment with CFS post-maturation and treatment with CFS during adipocyte differentiation and maturation. Both scenarios resulted in LPL-inhibition and decreased triglyceride accumulation; however the suppression of triglyceride accumulation was greater when treatment was conducted during differentiation and maturation. This does not seem to depend solely on LPL-activity as greater LPL-inhibition was observed when treatments were conducted post-adipocyte maturation. This indicates that bacterial metabolites may be influencing triglyceride accumulation through PPAR-mediated effects on cell differentiation and maturation additional to FIAF/LPL mediated events. Although these results are preliminary and need to be confirmed in vivo, they may also suggest that probiotics such as *B. longum* may impart superior anti-obesity effects when administered earlier in the life-cycle such as during infancy and childhood rather than when administered as adults. This is supported by evidence from another study in mice has shown that consumption of milk fermented with probiotic *Lactobacillus gasseri* SBT2055 (E. M. Hamad et al. 2009) may reduce adipose mass and adipocyte hypertrophy in lean mice but unable to do so in mice with pre-existing obesity.

In conclusion, the results from our study suggest that secreted bioactive compounds from the probiotic *B. longum* may assist in the prevention and management of diet-induced obesity by increasing intestinal FIAF levels, decreasing adipocyte LPL activity and fat storage in the adipocytes. Ours is one of the first few studies to report the species-specific effects of probiotics on adiposity that are mediated by FIAF.

IV. GENERAL DISCUSSION AND FUTURE DIRECTIONS

The normal human microflora influences various physiological, metabolic, and immunological functions throughout the body. A number of studies have revealed the role that the enteric microflora plays in human nutrition and disease (Fig. 4.1). The microflora is necessary for many digestive and metabolic functions that humans alone cannot perform, thus their involvement to energy homeostasis and disease is attracting much attention (Tilg et al. 2009; Ley et al. 2005). Only recently has it been recognized that the microbial ecology within the intestines significantly affects dietary energy harvest, metabolism, epithelial function and integrity, as well as the progression of various metabolic diseases including obesity (Musso et al. 2010; Turnbaugh and Gordon 2009; Cani et al. 2008).

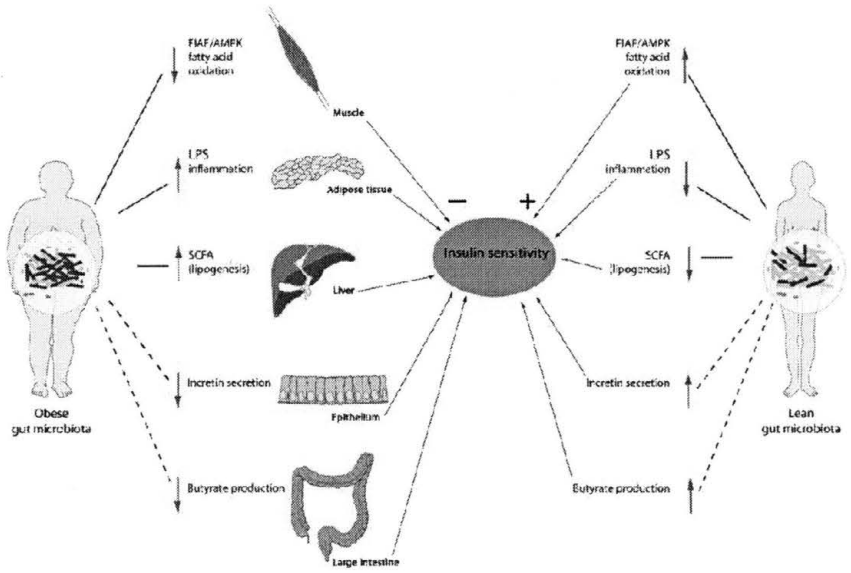


Figure 4.1: Possible relationships between gut microflora and human metabolism

Aside from the mechanisms listed above (Figure 4.1), yet another mechanism of action of gut microflora that may contribute to their role in obesity is their influence on digestive enzymes in the small intestine. Interestingly, only minimal investigative reports have revealed the microbial contribution to intestinal digestive activities within the gut and this has not been studied in the context of obesity. Human small intestine has a significant bacterial population and it is certainly plausible that these bacteria will impact host digestive enzymes either to divert diet-derived nutrients towards bacterial needs or as a physiological response to changes in the environment due to products of bacterial metabolism. The results of our study show that CFS from a probiotic *Lactobacillus casei* were able to increase the activity of intestinal brush border enzymes sucrase and maltase

and decrease the activity of pancreatic lipase, mediated by secreted bacterial components of varying molecular weights. CFS from another probiotic *Bifidobacterium longum* increased sucrase activity. CFS from a commensal bacterium *Bacteroidetes thetaiotaomicron* on the other hand decreased maltase and pancreatic lipase activity. This indicates that various species of gut microflora are capable of differentially modulating host digestive capabilities as a factor of not just bacterial enzymes but via impacting host enzyme activity. Another group has recently reported that in a gnotobiotic zebrafish model, gut microflora is able to influence host phospholipase and protease activity (Semova and Rawls 2010). This indicates that human gut flora can influence energy harvest by modulating host macronutrient digestion (carbohydrates, lipids and proteins). It remains to be seen what the net effect of this mechanism of energy harvest is on dietary caloric extraction and obesity. In future, our work in this area will focus on characterization of the secreted bioactives mediating host enzyme activity, initially based on molecular weight followed by identification via HPLC and MS. Some initial techniques we could employ in order to determine the compound(s) exhibiting these effects could be by subjecting our treatments to high heat, altering pH conditions, or the addition of a protease. Studies have shown that it is possible to solubilize proteins, lipids, and polysaccharides in the CFS from certain LAB by ammonium sulfate precipitation (proteins), cold enthanol extraction (polysaccharides) adsorption-desorption, and organic solvent extraction (lipids) (Kim et al. 2008; Pingitore et al. 2007). Another possible approach to further identify the compound increasing enzyme activity is to conduct our treatments using purified SCFAs instead of CFS. Studies have shown that SCFA treatments retain their effects regardless of pH adjustment to neutral, thus implicating that

the generated SCFA by our probiotics strains retain their integrity (Kwon and Ricke 1998). A recent study has shown that certain probiotics Lactobacilli sp. promote the increased expression of SGLT-1 and thus increase glucose uptake within the enterocytes (Rooj et al. 2010). These reports reinforce the idea of bacterial-probiotic cross-talk and their implication in host nutritional status, as well as their influences on enterocyte physiology. Therefore, we also hope to examine synergistic effects of probiotics and commensals in a co-culture system and influences on nutrient transporters in the gut. Also, we aim to further investigate the mode of enzyme activity regulation (transcriptional, post-transcriptional or post-translational).

The second part of our study focused on the ability of specific bacteria to influence energy-storage, specifically adipose fat deposition. We found that although the common probiotic *Lactobacillus casei* belongs to the phylum Firmicutes that has been reported to be enriched in obese individuals, it did not impact triglyceride storage in adipocytes, neither did it influence FIAF, a protein regulating LPL activity and ultimately triglyceride deposition in the adipose tissue. There have been some reports in literature about possible pro-obesity effects of Lactobacillus sp., as it has been used to promote higher body weight in farm animals (Armougom et al. 2009; Roault 2008) and has been found in higher numbers in feces of obese subjects (Armougom et al. 2009). However, these findings have currently not been accepted by the scientific community as a direct mechanistic role of Lactobacillus in obesity has not been demonstrated but only an indirect association shown. While increased disaccharidase activity in our studies may hint towards an obesogenic effect, decrease in pancreatic lipase activity will likely offset any net potential gain in energy. Moreover, in the complex gut ecosystem with thousands

of bacterial species and a dynamic flux of nutrients, individual species contributions while important, are hard to pinpoint. Nevertheless, considering that probiotic supplementation introduces a specific bacterial species into the gut at artificially high amounts, their effects on metabolism may be significant enough to warrant species-specific investigations aimed at understanding mechanisms of action and discovery of novel bioactive agents. For example, the other probiotic species investigated in our study, *Bifidobacterium longum* was able to significantly reduce triglyceride deposition in vitro, associated with increased FIAF and decreased LPL activity. However, a more in-depth examination of this effect is needed, as a decreased LPL may be involved in cardiovascular disease risk. Future studies in our lab will focus on identifying the secreted bioactive factor from *B longum* that up-regulates FIAF protein levels. Secondly, we aim to confirm influences of *B. longum* on triglyceride deposition in vivo, using the *C. elegans* as a model system, as it has been reported as a promising emerging model of obesity due to highly conserved and homologous energy metabolism pathways similar to humans.

The differential effects of the two probiotic strains examined in this thesis on dietary energy harvest and energy storage along with differences in bioactivity observed by the large and small molecular weight compounds present in the bacterial cell-free supernatants, it is possible that in future, probiotic-derived bioactives may be used as isolated compounds with targeted health benefits ('postbiotics'), rather than as live organisms which may be subject to greater biological variability, environmental susceptibility and unpredictability based on an individual's resident microbiome and genotype (Neish 2009; Kataria et al. 2009). In light of the potential of beneficial microbes

in influencing metabolic disease, the time is ripe for mining microbes as a source of novel bioactives for prophylactic or therapeutic application ('pharmabiotics').

REFERENCES

1. Aloulou A, Rodriguez JA, Fernanez S, Oosterhout D, Puccinelli D, Carrière: Exploring the specific features of interfacial enzymology based on lipase studies. Biochimica et Biophysica Acta 2006; 1761: 995-1013.

2. Armand M: Lipases and lipolysis in the human digestive tract: where do we stand? Curr Opin Clin Nutr Metab Care 2007; 10: 156-164.

3. Armougom F, Henry M, Vialettes B, Raccah D, Raoult: Monitoring bacteria community of human gut microbiota reveals an increase in *Lactobacillus* in obese patients and *Methanogens* in anorexic patients. PloS 2009; 9(4): e7125: 1-8.

4. Aronsson L, Huang Y, Parini P, Korach-Andre M, Hakansson J, Gustafsson JA, Pettersson S, Arulampalam V, Rafter J: Decreased fat storage by lactobacillus paracasei is associated with increased levels of angiopoietin-like 4 protein (ANGPTL4). PloS 2010; 5(9):e13078: 1-7.

5. Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI: The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci 2004; 101: 15718–15723.

6. Backhed F, Manchester JK, Semenkovich CF, Gordon JI: Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci 2007; 104: 979-984.

7. Booijink CCGM, Boekhorst J, Zoetendal EG, Smidt H, Kleerebezem M, de Vos WM: Metatranscriptome analysis of the human fecal microbiota reveals subject-specific expression profiles, with genes encoding proteins involved in carbohydrate metabolism being dominantly expressed. Appl Enivorn Microbiol 2010; 16(76): 5533-5540.

8. Byun Y, Singh VK, Yang VC: Low molecular weight protamine: a potential nontoxic heparin antagonist. Thrombosis Res 1999; 94: 53-61.

9. Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, Gibson GR, Delzenne NM: Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia 2007; 50: 2374-2383.

10. Cani PD, Delzenne NM: Gut microflora as a target for energy and metabolic homeostasis. Curr Opin Clin Nutr Metab Care 2007; 10: 729–734.

11. Cani PD, Delzenne NM, Amar J, Burcelin R: Role of gut microflora in the development of obesity and insulin resistance following high-fat diet feeding. Pathologie Biologie 2008; 56: 305–309

12. Cani PD, Delzenne NM: Interplay between obesity and associated metabolic disorders: new insights into the gut microbiota. Curr Opin Pharm 2009; 9: 737-743.

13. Cani PD, Delzenne NM: The role of the gut microbiota in energy metabolism and metabolic disease. Curr Pharm Des 2009; 15: 1546-1558.

14. Chae SY, Jang MK, Nah JW: Influence of molecular weight on oral absorption of water soluble chitosans. J Control Release 2005; 2;102(2): 383-394.

15. Collin MD, Gibson GR: Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. Am J Clin Nutr 1999; 69 (suppl): 1052S–1057S.

16. Cruz AG, Cadena RS, Walter EHM, Mortazavian AM, Granato D, Faria JAF, Bolini HMA: Sensory analysis: relevance for prebiotic, probiotic, and synbiotic product development. Inst Food Technol 2010; 9: 358-373.

17. Donkor ON, Henriksson A, Vasiljevic T, Shah NP: Proteolytic activity of dairy lactic acid bacteria and probiotics as determinant of growth and in vitro angiotensin-converting enzyme inhibitory activity in fermented milk. Lait 2007; 86: 21–38.

18. Dumas ME, Barton RH, Toye A, Cloarec O, Blancher C, Rothwell A, Fearnside J, Tatoud R, Blanc V, Lindon JC, Mitchell SC, Holmes E, McCarthy MI, Scott J, Gauguier D, Nicholson JK: Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. Proc Natl Acad Sci 2006; 103(33): 12511-12516.

19. Dutton S, Trayhurn P: Regulation of angiopoietin-like protein 4/fasting-induced adipose factor (Angptl4/FIAF) expression in mouse white adipose tissue and 3T3-L1 adipocytes. Brit J Nutr 2008; 100: 18-26.

20. Farooqi S, O'Rahilly S: Genetics of obesity in humans. Endocrine Rev 2006; 27(7): 710-718.

21. Fava F, Lovegrove JA, Gitau R, Jackson KG, Tuohy KM: The gut microbiota and lipid metabolism: implications for human health and coronary heart disease. Curr Med Chem 2006; 13: 3005-3021.

22. Freitas M, Tavan E, Cayuela C, Diop L, Sapin C, Trugnan G: Host-pathogens cross-talk. Indigenous bacteria and probiotics also play the game. Biol Cell 2003; 95: 503-506.

23. Gill SR, Pop M, DeBoy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE: Metagenomic analysis of the human distal gut microbiome. Science 2006; 312(2): 1355-1359.

24. Gonzales AM, Orlando RA: Role of adipocyte-derived lipoprotein lipase in adipocyte hypertrophy. Nutr Metabol 2007; 4: 22-30.

25. Güll I, Alves PM, Gabor F, Wirth M: Viability of the human adenocarcinoma cell line Caco-2: influence of cryoprotectant, freezing rate, and storage temperature. Sci Pharm 2009; 77: 133–141.

26. Haemer MA, Huang TT, Daniels SR: The effect of neurohormonal factors, epigenetic factors, and gut microbiota on risk of obesity. Prev Chronic Dis 2009; 6(3). http://www.cdc.gov/pcd/issues/2009/jul/09_0011.htm. Accessed October 2010.

27. Hamad EM, Sato1 M, Uzu K, Yoshida T, Higashi S, Kawakami H, Kadooka Y, Matsuyama H, Abd El-Gawad IA, Imaizumi K: Milk fermented by *Lactobacillus* gasseri SBT2055 influence adipocyte size via inhibition of dietary fat absorption in Zucker rats. Br J Nutr. 2009; 101: 716-724.

28. Hill JO: Understanding and addressing the epidemic of obesity: an energy balance perspective. Endocrine Soc 2006; 27(7):750–760.

29. Hooper LV, Midtvedt T, Gordon JI: How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr 2002; 22: 283-307.

30. Howell S, Kenny AJ, Turner AJ: A survey of membrane peptidases in two human colonic cell lines, Caco-2 and HT-29. Biochem J 1992; 284: 595-601.

31. Huin C, Schohn H, Hatier R, Bentejac M, Antunes L, Plénat F, Bugaut M, Dauça M: Expression of peroxisome proliferator-activated receptors alpha and gamma in differentiating human colon carcinoma Caco-2 cells. Biol Cell 2002; 94: 15-27.

32. Kataria J, Li N, Wynn J, Neu J: Probiotic microbes: do they need to be alive to be beneficial? Nutr Rev 2009; 9(67): 546-550.

33. Kersten S, Mandard S, Tan NS, Escher P, Metzger D, Chambon P, Gonzalezi FJ, Desvergne B, Wahli W: Characterization of the fasting-induced adipose factor FIAF; a novel peroxisome proliferator-activated receptor target. J Biol Chem 2000; 275: 28488-28493.

34. Kersten S: Regulation of lipid metabolism via angiopoietin-like proteins. Biochemical Soc 2005; 33(5): 1059-1062.

35. Kim Y, Whang JY, Whang KY, OH S, Kim SH: Characterization of the Cholesterol-Reducing Activity in a Cell-Free Supernatant of Lactobacillus acidophilus ATCC 43121. Biosci. Biotechnol. Biochem. 2008; 72 (6): 1483–1490.

36. Kleesen B, Blaut M: Modulation of gut mucosal biofilms. Brit J Nutr 2005; 93, Suppl 1: S35-S40.

37. Kondo S, Xiao J, Satoh T, Odamaki T, Takahashi S, Sugahara H, Yaeshima T, Iwatsuki K, Kamei A, Abe K: Antiobesity effects of *Bifidobacterium breve* strain B-3 supplementation in a mouse model with high-fat diet-induced obesity. Biosci Biotechnol Biochem 2010; 74(8): 1656-1661.

38. Kwon Y M, Ricke SC: Induction of Acid Resistance of Salmonella typhimurium by Exposure to Short-Chain Fatty Acids. App Environ Microbiol 1998; 64 (9): 3458-3463.

39. Ley RE, Backhed F, Turnbaugh PJ, Lozupone CA, Knight RD, Gordon JL: Obesity alters gut microbial ecology. Proc Natl Acad Sci 2005; 102: 11070-11075.

40. Ley RE, Turnbaugh BJ, Klein S, Gordon JI: Microbial ecology: human gut microbes associated with obesity. Nature 2006; 444: 1022-1023.

41. Long RT, Zeng WS, Chen LY, Guo J, Lin YZ, Huang QS, Luo SQ: *Bifidobacterium* as an oral delivery carrier of oxyntomodulin for obesity therapy: inhibitory effects on food intake and body weight in overweight mice. Internat J Obesity 2010; 34: 712-719.

42. Lye HS, Kuan CH, Ewe JA, Fung WY, Liong MT: The improvement of hypertension by probiotics: effects on cholesterol; diabetes; renin; and phytoestrogens. Int J. Mol Sci 2009; 10: 3755-3775.

43. Mandard S, Zandbergen F, Straten E, Wahli W, Kuipers F, Muller M, Kersten S: The fasting-induced adipose factor/angiopoitin-like protein 4 is physically associated with lipoproteins and governs plasma lipid levels and adiposity. J Biol Chem 2005; 281(2): 934-944.

44. Martínez I, Wallace G, Zhang C, Legge R, Benson AK, Carr TP, Moriyama EN, Walter J: Diet-induced metabolic improvements in a hamster model of hypercholesterolemia are strongly linked to alteration of the gut microbiota. Appl Environ Micorbiol 2009; 75: 4175-4184.

45. Martin FPJ, Sprenger N, Yap IKS, Wang Y, Bibiloni R, Rochat F, Rezzi S, Cherbut C, Kochhar S, Lindon JC, Holmes E, Nicholson JK: Panorganismal gut microbiome-host metabolic crosstalk. J Proteome Res 2009; 8: 2090-2105.

46. Martin FPJ, Wang Y, Sprenger N, Holmes E, Lindon JC, Kochhar S, Nicholson JK: Effects of probiotic lactobacillus paracasei treatment on the host gut tissue metabolic profiles probed via magic-angle-spinning NMR spectroscopy. J Proteome Res 2007; 6 (4): 1471–1481.

47. Mead JR, Irvine SA, Ramji DP: Lipoprotein lipase: structure, function, regulation, and role in disease. J Mol Med 2002; 80:753–769.

48. Mun S, Decker EA, Park Y, Weiss J, McClements DJ: Influence of interfacial composition on in vitro digestibility of emulsified lipids: potential mechanism for chitosan's ability to inhibit fat digestion. FOBI 2006; 1: 21–29.

49. Musso G, Gambino R, Cassader M: Gut microbiota as a regulator of energy homeostasis and extopic fat deposition: mechanisms and implications for metabolic disorders. Curr Opin Lipidol 2010; 21: 76-83.

50. National Institute of Health; www.nih.gov

51. Neish AS. Microbes in gastrointestinal health and disease. Gastroenterology 2009; 136:65-80.

52. Ogawa N, Satsu H, Watanabe H, Fukaya M, Tsukamoto Y, Miyamoto Y, Shimizu M: Acetic acid suppresses the increase in disaccharidase activity that occurs during culture of Caco-2 cells. Am Soc Nutr Sci 2000; 507-513.

53. Olejnik A, Lewandowska M, Obarska M, Grajek W: Tolerance of *lactobacillus* and *bifidobacterium* strains to low ph, bile salts and digestive enzymes. EJPAU 2005; 8(1): 1-12.

54. Pai R, Kang G: Microbes in the gut: a digestible account of host-symbiont. Indian J Med Res 2008; 128: 587-594.

55. Pan D, Zhang D: Function and safety assessment of Lactococcus lactis subsp. Lactis LB12 as potential probiotic strain. Afr J Biotechnol 2008; 7(22): 4037-4044.

56. Panesar PS, Kaur G, Panesar R, Bera MB: Synbiotics: potential dietary supplements in functional foods. IFIS 2010; http://www.foodsciencecentral.com/fsc/ixid15649. Accessed October 2010.

57. Pingitore EV, Salvucci E, Sesma F, Nader-Macías ME: Different strategies for purification of antimicrobial peptides from Lactic Acid Bacteria (LAB). Communicating Current Research and Educational Topics and Trends in Applied Microbiology 2007; A. Mendez-Vilas (Ed): 557-568.

58. Quezada-Calvillo R, Robayo-Torres CC, Ao Z, Hamaker BR, Quaroni A, Brayer GD, Sterchi EE, Baker SS, Nichols BL: Luminal substrate ''brake'' on mucosal maltase-glucoamylase activity regulates total rate of starch digestion to glucose. J Pediatr Gastroenterol Nutr 2007; 45: 32–43.

59. Quigley EM: Prebiotics and Probiotics; modifying and mining the microbiota. Pharmacol Res 2010; 61(3): 213-218.

60. Quigley EMM, Quera R: Small intestinal bacterial overgrowth: role of antibiotics, prebiotics, and probiotics. Gastroenterol 2006; 130: S78–S90.

61. Ramchandran L, Shah NP: Characterization of functional, biochemical and textural properties of snybiotic low-fat yogurts during refrigerated storage. Food Sci Technol 2010; 43: 819-827.

62. Rastall RA, Gibson GR, Gill HS, Guarner F, Klaenhammer TR, Pot B, Reid G, Rowland IR, Sanders ME: Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: An overview of enabling science and potential applications. FEMS Microbiol Ecol 2005; 145-152.

63. Raoult D: Obesity pandemics and the modification of digestive bacterial flora. Eur J Clin Microbiol Infect Dis 2008; 27: 631-634.

64. Resta SC: Effects of probiotics and commensals on intestinal epithelial physiology: implications for nutrient handling. J Physiol 2009; 587(17): 4169-4174.

65. Rooj AK, Kimura Y, Buddington RK: Metabolites produced by probiotic Lactobacilli rapidly increase glucose uptake by Caco-2 cells. BMC Microbiol. 2010; 10 (16): 1-10.

66. Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC, Crowley J, Yanagisawa M, Gordon JI: Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor; Gpr41. Proc Natl Acad Sci 2008; 105: 16767-16772.

67. Sanders ME, Marco ML: Food formats for effective delivery of probiotics. Annu Rev Sci Technol 2010; 1: 65-85.

68. Saulier DMA, Spinler JK, Gibson GR, Versalovic J: Mechanisms of probiosis and prebiosis: considerations for enhanced functional foods. Curr Opin Biotechnol 2009; 20: 135-141.

69. Shanahan F: Therapeutic implications of manipulating and mining the microbiota. J Physiol 2009; 587.17: 4175–4179.

70. Sim L: Structural and inhibition studies of human intestinal glucosidases. 2010; [Dissertation] Toronto, CA. University of Toronto.

71. Sonnenburg ED, Sonnenburg JL, Manchester JK, Hansen EE, Chiang HC, Gordon JI: A hybrid two-component system protein of a prominent human gut symbiont couples glycan sensing in vivo to carbohydrate metabolism. Proc Natl Acad Sci 2006; 23(103): 8834-8839.

72. Sonnenburg JL, Xu J, Leip DD, Chen C, Westover BP, Weatherford J, Buhler JD, Gordon JI: Glycan foraging in vivo by an intestine-adapted bacterial symbiont. Science 2005; 307: 1955-1959.

73. Sonnenburg JL, Chen CTL, Gordon JI: Genomic and metabolic studies of the impact of probiotics on a model gut symbiont. PLoS 2006; 4: 2213-2226.

74. Stappenbeck TS, Hooper LV, Gordon JI: Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. Proc Natl Acad Sci 2002; 99(24): 15451–15455.

75. Takemura N, Ozawa K, Kimura N, Watanabe J, Sonoyama K: Inulin-type fructans stimulated the growth of exogenously administered *Lactobacillus plantarum* No. 14 in the mouse gastrointestinal tract. Biosci Biotechnol Biochem 2010; 74(2): 375-381.

76. Tazoe H, Otomo Y, Karaki S, Kato I, Fukami Y, Terasaki M, Kuwahara A: Expression of short-chain fatty acid receptor GPR41 in the human colon. Biomed Res 2009; 30(3): 149-156.

77. Tilg H: Obesity, metabolic syndrome, and microbiota. J Clin Gastroenterol 2010; 44, Supp 1: S16-S18.

78. Tilg H, Moschen AR, Kaser A: Obesity and the microbiota. Gastroenterol 2009; 136: 1476-1483.

79. Turnbaugh PJ, Gordon JI: The core gut microbiome, energy balance and obesity. J Physiol 2009; 1587(17): 4153-4158.

80. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI: An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006; 444: 1027-1031.

81. Vankoningsloo S, Pauw A, Houbion A, Tejerina S, Demazy C, Longueville F, Bertholet V, REnard P, Remacle J, Holvoet P, Raes M, Arnould T: CREB activation induced by mitochondrial dysfunction triggers triglyceride accumulation in 3T3-L1 preadipocytes. J Cell Sci 2006; 119: 1266-1282.

82. Vankoningsloo S, Piens M, Lecocq C, Gilson A, De Pauw A, Renard P, Demazy C, Houbion A, Raes M, Arnould T: Mitochondrial dysfunction induces triglyceride accumulation in 3T3-L1 cells: role of fatty acid{beta}-oxidation and glucose. J Lipid Res 2005; 46: 1133-1149.

83. Vrieze A, Holleman F, Zoetendal EG, Vos WM, Hoekstra JBL, Nieuwdorp M: The environment within: how gut microbiota may influence metabolism and body composition. Diabetologia 2010; 53: 606-613.

84. Wolf G: Gut microbiota: a factor in energy regulation. Nutr Rev 2006; 64(1): 47-50.

85. Xiong M, Jing H, Zhiping L: Probiotics improve high fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells. J Hepatol 2008; 49: 821-830.

86. Yang SC, Chen JY, Shang HF, Cheng TY, Tsou SC, Chen JR: Effect of synbiotics on intestinal microflora and digestive enzyme activities in rats. World J Gastroenterol 2005; 11(147): 7413-7417.

87. Yang Y, Lowe ME: The open lid mediates pancreatic lipase function. J Lipid Res 2000; 14: 48-57.

88. Yin YN, Yu QF, Liu XW, Lu FG: Effects of four *Bifidobacteria* on obesity in high-fat diet induced rats. World J Gastroenterol 2010; 16(27): 3394-3401.

89. Zanderbergen F, Mandard S, Escher P, Tan NS, Patsouris D, Jatkoe T, Rojas-Caro S, Madore S, Wahli W, Tafuri S, Muller M, Kersten S: The G0/G1 switch gene 2 is a novel PPAR target gene. Biochem J 2005; 392: 313–324.

VITA

Reese Cotten was born in Houston, Texas on November 24th 1983, the son of Richard Ellis Cotten and Janice Lee Cotten. After receiving his primary education from Friendswood ISD, he went to Texas State University-San Marcos. He received the degree of Bachelor of Science in Family and Consumer Sciences, Nutrition and Foods from Texas State in May of 2007. He completed the Texas State Dietetic Internship and in August 2008, he entered the Graduate College of Applied Arts at Texas State University-San Marcos and began his studies in the Family and Consumer Sciences Department, Human Nutrition Graduate Program.

Permanent Address: Reese Cotten

2720 C.R. 155

Alvin, TX 77511

This thesis was typed by Reese E. Cotten.