# ELUCIDATING THE MODULATORY ACTIVITY OF PROBIOTIC *BIFIDOBACTERIUM LONGUM* TOWARDS ENTEROCYTIC FASTING INDUCED ADIPOCYTE FACTOR (FIAF), A REGULATOR OF FAT STORAGE

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ELUCIDATING THE MODULATORY ACTIVITY OF PROBIOTIC

*BIFIDOBACTERIUM LONGUM* TOWARDS ENTEROCYTIC

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A REGULATOR OF FAT STORAGE

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**DEDICATION**

*For Mom, Dad and Jonathan*

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## TABLE OF CONTENTS

**Page**

## LIST OF FIGURES

**Figure** **Page**

# CHAPTER I: INTRODUCTION

## *Obesity*

Over the past decade, the obesity rate in the United States has increased, affecting more than 35% of adults (National Center for Health Statistics, 2012; Ogden et al. 2012). Obesity is a serious health threat as it is a risk factor for many chronic diseases. These chronic diseases include, but are not limited to, coronary heart disease, type II diabetes mellitus and various forms of cancer, all which are leading causes of death in the U.S. (Centers for Disease Control and Prevention, 2010). The high prevalence of obesity and increased risk of related conditions has resulted in its recognition as a public health issue. Obesity results from an imbalance between energy intake via diet and energy expenditure via physical activity. By definition, obesity is the excessive accumulation of an individual's body fat as measured by a simple weight-for-height index referred to as BMI, body mass index (World Health Organization, 2012).

## *Factors regulating obesity*

Obesity is a complex disease involving the interaction of a number of causative factors including genetic and environmental factors and the imbalance between energy intake and expenditure (Tilg et al. 2009; Farooqi and O'Rahilly 2006).

Although these contributory factors are commonly accepted, the magnitude of their role in influencing obesity is only partly understood. The rapid increase in obese individuals over a short time span strikes out genetics as a likely major determinant but rather favors modifiable environmental factors, e.g. dietary nutrients, illness, and antibiotic therapy (Brownawell et al. 2012; Conterno et al. 2011;). A newly recognized environmental factor that has been shown to influence obesity is the composition of an individual's gut microflora and its ability to affect host metabolism (Backhed et al. 2004). Several studies have indicated that the interaction of host dietary components and gut microflora induce differential effects on carbohydrate and lipid metabolism and can thus contribute to increased fat storage and adiposity (Turnbaugh et al. 2006; Ley et al. 2005; Backhed et al. 2004).

***Human gut microbiota***

The importance of the human gut microbiome is underlined by its role in essential functions of host health and maintenance. The microbes that colonize the human intestines is estimated to comprise 300-500 different species (Scarpellini et al. 2010). The distribution of microbiota varies among anatomical sites but can be divided into three main sections: the stomach, small intestine (SI), and large intestine (colon). Bacterial concentrations within the stomach and ileum range from $10^1$ to $10^8$ colony forming units (cfu/ml) anre considered relatively low compared to that of the large intestine and colon (Wilson 2005). Bacteria in the small intestines of healthy persons consist of mainly facultative anaerobes: *Lactobacillus* sp., *Bacteroidetes* sp., *Clostridium* sp., *Bifidobacterium* sp., and *Streptococci*) (Quigley 2010). A rapid increase in bacterial concentrations and diversity may be observed upon reaching the colon (Fig. 3).

Numerous factors cause an influx in microbial distribution such as physiochemical dynamics (pH, oxygen content, and motility) of the gut and substrate availability (Quigley 2010). The human large intestines contains the most diverse microbial members than any other region of the human body with varying levels of all species, although not all have been identified, reaching as high as $10^{14}$ cfu/ml (Gill et al. 2006; Hooper et al. 2002).

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, the multifaceted bacterial-host interactions arising from the presence of these gut bacteria promote a symbiotic environment that influences host metabolism "beyond the gut''(Haemer et al. 2009; Wolf and Phil 2006; Freitas et al. 2003). 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). Since many studies have shown that microflora influences fat storage and adiposity, dietary

approaches using probiotics can thus contribute to inducing differential effects for favorable modulation of gut microflora (Sanders and Marco 2009; Yang et al. 2005).

***Gut flora and obesity***

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. An association has been established between the abundance of certain bacterial phyla in the human gut and the incidence of obesity (Musso et al. 2010; Vrieze et al. 2010; Cani and Delzenne 2009). Pioneering studies conducted by Jeffery Gordon and colleagues indicated that germ-free mice had 40% less total body fat compared to conventionally raised mice. This was despite the fact that germ-free mice had a 29% higher caloric intake compared to conventionally-raised mice. Transplantation of gut microbes from conventionally raised mice to the germ-free mice resulted in a 60% increase in body fat content and insulin resistance within two weeks (Backhed et al. 2007; Backhed et al. 2004). 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 (Backhed et al. 2007; Backhed et al. 2004). As mentioned earlier, these studies suggest that the presence of gut microflora contributes to obesity by influencing caloric extraction from the diet or dietary 'energy harvest'.

In a human study, analysis of distal gut microflora unveiled that an obese phenotype is related to an altered ratio of the two predominant bacterial divisions within the gut, Firmicutes and Bacteroidetes (Ley et al. 2005). The study determined that obese individuals exhibited a 50% reduction in the relative abundance of the phylum

Bacteroidetes and a corresponding increase in the phylum Firmicutes (Ley et al. 2005). This finding suggests a phylum-level positive correlation between the numbers of Firmicutes bacteria and obesity.

In addition to the phylum-level changes reported above, alterations at the genus/species level have also been reported. Numerous infant studies reveal bifidobacteria at a lower abundance in the microbiota of formula-fed infants in relation to breast-fed infants (Reinhardt et al. 2009). Children at 7 years of age exhibiting normal weight compared to overweight children were found to have higher numbers of *Bifidobacterium* spp. (Kalliomaki et al. 2008). Moreover, the study recognized that during the first year of life the quality and quantity of the microbiota varies as a result of *Bifidobacterium* spp. numbers. To reinforce this notion, studies have indicated that obese individuals have a higher concentration of *Lactobacillus* spp. in their gut than of lean or anorexic subjects (Armougom et al. 2009). Interestingly, bacterial-mediated responses have been shown to be species-specific; however, within the *Lactobacillus* and *Bifidobacterium* genera, metabolites with the capacity to exert differential effects on host metabolic functions have not been clearly elucidated (Grootaert et al. 2011; Aronsson et al 2010; Kondo et al. 2010; Mandard et al. 2006).

Dysbiosis, or imbalance of the gut microbiome causing an obese phenotype, has been recently termed “MicrObesity” (Cani et al. 2011). The rapidly mounting evidence to support the role of gut microflora in obesity signifies its potential as an emerging therapeutic target.

***Mechanisms of gut flora's role in obesity***

Key studies have proposed several primary mechanisms by which gut microbes modulate host adiposity and these are summarized in Figure 1 (Cani et al. 2009; Ley et al. 2005; Backed et al. 2004). Germ-free or conventionalized mice were fed a Western diet, high-fat and high-carbohydrate, for 8 weeks (Backhed et al. 2004). Germ-free mice had gained less weight and fat mass than conventionalized mice; however, germ-free and conventionalized mice feces were found to have similar energy content in contrast to the previous study by Gordon and colleagues. Both studies suggest that the gain in fat mass involves factors beyond efficient 'energy harvest' mediated by bacterial glycoside hydrolases. The increased processing of complex carbohydrates by microbial enzymes in the colon is followed by enhanced absorption of the monosaccharides and short-chain fatty acids (SCFAs) due to doubled density of the capillaries found in the villi of the small intestines upon conventionalization (Buck et al. 2009). Studies revealed that increased uptake of monosaccharides and SCFAs stimulated carbohydrate response element binding protein-, sterol response element binding protein-1- and G-protein

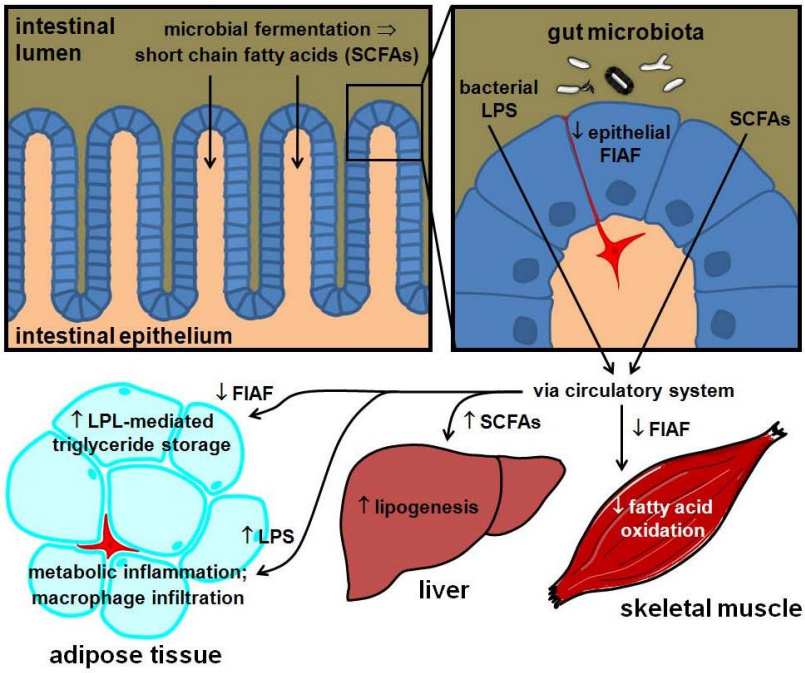


**Figure 1. Bacterial regulation of host metabolism and adiposity (J Pediatr Gastroenterol Nutr, 2009).**

coupled receptor-mediated hepatic and adipose tissue lipogenesis. Furthermore, conventionalization resulted in the elevated expression of acetyl-CoA carboxylase and fatty acid synthase, both which are necessary for de novo hepatic lipogenesis (Resta 2009; Tazoe et al. 2009). These changes result in a general accumulation of liver and adipose triglyceride storage.

The conventionalization of germ-free mice in a study by Backhed et al. 2007 also showed an increase in triglyceride deposition in adipocytes via a different mechanism mediated by a protein called fasting induced adipocyte factor (FIAF) and its influence on the activity of lipoprotein lipase (LPL). Microbiomes associated with mice resulted in selective suppression of intestinal FIAF, an inhibitor of LPL, thus promoting a general

increase in LPL activity, hydrolyzing triglyceride-rich lipoproteins to free fatty acids and consequently increased triglyceride accumulation in adipose tissue (Backhed et al. 2007; Backhed et al. 2004). Several studies have confirmed that FIAF influences plasma triglyceride levels by inhibiting LPL, a rate-limiting enzyme for plasma triglyceride hydrolysis (Kosta et al. 2005). FIAF has been reported to inactivate LPL via a transient interaction with its N-terminal domain and converts it to a stable inactivated form (Sukonina et al. 2006). The transcription of FIAF itself is controlled by PPARα, a peroxisome-proliferator activated receptor, which regulates shifts in cellular energy substrate preferences when nutrient environments change (Zandbergen et al. 2006). Thus, microbial regulation of host fat storage is in part mediated through intestinal expression of FIAF.

Data from recent studies suggests that the gut microbiota may also exert its effects through a mechanism mediated by bacterial lipopolysaccharides. Bacterial LPS are essential cell wall components of Gram-negative bacteria which have been reported to act as a trigger affecting obesity and insulin resistance via increased inflammation responses. Studies demonstrated by Cani et al. 2009 have shown that without altering energy intake, subcutaneous infusion of LPS in mice can cause weight gain and insulin resistance. LPS act by binding to the Toll-like receptor-4-CD14 complex inducing pro-inflammatory responses associated with obesity (Tsukumo et al. 2007). In addition, a high-fat diet has been reported to change gut microbiota by modulating plasma LPS levels and inflammation; a study in mice was shown to reduce the number of Bifidobacteria in the gut. Thus, a high fat diet may result in a reduction in the number of Bifidobacterial levels in the gut allowing higher LPS plasma levels (Cani et al. 2009).

Knowledge gained from these studies provides enormous insight into the ways that the gut microflora interacts with host-metabolism to influence obesity. Of the aforementioned mechanisms underlying microbial regulation of fat storage, FIAF/LPL-mediated effects appear to be an attractive target for dietary modulation as they play a central role in lipid metabolism.

### *FIAF as a therapeutic target*

*FIAF* is a human gene encoding for a secreted protein that plays an important role in glucose and lipid metabolism, angiogenesis and is strongly up-regulated by fasting in white adipose tissue and liver, as well as during adipogenesis (Mandard et al. 2006). Its abundance in plasma is increased by fasting and decreased by chronic high-fat feeding. In addition, FIAF plays an important role in the determination of adipose tissue size and plasma lipid levels (Kersten et al. 2000). Previous studies 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 storage and adiposity in the host (Backhed et al. 2004). It has been determined that 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; Backhed et al. 2004). 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. In addition, microbiota induce fat

deposition by influencing triglyceride storage within adipocytes (Tilg 2010; Backhed et al. 2007; Turnbaugh et al. 2006, Backhed et al. 2004). To further understand the contribution of FIAF to the relationship between gut microbiota and adiposity, comparisons were made using germ-free mice and germ-free knockout (FIAF -/-) mice. Results from the iinvestigation 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.

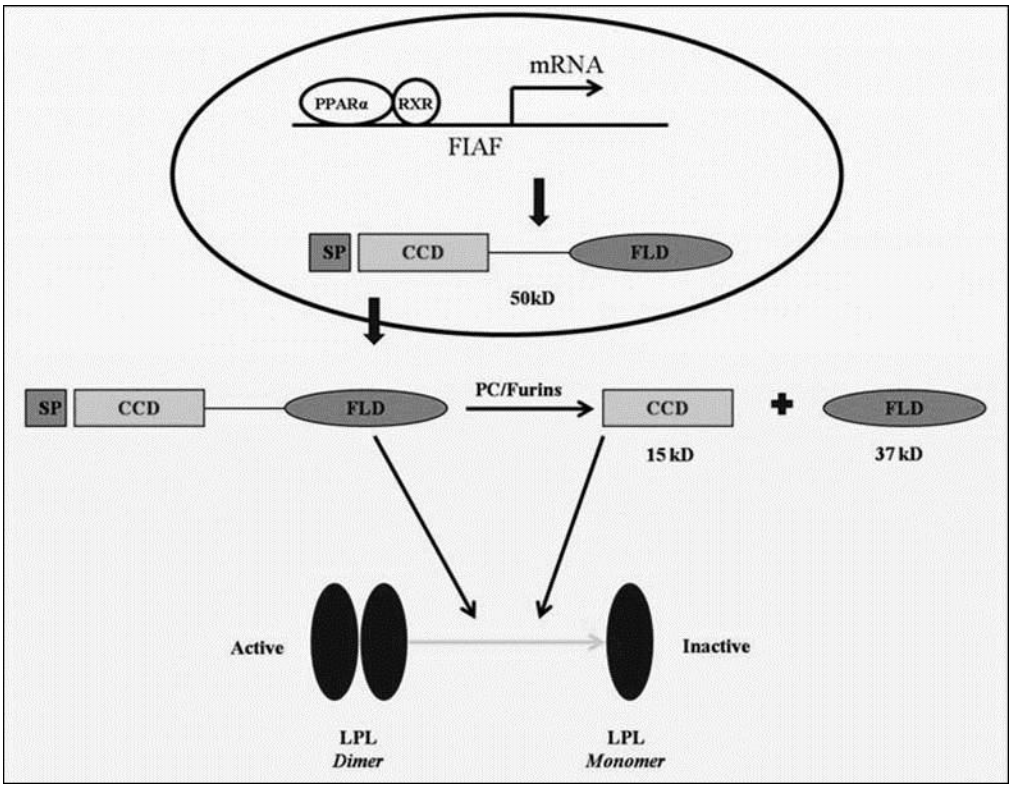


**Figure 2. Fasting-induced adipose factor (FIAF) synthesis and cleavage.** FIAF is synthesized in the cell as a 50 kDa full-length protein consisting of an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain. The full-length protein is secreted and upon secretion may be cleaved by proprotein convertases into 15 kDa N-terminal and 37 kDa C-terminal fragments. Circulating FIAF protein tightly binds LPL and converts the enzyme from dimers to monomers, thus rendering LPL activity.

FIAF is a PPARα target gene and its role in obesity is mediated by its ability to inhibit LPL (Kersten et al. 2000; Braissant et al. 1996). Recent advances report effects on LPL are mediated by the amounts of FIAF secreted as well as its post-translation cleavage (Lei et al. 2011; Cazes et al. 2006; Sukonina et al. 2006). In the cell, FIAF is synthesized as a full-length protein (50 kDa) consisting of an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain, Fig. 2 (Lei et al. 2011). Upon secretion, FIAF is cleaved by proprotein convertases, including furin, PC5/6, and paired basic amino acid-cleaving enzyme 4, into truncated 15 kDa N-terminal and 37 kDa C-terminal fragments (Lei et al 2011). Mechanistically, the circulating FIAF protein tightly binds LPL and converts the enzyme from dimers (active) to monomers (inactive), thus modulating the activity of LPL (Yin et al. 2009). Upon dislocation of the dimer molecule, FIAF is then released. The newly formed monomers of LPL remain stable but are incapable of reforming their active dimer configuration. Although cleavage of FIAF is not required for LPL-inhibition, it has been shown to enhance LPL-inactivation. Furthermore, studies indicate the N-terminal domain is pertinent for inhibitory action on LPL, whereas the C-terminal domain mediates antiangiogenic functions (Cazes et al. 2006; Sukonina et al. 2006).

Recent advances provide insight into the ways that the gut microflora interacts to influence obesity via FIAF-mediated mechanism. 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.

***Modulation of gut microflora by 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 (Roberfroid et al. 2010; Sanders and Marco 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). The majority of probiotic-containing foods generally result in an increase in the gut population of the genus Lactobacilli and Bifidobacteria associated with a multitude of reported health benefits. 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). Many efforts are now being focused in the direction of the gut microbiome and using 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 (Deibert et al. 2010; Corr and Gahan 2009). Although the mechanisms responsible for the beneficial effects of probiotic supplementation remain inadequately defined, the effects of supplementation are attributed to intricate microbe-microbe and microbe-host interactions (Musso et al. 2010). While the influence

that various gut-bacterial species including ‘probiotics’ have towards the gastrointestinal tract 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, *Lactobacillus* has been cause of controversy as it belongs to the obesity-related bacterial phylum Firmicute. Reports in literature have discussed possible pro-obesity effects of *Lactobacillus* spp., as it has been used to promote higher body weight in farm animals (Armougom et al. 2009) and in a separate study has been found in higher numbers in feces of obese subjects (Raoult et al. 2008). The growth promoting ability of this species suggests their use as probiotic supplements a concern for humans, and although both probiotic genera *Lactobacillus* and *Bifidobacterium* are used in commercially available products, manufacturers prefer *Lactobacillus* as it is more aerotolerant than *Bifidobacterium*, thus its viability is more readily maintained throughout food processing . It is important to note that these findings have only been highlighted as an indirect association, not as a direct mechanistic role of *Lactobacillus* in obesity. Conversely, *Bifidobacterium* spp. belongs to the phylum Actinobacter, with no reported link to obesity, and as mentioned earlier is an established resident in the human adult large intestine (Dumas et al. 2006; Gill et al. 2006). For example, a ‘bifidogenic’ diet in infants, promoted by breastfeeding, has been related to decreased risk of long-term obesity as an adult (Reinhardt 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. 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 mediated by host microflora. While there is a substantial body of knowledge with respect to the role of probiotics in gut health, research underlying species-specific mechanism in human health is limited.

***Preliminary Data***

Gut bacterial metabolites have been shown to influence fat deposition in adipose tissue by regulating the expression of a key regulatory factor called FIAF, a circulating inhibitor of the enzyme LPL (Yin et al. 2009; Mandard et al. 2006). FIAF levels are regulated by the transcription factor PPARα, increased during fasting and decreased during high chronic fat-feeding (Mandard et al. 2005; Zandbergen et al. 2005). Previous studies have shown that host microflora as well as alterations introduced by probiotic supplementation can influence metabolic parameters including cholesterol, triglycerides and fat storage (Pan and Zhang 2008; Wolf and Phil 2006). While both probiotic genera *Lactobacillus* and *Bifidobacteria* are used in commercially available products, the controversial status of the former in promoting adiposity and 'bifidogenic' properties of the latter are reasons for investigation on microbe-mediated mechanisms of obesity. As probiotics are often used for the enrichment of specific bacteria in the gut, we previously investigated if major genera of probiotics, *Lactobacillus* and *Bifidobacterium* species, could differentially modulate triglyceride deposition via FIAF/LPL-mediated mechanism. Our

previous investigation evaluated the influence of secreted bioactive compounds from representative probiotic strains from these two different genera, namely *Lactobacillus casei* and *Bifidobacterium longum*, on enterocytic FIAF synthesis and secretion. Specifically, it was determined that cell-free supernatants (CFS) from *L. casei* and *B. longum* could reduce triglyceride deposition via increased enterocytic FIAF expression. Our investigations examined the influence of CFS from *L. casei* and *B. longum* on the intracellular and secreted levels of FIAF in vitro in the human colonic epithelial cell line HT-29 by western blot.

### B. longum *CFS increases the intracellular levels of enterocytic FIAF in vitro*

Treatment of HT-29 cells with CFS from *L. casei* did not produce a significant change in the intracellular FIAF levels, with a value of 87.76%±11.38% of control (*P*=0.194) (Fig. 3). Treatment with *B. longum* CFS resulted in a significant increase of 47.16% ±20.23% (P=0.045) in intracellular FIAF protein levels compared to control (Fig. 3).

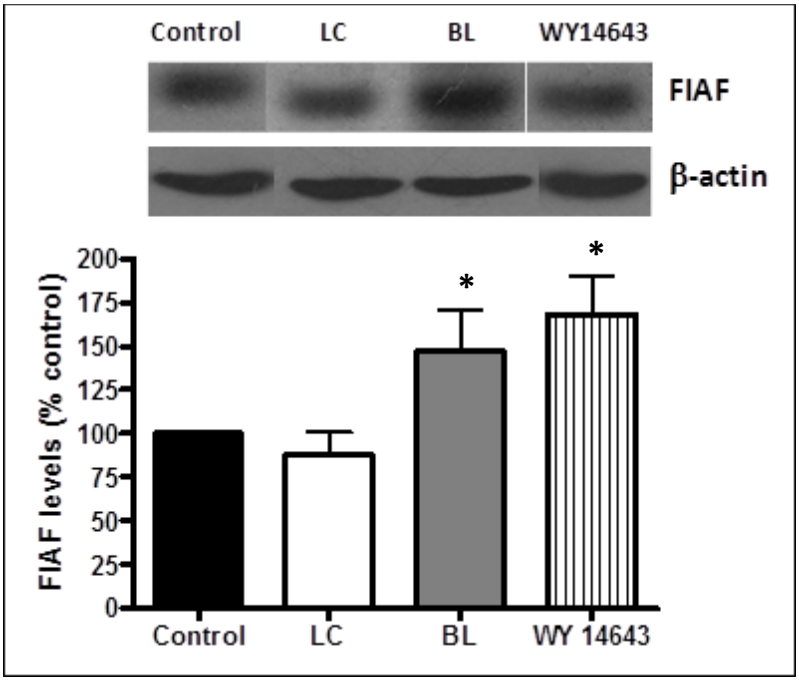


**Figure 3: CFS from *B. longum* increases the levels of intracellular enterocytic FIAF *in vitro*.** HT-29 cells were treated with bacterial CFS from *Lactobacillus casei* (LC) or *Bifidobacterium longum* (BL) for 24 hours and intracellular FIAF in cell lysates was detected by immunoblotting. Treatment 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. FIAF protein levels were normalized using β-actin as a loading control. Data shown are mean ± SEM of n=4. * indicates a significant difference from control at $p<0.05$.

### *CFS from* B. longum *increases the secreted levels of enterocytic FIAF in vitro*

Treatment of HT-29 cells with *L. casei* CFS did not result in a net change in secreted FIAF protein levels compared to control, with a value of 115.78%±24.39%, P=0.289 (Fig. 4). Treatment with *B. longum* CFS resulted in an 83.15%±17.51% increase in FIAF protein levels (P=0.001) compared to control. These data indicate that CFS from probiotic *B. longum* contains compounds with FIAF-modulatory activity, by which they could potentially influence dietary fat storage.

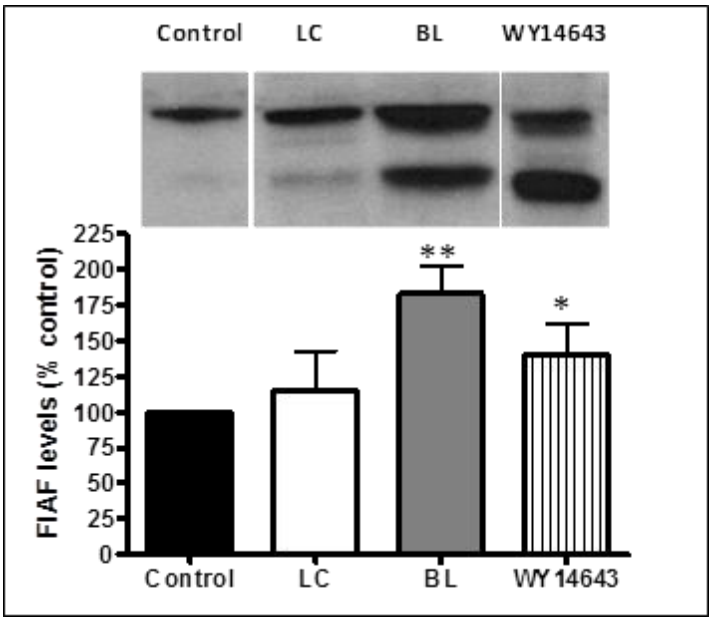


**Figure 4: CFS from *B. longum* increases the levels of secreted enterocytic FIAF *in vitro*.** HT-29 cells were treated with bacterial CFS from *Lactobacillus casei* (LC) or *Bifidobacterium longum* (BL) for 24 hours and secreted FIAF was detected by immunoblotting. Treatment with a PPAR-α agonist 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. Data shown are mean ± SEM of n=5. * and ** indicate a significant difference from control at $p<0.05$ and $p\leq0.001$, respectively.

We evaluated the influence of secreted bioactive components from *L. casei* and *B. longum* on FIAF levels *in vitro* as an indicator of their potential in regulating fat storage. Our preliminary data show that although *Lactobacillus* spp. belongs to the phylum Firmicutes, it did not significantly influence FIAF levels *in vitro*, suggesting that its use as a probiotic in foods likely does not pose an increased risk of obesity mediated via FIAF. Significant increases in both intracellular as well as secreted FIAF were observed upon treatment with CFS from *B. longum*. Secreted FIAF was visible as two distinct bands of approximately 50 kDa and 37 kDa, generated by post-translation proteolytic cleavage. The levels of both the full-length protein and the truncated form were increased in response to PPAR-agonist WY14643 and *B. longum* CFS. The cleavage itself is not required for LPL-inhibitory activity but may enhance it.

Preliminary data generated from our laboratory suggest that secreted bioactive compounds from the probiotic *B. longum* could assist in the prevention and management of diet-induced obesity by increasing enterocytic FIAF levels. Considering the rapid emergence of evidence for the modulation of adiposity by gut microflora including probiotics, it is important to understand the nature of the bacterial-derived bioactive compounds responsible. Thus, characterization of the secreted bioactive factors or metabolites that are responsible for modulating host adiposity may aid their application as prophylactic agents against diet-related obesity.

### *Objectives*

Gut microbial metabolites have been shown to influence diet-induced obesity by regulating the expression of FIAF. Although specific bioactive compounds responsible for mediating FIAF levels are not entirely known, it has been reported to be regulated by the transcription factor PPARα, increased during fasting and decreased during high chronic fat-feeding. Our laboratory has confirmed *B. longum* CFS as an inducer of FIAF expression in a human colonic epithelial cell line (HT-29); however, the bacterial factor or metabolite responsible for exerting these effects has not been established. The objective of this study is to elucidate the nature of the compound present in the *B. longum* CFS that possesses the observed bioactivity towards FIAF. This will be accomplished via a systematic exploration of key microbiological and biochemical parameters that may impact the ability of the *B. longum* CFS to increase the levels of FIAF *in vitro*. These include (1) the stage of bacterial growth, (2) effective concentration of the bioactive compound, (3) molecular weight of the bioactive compound, (4) susceptibility of the bioactive compound to (a) heat (b) freeze-thaw and (c) protease, and (5) direct bacterial adhesion to HT-29 cells.

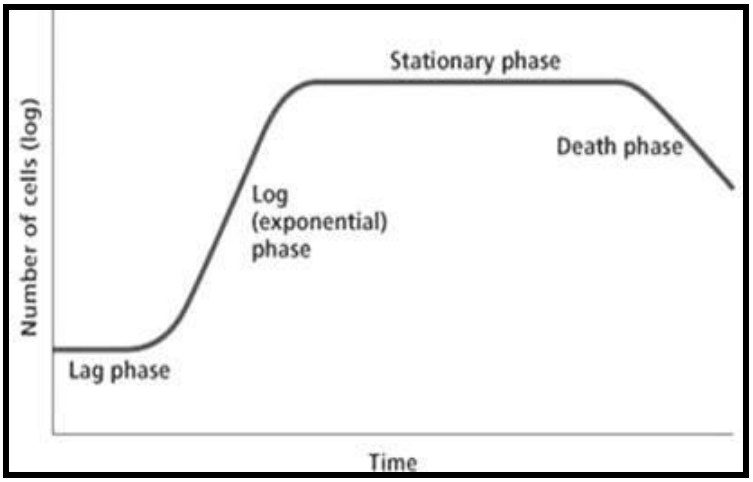


**Figure 5. Schematic bacterial growth curve shown as the number of bacterial cells per unit time (http://train-srv.manipalu.com/wpress/wp-content/uploads/2010/07/clip-image008-thumb108.jpg).**

Bacterial growth cultures are classified by four different phases (Fig. 5): lag, log or exponential, stationary and death. During the lag phase, bacteria adapt to environmental conditions by synthesizing RNA and enzymes. The exponential phase is dependent upon nutrient availability. Bacteria cell population doubles at a specific growth rate per unit time, producing metabolites assisting in essential functions such as cell maintenance and utilization of nutrients. Nutrient limitation and an accumulation of waste metabolites exhaust resources in the bacterial growth medium thus marking the stationary phase, where bacterial growth slows eventually resulting in bacterial death (Niel et al. 2002). Thus, metabolites produced during different phases of bacterial growth vary in proportions and types, determining the bacterial growth stage at which the bioactive metabolite is produced will provide insight regarding the nature of the compound (Rooj et al. 2010). Probiotic dosage varies among different food products and different bacterial

strains for efficient delivery of viable bacteria and biological functionality. Recommendations are typically 1-20 billion cfu/day for concentrations allowing colonization to occur; however, no consensus exists indicating the minimum concentrations for beneficial effects (Williams 2009). The efficacy of probiotics for therapeutic delivery would therefore be maximized after determining minimal concentrations for significant results. Details on molecular weight will provide insight into the type of compound. For instance, compounds of large molecular weight may represent a protein, enzyme, polysaccharide or nucleic acid; a compound of small molecular weight possibly indicates a short chain fatty acid, peptide or quorum signaling molecule. Proteins and enzymes are known to denature and unfold when exposed to heat and/or freeze-thaw. Susceptibility to these conditions as well as to protease-hydrolysis would therefore indicate the candidate factor to be a protein or an enzyme. Moreover, they will help ascertain resilience to commercial processing methods. Finally, investigating the impact of bacterial adhesion on in vitro FIAF levels will help compare the impact of direct bacterial-host cell interaction to that exerted by cell-free supernatants. As most probiotic bacteria are non-colonizers and only exert their effects transiently, this investigation will help understand the significance of picking an adherent versus non-adherent strain of bacteria and whether *Bifidobacterium longum* acts transiently or directly to mediate the FIAF/LPL mechanism (Lodemann et al. 2010).

CFS from each of the above described variations will be tested for their impact on FIAF levels *in vitro* using HT-29 cells. Various treatments for characterization of CFS from *B. longum* will assist in examining the biochemical nature of the modulatory factor prior to its detailed analysis for identification. Previous studies investigated the influence

of gut-bacterial metabolites on influencing fat deposition in adipose tissue by regulating the expression of FIAF; however, species-specific identification of the bacterial factor or metabolite responsible for exerting these effects have not been established (Grootaert et al. 2011; Aronsson et al 2010; Kondo et al. 2010; Mandard et al. 2006).

The proposed research will provide preliminary characterization of the secreted bioactive factor of probiotic *B. longum* responsible for modulation of enterocytic FIAF levels. Characterization may aid in its application as a prophylactic agent against diet-related obesity. Compounds with maximum bioactivity may potentially be used in a purified form, "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 (Kataria et al. 2009; Neisha 2009).

# CHAPTER II: MATERIALS AND METHODS

## *Bacterial Strain*

The probiotic strain used in this investigation was *Bifidobacterium longum* (*B. longum*, ATCC 15707) obtained from American Type Culture Collection (ATCC, Manassas, VA).

## *Bacterial Culture*

*B. longum* was cultured anaerobically in MRS broth (Oxoid) to an optical density at 600 nm (OD600) of 0.7-0.8, measured using a BioMate 3 spectrophotometer (Thermo Scientific). As assessed by viable plate counts, this OD corresponds to bacterial numbers of ~$10^8$-$10^9$ cfu/ml, representative of gut microflora density in the human small intestine (Rambaud et al. 2007).

## *CFS preparation*

CFS containing secreted bioactive compounds from *B. longum* (BLCFS) was routinely prepared by centrifugation (4000g, 10 min, 4$^o$C) of log-phase cultures (OD600~0.7-0.8) of *B. longum* in MRS. CFS pH was adjusted to 7.0 using 2 M NaOH, followed by filter-sterilization through a 0.2 μm sterile syringe filter (Corning). Un-inoculated bacterial growth medium (MRS) was used as a control. Prepared bacterial

CFS were stored in single-use aliquots at -20$^{o}$C until needed, and care was taken to avoid subjecting samples to freeze-thaw.

### *Variations of BLCFS to elucidate the nature of the secreted bioactive compounds*

#### *Stationary-phase BLCFS*

To assess the effect of bacterial growth stage on CFS bioactivity, CFS were also prepared from *B. longum* grown to stationary phase (OD600 of 1.2-1.4) for some experiments, in addition to the routinely used log-phase CFS. Other parameters such as growth medium, centrifugation conditions, pH and storage conditions were the same as log-phase CFS. All other BLCFS variations outlined below were prepared using log-phase CFS.

#### *Size-fractionation of BLCFS*

To identify the bioactive molecular weight range of BLCFS, supernatant was sequentially fractioned using Vivaspin Columns (Sartorius Stedim Biotech, Göttingen, Germany) with the following molecular weight cut-offs: 100 kDa, 50 kDa, 30 kDa, 10 kDa and 3 kDa. These were used to generate BLCFS fractions with the following molecular weight ranges: >100 kDa; 50-100 kDa; 30-50 kDa; 10-30 kDa; 3-10 kDa and <3 kDa. Each fraction was filter-sterilized through a 0.2 μm sterile syringe filter from Corning and stored at -20$^{o}$C in single-use aliquots as described above.

#### *Heat -denaturation of BLCFS*

For some experiments, the >100 kDa and the 50-100 kDa BLCFS fractions were pooled together into a >50 kDa fraction. This fraction was subjected to heat inactivation

in a ThermoFisher heating block at 95$^{o}$C for 20 minutes. Heat inactivated >50 kDa BLCFS was then centrifuged, and the supernatant transferred to a new tube and stored at -20$^{o}$C until needed.

***Freeze-thaw of BLCFS***

Freeze-thaw treatment of >50 kDa BL CFS was carried out by subjecting it to three rounds of freezing in liquid nitrogen followed by thawing at room temperature.

***Pepsin-hydrolysis of BLCFS***

Pepsin treated BLCFS was prepared by incubation of the unfractionated CFS or the >50 kDa CFS with immobilized pepsin from Thermo Scientific (cross-linked to agarose). Briefly, 250 µl of a 50% slurry of immobilized pepsin was equilibrated by two washes in the digestion buffer (20 mM sodium acetate buffer, pH 4.5) and then re-suspended in 500 µl of the same digestion buffer. Whole BLCFS or >50 kDa BLCFS was concentrated to a total protein value of 10 mg/ml by centrifugal ultrafiltration and 1 ml of each was added to the equilibrated immobilized pepsin and incubated for 4 hours at 37$^{o}$C with frequent mixing of the gel suspension. The immobilized pepsin gel beads were then removed by centrifugation and the supernatant containing the crude digest was decanted to a new tube. The immobilized pepsin was then washed with 1.5 ml of 10 mM Tris-HCl, pH 7.5. This wash was added to the crude digest for a total volume of ~3.0 ml of pepsin-hydrolyzed BLCFS. The hydrolyzed CFS were stored at -20$^{o}$C until use.

Variations of un-inoculated bacterial growth medium (MRS) prepared similarly as BLCFS were used as the respective controls in corresponding experiments.

***Cell Culture***

HT-29 cells were used as the *in vitro* model of the intestinal epithelium to investigate the bioactivity of BLCFS and its variations on enterocytic cells, specifically their impact on the secreted levels of FIAF. HT-29 cells were purchased from ATCC and maintained in high glucose-DMEM (Cellgro) supplemented with 10% FBS, 1% L-glutamine, 1% NEAA, and 1% antibiotic/antimycotic solution in tissue culture-treated 25 $cm^3$ flasks (Corning).

***Investigation of BLCFS effects on FIAF***

***i. Treatment of HT-29 cells with BLCFS***

For the purpose of experiments, near-confluent (90%) HT-29 cells were switched to serum-free DMEM and treated with whole BLCFS or its variations described above, along with the experimental controls, for 24 hours. Treatment groups were categorized as follows: No treatment (serum-free DMEM), Positive control (50 µM PPAR-α agonist WY14643), 20% (v/v) BLCFS or its variations and 20% (v/v) uninoculated MRS.

***ii. TCA-precipitation of secreted proteins***

At the end of the 24 hour treatment period, conditioned medium from each treatment group described above was collected in phenylmethylsulfonylfluoride (PMSF)-containing tubes with 3 µl PMSF per 1 ml of conditioned media and centrifuged at 5500 rpm for 5 mins to remove non-viable cells and debris. PMSF was prepared at a concentration of 50 mg/1 ml of ethanol. The conditioned medium was then transferred to fresh tubes followed by a 12.5% TCA precipitation for 2 hours on ice and centrifuged for 15 mins at 12,000g.

Following centrifugation, protein pellets were washed twice with acetone (200 µl) and centrifuged for 15 mins at 12,000 g. Pellets were air-dried for 30 min to remove acetone and re-suspended in 300 µl of re-hydration buffer containing 0.125 M Tris and 4% SDS.

***iii. Protein concentration determination***

Protein concentrations in the TCA-precipitated samples were determined by the Bio-Rad DC protein assay (microplate method) using BSA (1-2 mg/ml) as reference. 25 µg of total protein were used for FIAF-detection by western blot.

***iv. FIAF western blot analysis***

TCA-precipitated proteins from conditioned media prepared as described above were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked using 5% blot (Santa Cruz Biotechnology, Santa Cruz, CA) dissolved in Tris-glycine, 1X buffer (TBS), pH 8.4 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. Incubation with secondary HRP-conjugated anti-goat IgG (1:1000) antibody (R&D systems) was conducted in 5% blotto (Biorad)/TBS with 0.05% Tween for 1 hour at room temperature. Blocking as well as antibody incubations were followed by washings in TBS with 0.05% Tween (4 x 10 min). Immunoreactivity was detected using an enhanced chemiluminescent plus ECL substrate kit (PerkinElmer). The intensity of the FIAF bands was quantified using GE ImageQuant© TL software.

***v. Statistical analysis***

Secreted FIAF levels detected by western blotting upon treatment with BLCFS or its variations were expressed as a percentage of the FIAF levels detected in response to the un-inoculated MRS control. Values shown are the mean ± SEM of three independent experiments unless otherwise indicated. Data were analyzed using the Student's t-test comparing each CFS treatment with control and *P*-values < 0.05 were considered significant.

***Investigation of* B. longum *adhesion on FIAF***

To assess the influence of bacterial adherence on enterocytic FIAF levels, an adhesion assay was performed with *B. longum* and HT-29 cells in T-25 flasks. HT-29 cells were maintained as described above and were switched to serum-free DMEM medium free of antibiotics and antimycotics and containing 25 mM HEPES, prior to conducting the adhesion assay. For adhesion assays, *B. longum* was grown anaerobically in MRS as above and then sub-cultured into serum and antibiotic/antimycotic-free DMEM containing 25 mM HEPES and grown to an OD 600 of 0.7-0.8, representing the log phase of growth. 2 ml of the *B. longum* cell suspension was added to the HT-29 cells in a T-25 flask in a total volume of 5 ml. After two hours, any un-adhered bacterial cells were removed by washing the HT-29 cells with PBS and fresh cell culture medium was added. The adhered bacteria and cells were incubated for a period of 24 hours, following which the conditioned medium was collected for FIAF detection by western blotting. The number of adhered bacteria (CFU/ml) were determined by plate-counting on MRS agar, after detaching the bacteria from HT-29 cells by trypsin, and performing serial dilutions.

***Detection of proteins from BLCFS and HT-29 conditioned medium***

TCA-precipitated proteins from BLCFS as well as conditioned media prepared as described above were separated by SDS-PAGE on 12% polyacrylamide gels, and detected by staining using the Blue-BANDit protein stain (Amresco) according to the manufacturer's protocol. Following SDS-PAGE, gels were subjected to pre-washing for 15 min with deionized water followed by completely submerging the gel for 90 min in the Blue-BANDit stain. Deionized water was used to destain gels for 30 min to reveal blue protein bands against a clear background.

# CHAPTER III: RESULTS

## *Increase in FIAF levels is induced by bioactive compounds present in the log-phase BL CFS*

The effect of bacterial growth stage on FIAF levels were analyzed using HT-29 intestinal epithelium cells. The effects of *B. longum* log and stationary phase culture supernatants were examined (Fig. 6). Treatment of HT-29 cells with *B. longum* log phase culture supernatants significantly increased FIAF levels by 94.85%± 30.25% compared to control (P=0.017). Cells treated with *B. longum* stationary phase culture supernatants displayed no significant difference in FIAF levels (115.25%±33.04% of control, P=0.352). The results indicate that the increase in FIAF levels is due to bioactive compounds secreted or altered by *B.longum* during exponential phase of growth.

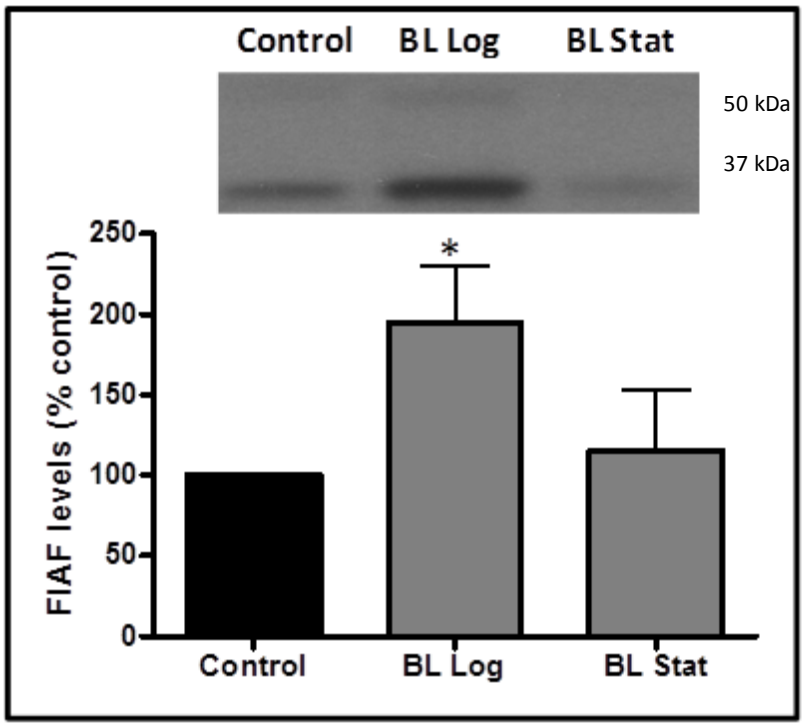


**Figure 6: Increase in FIAF levels is induced by bioactive compounds present in the log-phase BLCFS.** HT-29 cells were treated with cell-free supernatants from *Bifidobacterium longum* (BL) in the log or stationary phase of growth, for 24 hours and secreted FIAF was detected by immunoblotting. Results are presented as % control, relative to treatment with uninoculated bacterial growth medium MRS. Data shown are mean ± SEM of n=4. * indicates a significant difference from control at $p<0.05$.

***Increase in FIAF levels is dependent on the concentration of the BLCFS***

To determine if the effect of BLCFS on FIAF was concentration dependent, we treated HT-29 intestinal epithelium cells with three different concentrations of BLCFS: 10% (v/v), 20% (v/v), and 30% (v/v). Treatment of HT-29 cells with *B. longum* CFS at a concentration of 10% (v/v) resulted in no significant difference in FIAF levels with respect to control (112.02%±16.87%, P=0.324). *B. longum* CFS at a concentration of 20% (v/v) and 30% (v/v) resulted in a significant increase in FIAF levels by 142.07%±81.06%, P=0.045 and 211.76%±67.35%, P=0.013 respectively, relative to the control (Fig. 7). A concentration of BLCFS of at least 20% (v/v) is able to increase in FIAF levels. The FIAF levels in response to 20% (v/v) and 30% (v/v) BLCFS were significantly different compared to those in response to 10% (v/v) treatment; P=0.030 and 0.007 respectively; but there was no significant difference in the response to 20% (v/v) and 30% (v/v) concentrations (P=0.298).

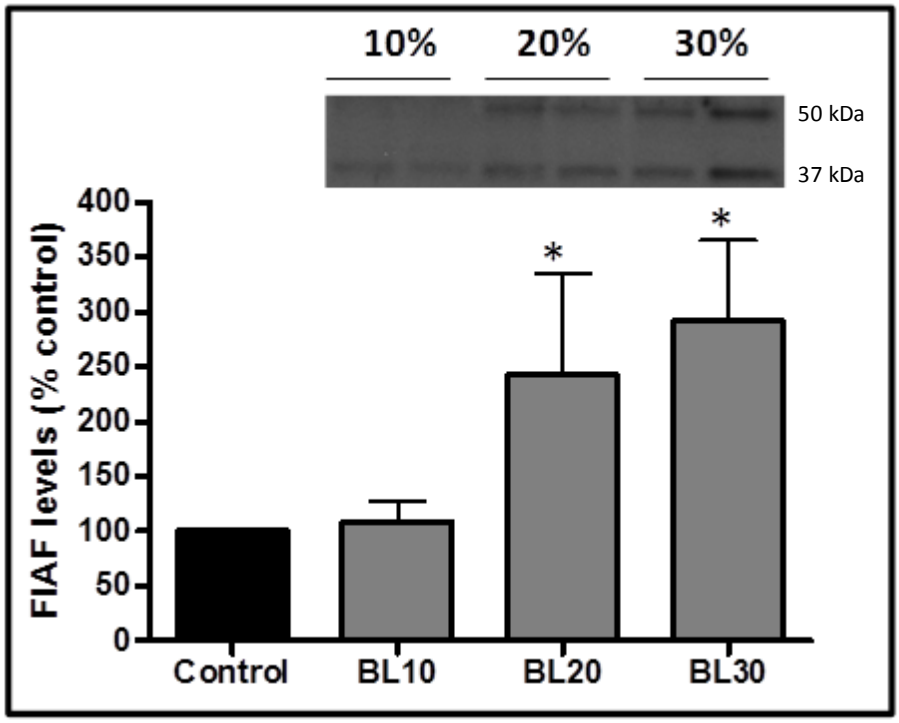


**Figure 7: Increase in FIAF levels is dependent on the concentration of the BLCFS**. HT-29 cells were treated with cell-free supernatants from *Bifidobacterium longum* (BL) at a concentration of 10% (v/v), 20% (v/v) or 30% (v/v) for 24 hours and secreted FIAF was detected by immunoblotting. Results are presented as % control, relative to treatment with the corresponding concentration of uninoculated bacterial growth medium MRS. Data shown are mean ± SEM of n=6. * indicates a significant difference from control, and † indicates a significant difference from effect of 10% (v/v) treatment, at $p<0.05$.

***Increase in FIAF levels by BLCFS is caused by bioactive compounds of molecular weight > 50 kDa***

For preliminary investigation of the nature of the secreted bacterial factor or metabolite responsible for an increase in FIAF levels, *B. longum* CFS were fractionated into various molecular weight ranges: >100 kDa; 50-100 kDa; 30-50 kDa; 10-30 kDa; 3-10 kDa and <3 kDa. *B. longum* CFS fractions were analyzed for their effect on FIAF expression using HT-29 intestinal epithelium cells. Treatment of HT-29 cells with the *B. longum* CFS >100 kDa and 50-100 kDa significantly increased FIAF by 28.49 ± 6.12% ($p=0.01$) and 51.57% ± 11.19% ($p=0.01$), respectively (Fig. 8). Subsequent fractions from *B. longum* CFS had no significant effect on FIAF with FIAF levels relative to the control as follows; 30-50 kDa: 116.56%±25.41%, $P=0.311$; 10-30 kDa: 119.56±16.85%, $P=0.194$; 3-10 kDa: 74.26%±15.50%, $P=0.123$; <3 kDa: 109.46%±7.65%, $P=0.165$. Based on the molecular weight range of BLCFS resulting in significant increases in the levels of FIAF (>100 kDa and 50-100 kDa), the factor may be a protein, enzyme, polysaccharide or nucleic acid. Interestingly, treatment with only the >50 kDa fractions resulted in the secretion of both the full-length FIAF protein (50 kDa) along with its truncated 37 kDa fragment which may have a role in LPL regulation as cleavage has been shown to enhance LPL-inactivation (Sukonina et al. 2006; Cazes et al. 2006).

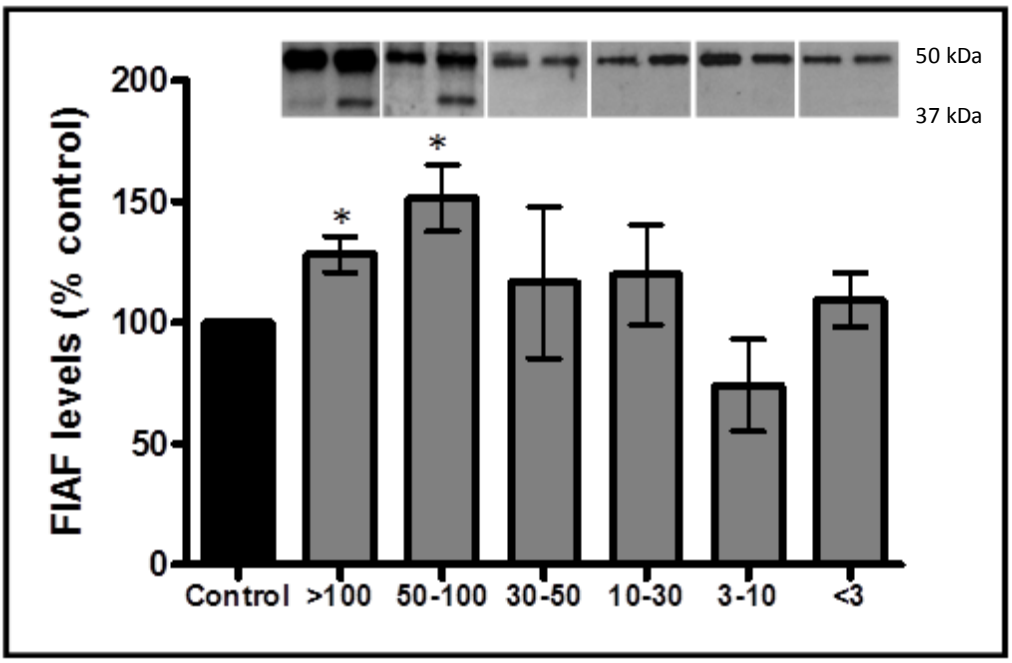


**Figure 8: Increase in FIAF levels by BLCFS is caused by bioactive compounds of molecular weight > 50 kDa.** HT-29 cells were treated with cell-free supernatants from *Bifidobacterium longum* (BL) separated into fractions of discrete molecular weights as described in the methods section, for 24 hours and secreted FIAF was detected by immunoblotting. Results are presented as % control, relative to treatment with uninoculated bacterial growth medium MRS of the corresponding molecular weight range. Data shown are mean ± SEM of n=3.* indicates a significant difference from control at $p<0.05$.

***FIAF-modulatory factors in the BLCFS are heat-resistant but susceptible to freeze-thaw***

To investigate whether the >50 kDa candidate factor could potentially be a protein or enzyme, the >50 kDa fraction of BLCFS was subjected to heat-treatment (HT) or freeze-thaw (FT) as described in methods. Heat-treatment did not result in loss of modulatory activity, with both BL>50 and the BL>50HT treatments inducing significant increases in secreted FIAF levels of 25.91%±11.48%, P=0.027 and 60.64%±28.02%, P=0.031with respect to the control, respectively. Subjecting the BL>50 fraction to freeze-thaw led to loss of FIAF-modulatory activity, and treatment of HT-29 cells with the >50 kDa FT fraction of *B. longum* CFS had no significant effect on FIAF levels, with a value of 96.63%±9.80%, P=0.369 with respect to control (Fig. 9). Thus, the bioactive compound from *B. longum* CFS of >50 kDa is heat-stable but loses its ability to increase the levels of secreted FIAF upon freeze-thaw.

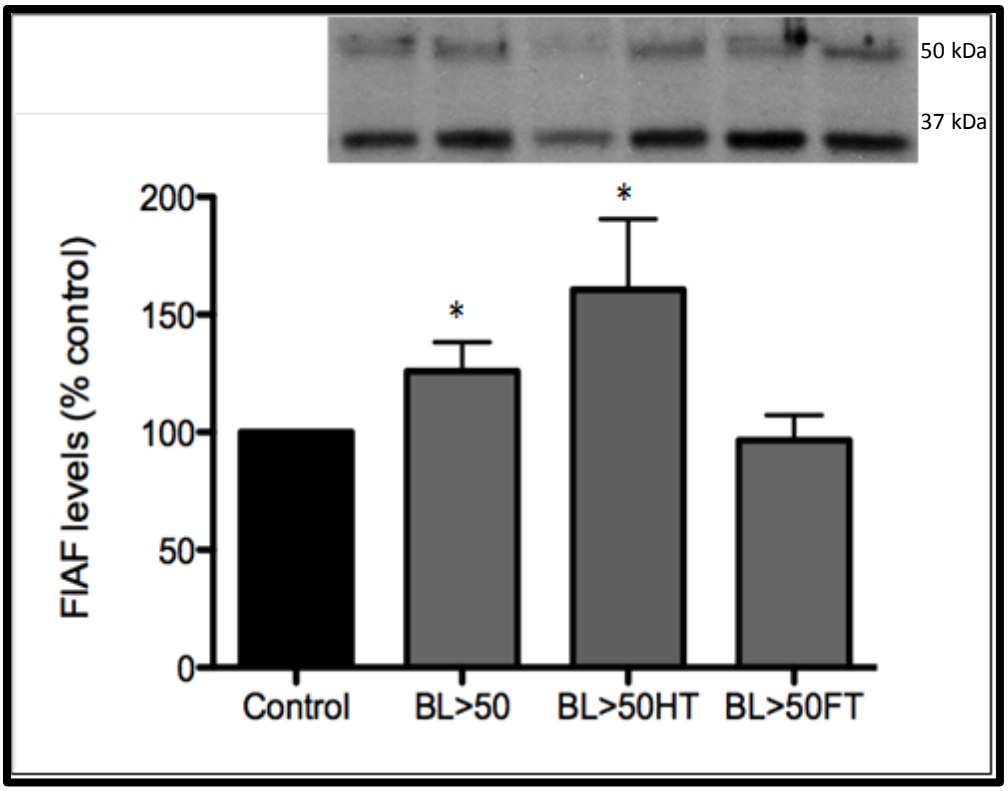


**Figure 9: The FIAF-modulatory factors in the BLCFS are heat-resistant but susceptible to freeze-thaw.** HT-29 cells were treated with cell-free supernatants from *Bifidobacterium longum* (BL) of >50 kDa that were subjected to heat-treatment (HT) or freeze-thaw (FT) as described in the methods section, for 24 hours, and secreted FIAF was detected by immunoblotting. Results are presented as % control, relative to treatment with uninoculated MRS of >50 kDa subjected to heat or freeze-thaw. Data shown are mean ± SEM of n=8. * indicates a significant difference from control at $p<0.05$.

***Pepsin-treatment renders the whole BLCFS and the >50 kDa fraction ineffective at increasing secreted FIAF levels***

To further characterize whether the bioactive factor present in the BLCFS could potentially be a protein, pepsin was used to hydrolyze any proteins present in the whole CFS from *B. longum* as well as the >50 kDa CFS fraction of *B. longum*. While both whole BLCFS (BL) and >50 kDa BLCFS (BL>50) that had not been pepsin-treated produced increases in secreted FIAF levels, as expected, of 79.21%±21.52% , P=0.009 and 98.96%±17.50% , P=0.0001 respectively, their pepsin-treated versions failed to do so, with relative FIAF levels being 97.85%±15.60%, P=0.450 and 100.70%±10.15%, P=0.477 for pepsin-treated whole CFS (BLPT) and pepsin-treated >50 kDa fraction (>50BLPT) respectively (Fig. 10). The above data provides further evidence to support that the candidate factor causing an increase in FIAF levels is a protein present in the CFS derived from *B. longum*.

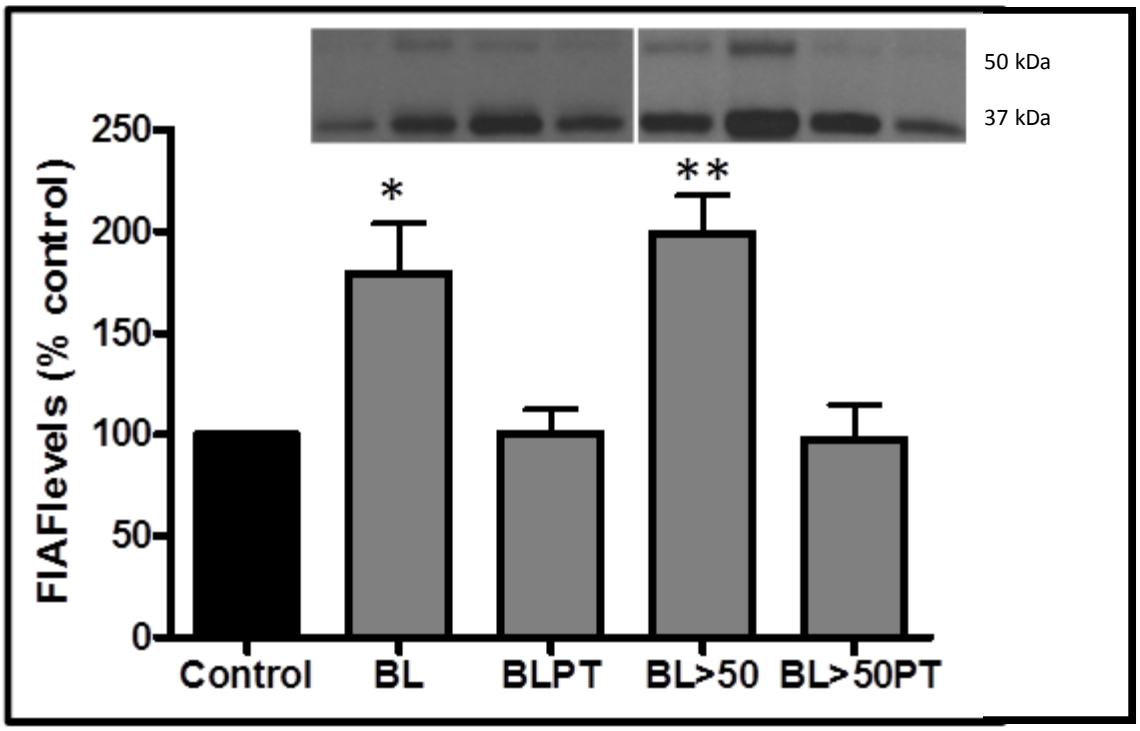


**Figure 10: Pepsin-treatment renders the whole BLCFS and the >50 kDa fraction ineffective at increasing secreted FIAF levels.** HT-29 cells were treated with whole cell-free supernatants from *Bifidobacterium longum* (BL) or those of >50 kDa that had been subjected to pepsin-hydrolysis as described in the methods section, for 24 hours, and secreted FIAF was detected by immunoblotting. Results are presented as % control, relative to treatment with uninoculated pepsin-hydrolyzed whole or >50 kDa MRS. Data shown are mean ± SEM of n=4 for BL and BLPT and n=7 for BL>50 and BL>50PT. * and ** indicate a significant difference from control at $p<0.05$ and $p\leq0.001$respectively.

***SDS-PAGE analysis of BLCFS and conditioned medium from BLCFS-treated HT-29 cells reveals protein bands ranging from ~30-100 kDa***

To corroborate the hypothesis that the candidate factor is a protein, CFS from *B. longum* were analyzed by SDS-PAGE to confirm that proteins of the expected molecular weight (>50 kDa) can be detected. BLCFS revealed several secreted protein bands ranging from ~30-100 kDa (Fig. 11), most prominently visible in the >50 kDa fractions. Heat-resistant protein bands were visible at ~70 kDa. The protein bands did not exhibit any visible change upon freeze-thaw. Protein bands consistent with the molecular weight range of secreted FIAF were visible in the conditioned medium from HT-29 cells upon treatment with the >50 kDa fraction. These bands were also visible in the conditioned medium collected from HT-29 cells after treatment with heat-treated BLCFS but not in the conditioned medium from cells treated with BL CFS that had been subjected to freeze-thaw. The results from SDS-PAGE thus are consistent with the immunoblotting experiments indicating that the candidate factor may be a freeze-thaw sensitive protein of molecular weight >50 kDa.

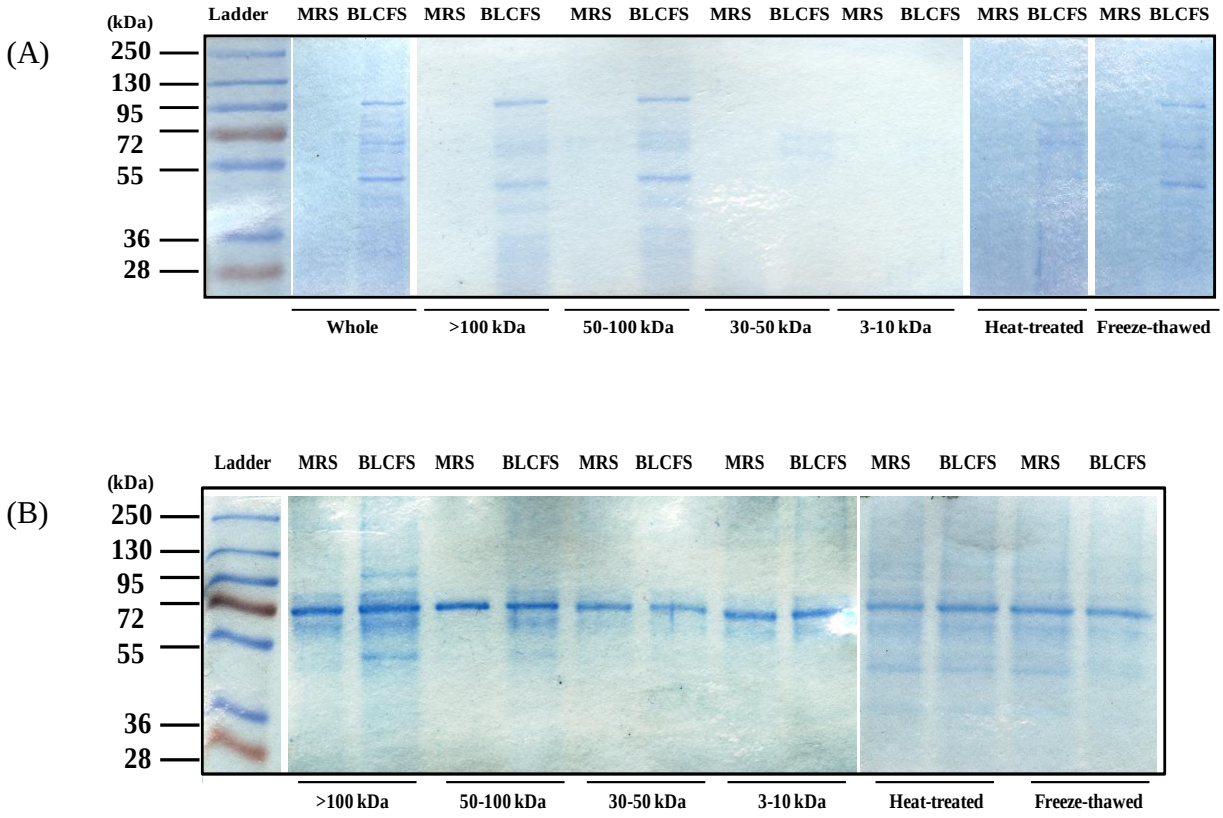


**Figure 11**. **SDS-PAGE analysis of secreted proteins** in (A) cell-free supernatants from *Bifidobacterium longum* and (B) conditioned medium from HT-29 cells collected after treatment with BL CFS fractions and heat- or freeze-thaw treated BL CFS.

***BLCFS, but not adherent* B. longum *cells, were able to increase secreted FIAF levels in vitro.***

To determine the role of direct bacteria-host cell interaction in modulating FIAF levels, FIAF levels were assayed following adhesion of live *B. longum* cells to HT-29 cells. Treatment with live bacteria increased FIAF levels by 27.23% with respect to control, compared with an 83.15%±17.51% increase in FIAF protein levels (P=0.001) with BLCFS (Fig. 12). The number of adhered bacteria was (9.5±1.2) x $10^6$ cfu/ml. The results indicate that direct adhesion of *B. longum* does not enhance its effects on FIAF, suggesting that a secreted bacterial component rather than a cell-cell interaction is responsible for the observed effect.

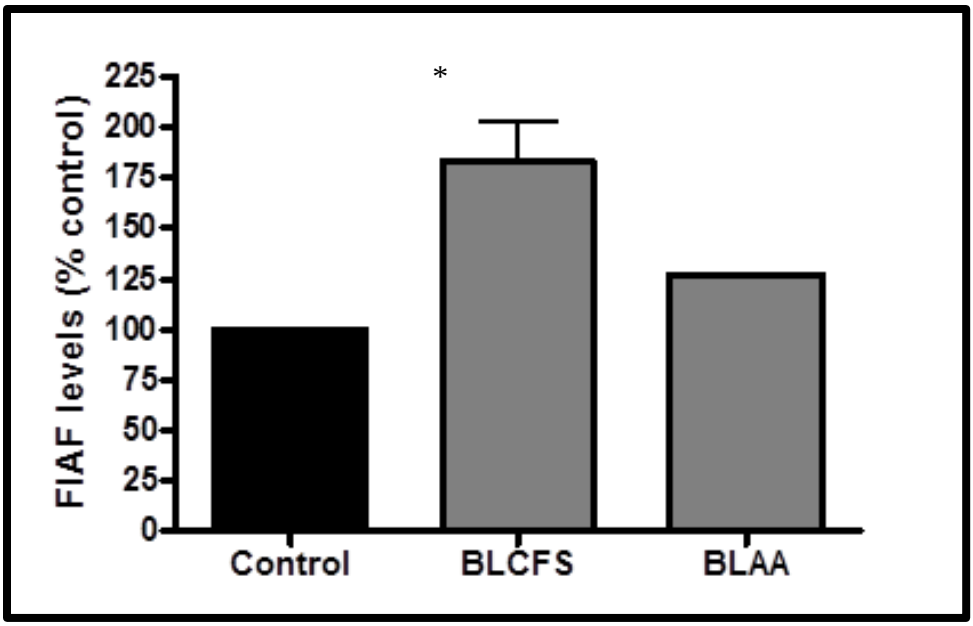


**Figure 12**. **BLCFS, but not adherent *B. longum* cells, were able to increase secreted FIAF levels *in vitro*.** HT-29 cells were treated with whole cell-free supernatants from *Bifidobacterium longum* (BL) or live bacteria in an adhesion assay (AA) as described in the methods section, for 24 hours, and secreted FIAF was detected by immunoblotting. Results are presented as % control, relative to treatment with uninoculated MRS. * indicates a significant difference from control at $p<0.05$.

# CHAPTER IV: DISCUSSION AND FUTURE DIRECTIONS

In light of the role of FIAF in microbe mediated obesity, probiotic strains from both genera *Lactobacillus* and *Bifidobacterium*, namely *L. casei* and *B. longum* were evaluated previously based on their usage as probiotic strains in foods and also their presence in the human intestinal tract (Dumas et al. 2006; Gill et al. 2006). Interestingly, *Lactobacillus* spp. belongs to the obesity-related phylum Firmicutes whereas *Bifidobacterium* spp. 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. We evaluated the influence of *B. longum* on the novel biomarker of microbe-mediated effects on obesity, namely FIAF (Reinhardt et al. 2009).

Our preliminary data showed that although *Lactobacillus* spp. belongs to the phylum Firmicutes, *L. casei* did not significantly influence FIAF levels *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* spp. which is the predominant member of Firmicutes may be contributing to an obese phenotype rather than *Lactobacillus* spp.

Although *Bifidobacterium* spp. 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. Our results showed 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) to suppress high-fat diet-induced obesity in mice by increasing intestinal FIAF gene expression.

Characterization of the probiotic-derived factors or metabolites that are responsible for inducing FIAF may aid their application as prophylactic agents against diet-related obesity; therefore, in this study we investigated the nature of the bioactive factor responsible for mediating FIAF modulatory activity. The present findings demonstrate that the bacterial factors produced during exponential growth of the *B. longum* cultures increase FIAF levels *in vitro*, but this was not observed in stationary phase cultures (Fig. 5). Exponentially grown bacterial cultures of log phase secrete metabolites into the growth medium for extracting energy from nutrients and health promoting factors. A number of studies have established production of health-promoting compounds by intestinal bacteria, such as fatty acids like conjugated linoleic acid (CLA) by conversion of linoleic acid by *Bifidobacterium* spp. CLA has been shown impart antimicrobial properties to influence microbial populations and modulate host metabolism of fatty acids within the liver and adipose tissue (O'Shea et al. 2011). A study in obese rats showed supplementation with CLA significantly decreased body fat and triglyceride levels (Zhou et al. 2008). In addition, CLA has been proposed to impact lipid metabolism by modulating activation of transcription factors such as PPARs. Bacteriocins produced by

various bacterial species also possess antimicrobial properties for immunity against pathogens. Bacteriocins include microcins of low molecular weight (<5 kDa) and colicins of high molecular weight range (25-80 kDa). Moreover, it was reported that decreases in nutrient availability, such an occurrence during stationary bacterial growth phase, affects the production of colicins (O'Shea et al. 2011).

Determination of concentration dependency served as a measure to maximize cell viability and metabolic function as well as determining a safe range for dosage within commercially prepared products. Our studies indicate FIAF levels are significantly increased at a concentration of 20% (v/v) and 30% (v/v); although viability assays (data not shown) suggest treatments at a concentration no more than 20% (v/v) due to decreased overall HT-29 intestinal epithelium cell health; therefore, remaining experiments utilized treatments at a concentration of 20% (v/v). Few studies have been conducted to determine minimal concentrations necessary for beneficial effects; although studies by Klingberg et al. (2005) and Lindfors et al. (2008) have reported probiotic supplementation as dose-dependent. Commercially available probiotic-containing products vary in dosage and are strain-specific with many products containing a multitude of strains. Commane et al. (2005) concluded that the concentration of bacterial numbers was not the determining factor but rather the concentration of specific metabolites.

We have indicated that the increase in intestinal FIAF protein levels by *B. longum* metabolites is primarily contributed to a large molecular weight molecule, specifically >100 kDa and 50-100 kDa. Interestingly, metabolites of >100 kDa and 50-100 kDa are the only fractionated molecular weight ranges able to produce FIAF expression of the

full-length protein and the cleaved truncated N-terminal domain. Recent advances report effects on LPL are mediated by the amounts of FIAF secreted as well as its post-translational cleavage (Lei et al. 2011). Although cleavage of FIAF is not required for LPL-inhibition, it has been shown to enhance LPL-inactivation. Furthermore, studies indicate the N-terminal domain is pertinent for inhibitory action on LPL, whereas the C-terminal domain mediates antiangiogenic functions (Cazes et al. 2006; Sukonina et al. 2006). Therefore, we pooled *B. longum* CFS fractions of >100 kDa and 50-100 kDa, resulting in a molecular weight fraction of >50 kDa. Large molecular weight metabolites suggest that the active metabolite is possibly a large peptide, protein, enzyme, and/or polysaccharide. Several proteomic studies have analyzed and listed secreted proteins from *Bifidobacterium* spp. in this molecular weight range; however, a majority of them are of unknown function and significance (Vitali et al. 2008). Fujiwara et al. (1999) reported *Bifidobacterium* spp. BL2928 to produce a > 100 kDa bacterocin-like protein in its supernatant, which competitively inhibits pathogen-binding. Lee et al. (2009) demonstrated that CLA-produced from *L. plantarum* PL62 exerted anti-obesity effects in diet-induced mice, reducing body weight without reducing energy intake. They also provided evidence correlating reduced leptin concentrations with enhanced CLA concentrations in individuals consuming varying bacterial numbers of *L. rhamnosus* PL60 (Lee et al. 2009). Of 13 *Lactobacillus* species analyzed for putative extracellular proteins, the largest exoproteomes predicted were of *L.casei* and *L. plantarum*. Most of the predicted exoproteomes comprised anchoring and secreted proteins produced from those strains. These proteins have been established in metabolic processing functions such as transport, signal transduction and biosynthesis of exopolysaccharides

(Kleerebezem et al. 2010). *Bifidobacteria* are commonly used in probiotic products and known to impart 'bifidogenic' properties from breastfeeding; recently discoveries unveiled a gene cluster encoding a transporter and novel hydrolase specifically for digesting human oligosaccharides (Kitaoka et al. 2005).

In the heat and freeze-thaw susceptibility experiments, the regular pooled fractions of >50 kDa increased the levels of FIAF as expected. Heat-treatment did not affect FIAF modulatory activity, but the effect on FIAF was lost upon freeze-thaw treatment. These two tests serve as indicators that the factor may be a protein. Other studies have explored the effect of heat on host gene modulation including FIAF. In a study of mice fed high-fat chow, heat-killed *L. paracasei* F19 were not able to affect FIAF levels (Aronnson et al. 2010). In another study, metabolites produced by Lactobacilli exposed to Caco-2 cells were reported to rapidly upregulate SGLT-1, and the effect was diminished by heat-denaturation (Rooj et al. 2010).

To further confirm if our modulatory factor could potentially be a protein, immobilized pepsin was used to hydrolyze any proteins present in the whole CFS as well as the >50 CFS fraction of *B. longum*. In both instances, treatments were unable to increase FIAF levels, indicating that the factor responsible for this is potentially a protein. Extracellular proteins have been reported to be secreted and released into the host environment by probiotic bacteria to mediate certain beneficial effects on human health by direct interaction with epithelial cells (Sanchez et al. 2008).

Our investigation reports *B. longum* exerting its effects via components present in its cell-free supernatants, rather than via direct bacteria-host cell interactions. The need for

colonization by a probiotic strain to exert beneficial effects remains a topic open for debate, although the general consensus is that adhesive ability is not a pre-requisite and probiotics exert their metabolic effects on the host transiently passing through the gut (Izquierdo et al. 2008). Similar to our observations, a study comparing the effect of co-culture and CFS from *L. paracasei* F19 on FIAF found that heat-killed F19 cells generated no effect on FIAF but heat-treated cell free supernatants continued to do so (Aronsson et al. 2010). Another study (Wang et al. 2011**)** reported a 50 kDa heat-stable putative protein present in cell-free supernatants from *B. infantis,* exhibiting anti-inflammatory activity in Caco-2 cells. In our study, absence of a marked effect with live *B. longum* cells could be due to poor adhesivity of our *B. longum* strain (Izquierdo et al. 2008), resulting in insufficient concentrations of the bioactive metabolite. Alternatively, the bioactive compound generation could be specific to components within the bacterial growth medium MRS, which has also been previously reported to exert influences on mammalian cells, such as immunomodulation (Foligne and Pot 2009).

In conclusion, the results from our study suggest that secreted bioactive compounds of large molecular weight, specifically >50 kDa, from the probiotic *B. longum* produced during exponential growth may assist in the prevention and management of diet-induced obesity by increasing intestinal FIAF levels. We provide preliminary characterization of this heat-stable factor by *B. longum* and not *L. casei.*

Based on the overall properties of the compound: (1) produced during exponential phase (2) non-linear concentration effect (3) molecular weight of >50 kDa (4) heat-stability (5) freeze-thaw susceptibility (6) protease susceptibility (7) effect caused by CFS and not adhesion, we can hypothesize that the candidate factor is a protein released by *B.*

*longum* for nutrient utilization for growth and multiplication. Due to the ability of the BLCFS to increase the levels of not only the full-length FIAF but also the truncated band similar to the PPAR-α ligand WY 14643, it is possible that the bioactive compound is either generating PPAR ligands during nutrient utilization in the growth medium, serving as a co-activator protein for PPAR-α or activating PPAR-α via signaling leading to its phosphorylation. *B. longum* has also been reported to secrete a eukaryotic-like protease inhibitor called serpin (Ivanov et al. 2006), which may assist in increasing FIAF levels by preventing its protease-mediated degradation. As far as effects on cleavage are concerned, they may be mediated via furin-like proteins called subtilisins, present in many bacteria and predicted to be present in *B. longum* via bioinformatic analysis (UNIPROT). With respect to specificity to MRS medium, and the fact that the un-inoculated MRS medium itself is able to increase FIAF levels when compared with untreated HT-29 cells, it is possible that bacterial growth and metabolism is able to enhance the concentration of an FIAF-modulatory compound already present in MRS. In our investigations, the use of MRS-medium treated similarly to each variation of the CFS ensures that we are adequately able to account for any effects that are due to medium alone, as recommended previously in literature (Foligne and Pot 2009). Other possibilities include extracellular lectins (Lakhtin et al. 2007).

The information generated from this study can be applied for development of rationally designed nutritional strategies with respect to probiotics, as an additional tool in the management of diet-related obesity, in combination with standard diet and lifestyle changes. *B. longum* is often used in probiotic foods and supplements but it suffers from a significant loss of viability due to low acid and bile tolerance in the gut (Izquierdo et al.

2008). It is also a low-adhesive strain and can thus not colonize the gut for extended periods of time (Izquierdo et al. 2008). Moreover, it does not perform well in multi-strain scenarios (Chapman et al. 2011), especially in combination with Lactobacilli, as commonly observed in probiotic-containing foods nowadays. Use of *B. longum* derived factors at the effective dose in the food product rather than bacteria themselves may help circumvent many of these issues, allowing development of better functional foods. The finding that the effects on FIAF were observed by bioactives produced during active growth phase is important to note for product formulation. Product efficacy may be improved as a synbiotic combining *B. longum* with its growth substrates. An individual's diet may also play a role in providing a 'bifidogenic' environment such as prebiotic-containing foods and supplements (Reinhardt et al. 2009). In infants, breastfeeding would play an important role. Bifidogenic foods to encourage the growth of existing *B. longum* in the gut may also exert FIAF-modulation. The heat-stability of the compound would be a huge asset as it would expand the range of food products to which it can be added, contrary to just cold dairy products where the bulk of probiotics are used. Use of CFS rather than live cells may also help reduce the risk of immune reactions to bacterial cell wall components.

The prebiotic approach to enhance Bifidobacterium numbers in the gut has already been shown to be effective in modulating LPS-mediated effects of gut bacteria on obesity (Cani et al. 2011). Oligofructose supplementation was able to ameliorate high-fat diet induced obesity related to reduction in the numbers of Bifidobacteria. Improved insulin secretion induced by glucose, and normalization of inflammatory markers, linking its affect with endotoxemia, were also observed (Cani et al. 2011).

Further investigations will be conducted to ensure that this delivery method of probiotics and anti-obesity compounds complements the residing microbiome. A new study conducted by Kondo et al. (2010) in rats with diet-induced obesity 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* spp. 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. 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 (Aronsson et al. 2010). The study further determined that the metabolite responsible for the induction was, in fact, secreted as well as heat-stable. 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 *Lactobacillus 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 (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 LPL activity. These effects on adipocytes could not, however, be replicated in mice in which obesity was already present prior to lactic acid bacteria supplementation.

A study conducted using synbiotics revealed another possible approach in weight management and the treatment of obesity. A synbiotic powder containing inulin, *Bifidobacterium* spp. and *Lactobacillus* spp. 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 Yin et al. (2009) suggested 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.

Future studies in our lab will focus on identification of the protein via mass spectroscopy. Studies have shown that it is possible to solubilize proteins, lipids and

polysaccharides in the CFS from certain lactic acid bacteria by ammonium sulfate precipitation (proteins), cold ethanol extraction (polysaccharides), adsorption-desorption, and organic solvent extraction (lipids) (Kim et al. 2008; Pingitore et al. 2007). We also aim to further investigate the influence of probiotic mediated regulation of the varying forms of FIAF (full-length protein, truncated N-terminal domain, and C-terminal fibrinogen-like domain) and effects on diet-induced obesity.

We will also be testing independent MRS components to determine whether they have marked effects on FIAF levels. MRS medium contains over ten different components, which may or may not interact with the bioactive factor to indirectly affect FIAF.

We also aim to confirm our current findings *in vivo*. *C.elegans* have been widely used a model to study energy homeostasis, including lipid metabolism due to the conservation of their metabolic pathways, genes encoding proteins homologous to human lipogenic and lipolytic enzymes, and their transparent bodies allow direct visualization of triglyceride deposition by staining. Dietary components metabolized by *C.elegans*, such as fatty acids, are synthesized de novo from acetyl CoA and subsequently incorporated into triacylglycerols (TAGs) or phospholipids or modified to form polyunsaturated fatty acids. TAGs are stored as lipid droplets in the intestine and hypodermis sites, which allow quantification (Watts 2009). Furthermore, recent development of a $^{13}C$ isotope assay has allowed quantification of fat synthesis to determine the contribution of dietary fat absorption in relation to fat stored (Perez and van Gilst 2008). A study reported that lipid droplet expansion is accompanied by triglyceride increases resistant to fasting- or triglyceride lipase-triggered lipolysis, suggesting a model for comparison to the human

LPL function (Zhang et al. 2010). Their similarities to human metabolism make them as excellent models. For example, mammalian PPARα is comparable to the role of NHR-9 (Nuclear hormone receptor) in regulating β-oxidation, lipid synthesis and expression of other genes involved in dietary responses (Mullaney and Ashrafi 2009). Another study in *C.elegans* has recognized many regulator proteins and downstream effector genes involved for lipid homeostasis (Walker et al. 2010). *C. elegans* encodes proteins homologus to mammalian fatty acid transport proteins associated with obesity and insulin resistance: fatty acid transport protein (FATP), fatty acid binding proteins (FAB-Ps), acyl-CoA binding proteins (ACBPs), arnintine-palmitoyl transferases (CPTs) and ATP-binding cassette (ABC) transporter proteins (Ashrafi 2007). *C. elegans* has also been reported to have eight digestive enzymes belonging to the α/β hydrolase lipase family. Other relevant cellular and homeostatic mechanisms include sugar transporters, kinase energy-sensors (e.g. AMPK and TOR), sterol regulatory element-binding proteins and regulatory processes such as insulin and adiposity-regulation (Mullaney and Ashrafi 2009). A recent study determined the effects of resistant starch, fermented resistant starch, and SCFAs on intestinal fat deposition in *C. elegans* (Zheng et al. 2010 ). The model reflected the fat deposition in response to bioactive components and revealed reduced fat deposition most notably by amylose starch, suggesting increased energy expenditure or decreased caloric intake. *C. elegans* is thus a promising model for determining the effects of probiotics *in vivo*.

The improvement of the intestinal enzyme activity, mucosal health, microbial ecology and body weight by the ingestion of selected probiotics and prebiotics to influence the gut microbial ecology is a promising alternative means to combat obesity.

Furthermore, 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 (Kataria et al. 2009; Neish 2009).

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## VITA

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