METHOD DEVELOPMENT FOR INVESTIGATING PROBIOTIC EFFECTS ON FAT STORAGE IN A *C. ELEGANS* MODEL

by

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DEDICATION

For Mom, Dad, Brenna, Memaw, and Papaw

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CHAPTER I: INTRODUCTION

Obesity

Obesity is one of the most concerning and prevalent public health issues of the 21st century. Obesity, which is commonly defined as having a Body Mass Index over 30, has risen to epidemic proportions over the last 50 years. The World Health Organization estimates that around 8% of the entire global population, or about 300 million people, are obese (www.who.com). Statistics regarding obesity are even more staggering when considering the population of the United States alone, with ~33% of Americans considered as obese (www.cdc.gov). On June 18th, 2013, in an effort to "help change the way the medical community tackles this complex issue" the American Medical Association adopted a new policy which now recognizes obesity as a disease (www.ama-assn.org). This momentous decision reflects the gravity of this issue in today's society, and the need for novel interventions targeted at obesity (Must et al., 1999).

In its most basic essence, obesity is an energy homeostasis disorder in which energy consumption is greater than expenditure. This imbalance is typically associated with increased triglyceride levels, an increased LDL: HDL ratio, and increased cholesterol levels, eventually resulting in the accumulation of adipose deposits throughout the body (Turnbaugh et al., 2006). Over a lifetime, the accumulation of lipids can lead to a number of different metabolic conditions, including type II diabetes, stroke, atherosclerosis, dyslipidemia, and hypertension (Must et al., 1999). According to the Center for Disease Control, there are an estimated 112,000 obesity-related deaths each year in the United States (www.cdc.gov).

When considering both the etiology and interventions for obesity, it is very difficult to establish a causative relationship with any one nutrient or lifestyle due to its multifactorial nature. Although a large number of correlative studies have been published showing associations between obesity and several different nutritional and lifestyle variables, less information exists about causative relationships (Lee et al., 2008; Giles-Corti et al., 2003). This is, in part, due to the complex multifactorial nature of obesity, making it difficult to attribute obesity to one specific etiologic source. Correlative information is useful in determining risk factors; however, there is a large unmet need to discover causative relationships between specific environmental factors underlying weight gain or weight loss. Sometimes these causative findings can change the nature of therapeutic interventions by facilitating new drug discovery. It is thus important that researchers are exhaustive in their efforts regarding obesity-prevention and treatment. Investigating all avenues of possible therapies will surely reveal new opportunities beyond traditionally utilized nutritional, lifestyle or pharmaceutical approaches. One such novel avenue of research, focusing on the human gut microbiota, is beginning to show great implications for modulating human lipid metabolism and obesity.

Human gut microbiota

Gut microbial composition has a great influence on the absorption and metabolism of many nutrients in the diet. This has been understood for quite some time, and despite a large amount of attention the field has been receiving recently, the connection between diet, weight, and gut microbiota is still ambiguous. There are estimated to be 100 times more bacterial cells in the human microbiota than actual human mammalian cells in an adult body (Qin et al., 2010). The amount of bacteria in the human

gut is estimated to be around 10^{14}, and with this in mind, their ability to influence the digestion and absorption of nutrients should not be especially surprising (Delzenne et al., 2011). The typical adult microbiota contains some 1,000-1,500 bacterial species; however, some specialists estimate that only 160 of these species make up the core microbiota present in almost all humans, while the presence of other species can vary greatly (Qin et al., 2010). The intestinal microbiota of humans, as well as rodents, are composed of several different phyla, including *Bacteroidetes, Firmicutes,* and *Actinobacteria,* however, it has been shown that >90% of bacterial types are members of either *Bacteroidetes* or *Firmicutes* (Eckburg et al., 2005). In the search for new obesity-related research directions with translational and therapeutic potential, many researchers have most recently turned towards the gut microbiota and the effects of altering the ratios of the different species and phyla of bacteria.

Gut microbiota and obesity

The ratio of two major phyla seems to play a major role in determination of the phenotype of their host, as researchers have observed lean and obese individuals carry different amounts of *Bacteroidetes* and *Firmicutes.* In these observations, lean individuals seem to carry a higher amount of *Bacteroidetes,* while obese individuals tend to carry higher relative levels of *Firmicutes* (Ley et al., 2006; Turnbaugh et al., 2006). These researchers also observed a shift in the levels of these phyla as a function of diet. They noticed an increase in the relative abundance of *Bacteroidetes* as obese individuals lost weight either on a fat- or carbohydrate-restricted low-calorie diet (Turnbaugh et al., 2006). The same researchers also found that germ-free mice conventionalized with an

"obese microbiota" gained significantly more weight than germ-free mice conventionalized with a "lean microbiota" (Turnbaugh et al., 2006).

Another intervention study highlights the role of energy intake in defining microbial composition. Jumpertz et al. (2011) compared a 2400 Kcal diet against a 3400 Kcal diet in 12 lean and 9 obese individuals in a randomized trial. Subjects on the higher calorie diet show a significant increase in the relative abundance of *Firmicutes* and a corresponding decrease in the amount of *Bacteroidetes* (Jumpertz et al., 2011). Also, when fecal energy composition was measured, a 20% increase in the presence of *Firmicutes* correlated to a decreased fecal caloric content. This translated to an increased energy intake of ~150 Kcal, displaying the apparent role of human gut microbial composition in influencing intestinal energy absorption (Jumpertz et al., 2011). While the sample size of this study is small, it certainly does provide a basis to investigate the effect of gut microbiota on energy intake.

Lastly, Bervoets et al. (2013) recently observed an elevated *Firmicutes:Bacteriodetes* ratio when comparing overweight/obese children to lean children. Such observations raise several questions. For instance, is there a causative role for the gut bacterial composition in determining a lean or obese human phenotype? If so, then it could be hypothesized that by altering the composition of obesity-related microbes in the gut, one could take a proactive step in either treatment or prevention of obesity. Research into the field over the past few years certainly supports the idea that body weight alters the gut microbiota composition, such as the ratio of *Bacteroidetes:Firmicutes* at a phylum level, and the numbers of Bifidobacterium sp., at a species level (An et al., 2013; Kalliomaki et al., 2008). It is therefore reasonable to

assume that defined alterations of the gut microbial composition through dietary approaches such as probiotic supplementation, could lead to alteration of body weight.

Known mechanisms of gut microbiota's influence on obesity

It is now understood that the bacterial population in our gut affects several different functional systems in our body, and are neurological, metabolic, immune, and of course, gastrointestinal effectors (Oelschlaeger, 2010). Some of the known mechanisms behind the effect of the gut microbiota on weight gain involve intestinal absorption of monosaccharides, short-chain fatty acid production, microbial regulation of host genes, and fermentation of indigestible dietary polysaccharides (Cani and Delzenne, 2009). The intestinal microbiota works synergistically with the host providing many functions, such as the production of enzymes capable of breaking down nutrients that humans lack the capabilities to digest (Xu et al., 2007). For example, *Bacteroides thetaiotaomicron*, which is an anaerobe found in the human colon, contains over 60 of these enzymes (Sonnenburg et al., 2005). Sonnenburg et al., (2005) have also shown that *B. thetaiotaomicron* can increase the levels of the sodium/glucose transporter in the human mucosal epithelium.

It has been shown that intestinal microbiota plays a role in several different intestinal biological functions, including immunity, defense against pathogens, and microvilli development (Cani and Delzenne, 2009). It is also known that the dominant members of bacteria in our gut, *Bacteriodetes* and *Firmicutes*, are able to ferment dietary fiber to generate short-chain fatty acids and monosaccharides, which can enhance the energy harvested from the diet. Some studies suggest that these fatty acids and

monosaccharides can account for up to 10% of total daily caloric intake (Samuel & Gordon, 2006).

Additionally, the gut microbiota can alter energy uptake through the modification of the expression of genes that are involved in macronutrient metabolism (Sanz et al., 2012). For example, microbes that are associated with obese mice result in an increase in Lipoprotein Lipase (LPL) activity through suppression of intestinal Angiopoietin-like 4 (ANGPTL4)-expression (Fava et al., 2006). ANGPTL4 is a peptide that is an inhibitor of circulatory LPL. It is strongly up-regulated by adipogenesis, as well as fasting, in white adipose tissue and the liver (Mandard et al., 2006). It is a key regulator of triglyceride clearance, and shows potential as a new pharmacological target (Koster et al., 2005). As will be discussed later, it has been a target of interest in our laboratory as well.

Prebiotics/probiotics and obesity

Investigations into the mechanisms behind the effect of the gut microbiota on energy intake, as well as observing different bacterial phenotypes in obese and lean individuals, have led researchers to propose that obese individuals have gut microbiota that is more efficient at extracting energy from certain diets compared to the microbiota of lean individuals. We also know that probiotic supplementation can lead to changes in the ratio of certain bacterial species within our gut, such as an increase in the number of *Lactobacillus* sp, and *Bifidobacterium* sp, which are used most commonly as probiotics, decrease in the numbers of *Clostridium* sp, and even changes at the phylum level, for e.g. in *Bacteriodetes* during consumption periods of probiotic-fermented milk (Tatsuya et al., 2015). These observations allude to the possibility of using gut-microbiota altering

interventions as a new strategy for managing adiposity. Attractive candidates for this approach would be pre- and probiotic-containing foods and supplements, considering their long history of use for enriching some beneficial groups of bacteria in the human gut.

Probiotics are defined as live organisms, which, when administered in sufficient amounts, can have a beneficial effect on the host's health (Joint FAO, 2001). Prebiotics are defined as selectively fermented ingredients that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health (Gibson et al, 2010). Mostly, bacteria used as probiotic supplements fall within the *Bifidobacterium* and *Lactobacillus* genre, due to their reported health benefits and their ability to withstand gastric and bile digestion (Cruz et al., 2011; Teitelbaum and Walker, 2002). Within this genre, the most common species utilized as probiotics are *Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium bifidum, Bifidobacterium infantis, and Bifidobacterium longum* (Cruz et al., 2011).

Probiotic supplementation has been shown to modulate lipid metabolism through several different mechanisms. For example, the lipopolysaccharides (LPS) which form the outer membrane of gram-negative bacteria are slowly absorbed in the gut, and serve as pro-inflammatory compounds that contributor to the link between obesity and insulin resistance (Wellen et al., 2005; Goto et al., 1994; Creely et al., 2007). Probiotic supplements are typically gram-positive bacteria, and consumption of these bacteria can positively shift the prevalence of gram-negative bacteria to gram-positive and decrease the prevalence of pro-inflammatory compounds (Nunez et al., 2014). This was recently shown in a study when obese or non-obese rodents fed either milk fermented with *L.*

casei, or *L. casei* as a suspension in water, displayed a reduction both weight and inflammatory markers in obese rodents (Nunez et al., 2014). Another *in vitro* study performed by Riedel et al. (2006) examined the anti-inflammatory properties of several different *Bifidobacterium*, including *B. longum* S17, *B. infantis* NCC2705, displayed an inhibitory effect on LPS-mediated NF-κB expression in HT-29 intestinal cell lines when compared to LPS treatments or treatment with *E. coli* cell-free supernatant.

Other studies of the probiotic effect on lipid metabolism examine the cholesterol-lowering effect of certain probiotic strains on humans and animals in attempts to combat hypercholesterolemia, which is most often accompanied with obesity. There have been several mechanisms identified, such as assimilation of cholesterol by probiotics, conversion of cholesterol into coprostanol, enzymatic deconjugation of bile acids by bile-sale hydrolase of probiotics, assimilation of cholesterol by probiotics, co-precipitation of cholesterol with deconjugated bile (Lambert et al., 2008; Pereira et al., 2002; Lye et al., 2010; Liong et al., 2006). In rats and chickens, probiotic bacteria have shown a hypocholesterolemic effect, and decreased HMG-Coa reductase activity (Fukushima & Nakano 1995; Endo et al., 1999).

The role of *Bifidobacterium,* which comprises up to 3-5% of the human microbiota, has been implicated in lipid metabolism in several instances (Riedel et al., 2006; An et al., 2011; Million et al., 2013). Specifically, there is new research emerging suggesting antiobesogenic properties of *Bifidobacterium*. An et al. (2011) examined the effect of *B. longum* SPM 1205, *B. longum* 1207, and *Bifidobacterium pseudocatenulatum* SPM 1204 on Spragye-Dawley rats fed a high-fat diet, and observed a reduction in body weight, body fat percentage, triglyceride, glucose levels, total cholesterol, HDL-

cholesterol LDL-cholesterol, and significantly increased fecal lactic-acid bacteria counts. A 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 (Kondo et al., 2010). Significantly, it has been established that a high-fat diet diminishes the levels of *Bifidobacterium* spp. within the intestine. Another study involving 134 obese, 28 overweight, 76 lean, and 15 anorexic individuals examined the correlation between fecal bacterial concentration and BMI, finding a negative correlation between obesity and *B. animalis* which was found in much smaller amounts in obese people (Million et al., 2013). Lastly, another study examined the effect of *B. longum* on the accumulation of lipids in the liver of rats fed a high-fat diet, and found that *Bifidobacterium* significantly attenuated hepatic fat accumulation, while displaying increased *Bifidobacterium* levels in fecal samples (Xu et al., 2011).

To date, the typical model used to study the effects of probiotics on lipid metabolism has been a mouse model fed a high-fat, Western-style diet, supplemented with different probiotic bacteria, such as *Bifidobacterium* and *Lactobacillus*. However, other models are emerging as possible candidates for models in probiotic-host interaction studies.

Rodent studies as a benchmark for probiotic studies

New findings concerning the role of the gut microbiota including probiotics in human metabolism and obesity has generated a large amount of interest in this new area of research. As with majority of biomedical research, scientists are using the rodent

model as a benchmark for probiotic research. Rodent studies have been extremely effective in revealing information about host-microbe interactions and have utilized germ-free/gnotobiotic mice to which specific bacteria of interest have been introduced. Backhed et al. (2004) have shown that germ-free mice conventionalized with distal intestinal microbes have a markedly larger increase in body fat within 10-14 days when compared to germ-free mice, even when less food is consumed by the conventionalized mice. They were also able to show a suppression of intestinal Angiopoietin-like 4 (*ANGPTL4)*-expression, induction of hepatic *de novo* lipogenesis, and increased monosaccharide absorption from the intestinal lumen (Backhed et al., 2004). Increased intestinal glucose absorption and energy extraction from non-digestible polysaccharides would potentially lead to higher insulin levels. Increased glucose and insulin levels are both important in regulation of lipogenesis, and promotion of hepatic *de novo* lipogenesis through increased expression of lipogenic enzymes, such as acetyl-CoA Carboxylase and Fatty Acid Synthase (Cani and Delzenne, 2009).

Rodent models have many benefits of use, such as an extremely similar genome and strikingly similar anatomy and physiology. Their efficacy in biological research as an animal model is unmatched in terms of the ability to study the mechanisms underlying diseases. For research exploring probiotic-host interactions, gnotobiotic mice are often used, giving a great deal of control to the researcher by granting the ability to colonize their gut with selected microorganisms that they wish to investigate. The use of gnotobiotic mice also provides the ease of comparison between germ-free, conventionalized, or conventionally raised mice. However, the use of this rodent model is not without its drawbacks. One such limitation is that by virtue of being born and raised

in a germ-free environment, they do not undergo the normal microbial succession observed in conventionally reared animals, due to which they may exhibit an atypical physiological response to microbial introduction in the gut. In addition, the high cost associated with the infrastructure and personnel required to work with gnotobiotic mice is not feasible for smaller labs with limited resources.

C. elegans as a model organism

A viable alternative to rodent models can be offered by small model organisms such as the nematode *Caenorhabditis elegans. C. elegans* is a microscopic, soil-dwelling nematode found in temperate climates worldwide. As a laboratory specimen, *C. elegans* poses as a novel solution to some spatial, financial, and ethical limitations posed by rodent animal models. This nematode has also recently emerged as a key model for studies involving lipid regulation and obesity (Brooks et al., 2009; Jones and Ashrafi, 2009). *C. elegans* is a well-studied organism that offers numerous benefits as a model organism such as a completely sequenced genome, a short lifespan, a transparent body amenable to staining, reporter systems (e.g. GFP) and microscopy; and elucidation of gene function through RNA-mediated interference (RNAi) (Corsi, 2006). It is also attractive as a model to study microbe-host interactions as it naturally consumes bacteria as part of its diet. In laboratory conditions, *C. elegans* consume a diet of a select few *E. coli* strains, such as *E. coli* OP50 (OP50), using a pump-like organ called the pharynx. In natural environmental (soil) conditions, it is likely that *C. elegans* have a wide variety of bacterial food sources considering the low abundance of *E.coli* in this habitat. However, under monoxenic laboratory conditions the nematode greatly prefers *E. coli* over other bacteria for unknown reasons (Shtonda and Avery, 2006).

C. elegans share many basic metabolic pathways with mammals, and due to their quick reproductive rate and simple growth requirements, they serve as an ideal organism for a low-cost *in vivo* model. Their usefulness is not without limitations, but many of the quintessential components that regulate metabolism in humans are largely conserved in *C. elegans*, such as fat and carbohydrate pathways, synthesis of proteins, and neuroendocrine regulators of growth, reproduction, and metabolism (Jones and Ashrafi, 2009; Watts, 2009).

Both mammals and *C. elegans* have a balance between fat storage and fat utilization that is highly dependent on similar transcriptional and translational regulatory mechanisms. For example, *C. elegans* have a homologue for Sterol Regulatory Element Binding Protein called *sbp-1*, which regulates some common transcriptional targets, such as acetyl-coA carboxylase, ATP-citrate lyase, glycerol-3-phosphate acyltransferase, fatty acid synthase, and malic enzyme (McKay et al., 2003). Another example of an analogous protein is nhr-49, which has the mammalian equivalent of PPARα. This nuclear hormone receptor is integral in the fasting response, and deletion of it in a *C. elegans* model has profound changes on lipid metabolism (Atheron et al., 2008).

C. elegans derive fatty acids through their bacterial diet, as well as by *de novo* synthesis from acetyl CoA, through the use of the two multifunctional enzymes acetyl-CoA Carboxylase and Fatty Acid Synthase (Watts 2008). These enzymes have human-based analogs, and studies involving their expression may be broadly informative in humans. Also similar to humans, fatty acids are used as storage molecules after esterification to glycerol to form triglycerides, but they also comprise the hydrophobic portion of all cellular membranes in *C. elegans*, having important roles in cellular

signaling, selective permeability, and membrane fluidity (Watts 2008). Interestingly, the nematode cannot synthesize cholesterol, so it must be obtained through dietary sources. The small amount of cholesterol required leads some researchers believe it is not necessary for cellular fluidity, only for sterol signaling (Kim et al., 2009).

C. elegans life cycle

In addition to their metabolic similarities with humans, *C. elegans* are appealing as a model for many reasons. This is in part due to their very rapid life cycles. In addition, they require a relatively small amount of space to maintain, and their culture conditions are similar to bacteria in that both can be cultured on both agar plates and in liquid culture. Hatchling *C. elegans* are 0.25 mm long and grow to a length of 1.4 mm within 5 days, which translates to a 6-fold increase in length and a 100-fold increase in volume (Knight et al., 2002). Four different molts define its five stages of life, and other than a brief period of lethargus just before its ecdysis, there is no real halt of its continuous growth. The life cycle of *C. elegans* is comprised of an embryonic stage, four larval stages (L1-L4) and adulthood. Each larval stage ends with a molting period. As displayed in Figure 1, it takes anywhere from 50-60 hours to reach an adult reproductive stage from a newly laid egg.

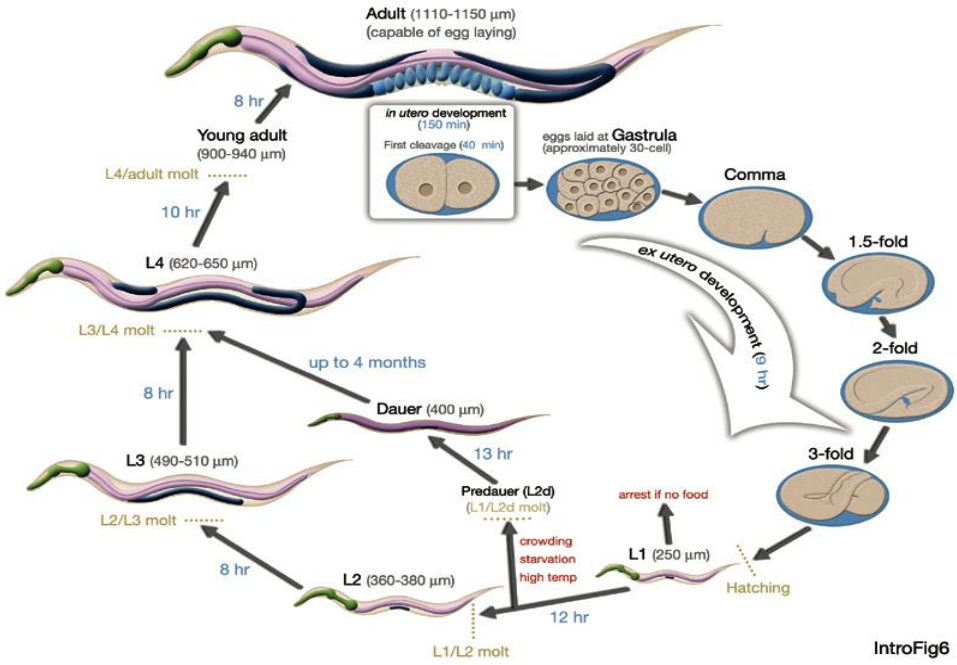


Figure 1. The life cycle of *C. elegans* grown at 22°C. The various life stages are labeled, along with the length of the worm (in parentheses) at each life stage. Reproduced from (Altun Z, and Hall DH, 2005).

C. elegans dietary habits

Interestingly, there is not a large amount of data regarding *C. elegans* and a probiotic diet. This is most likely due to the fact that the nematodes prefer specific bacterial strains, and will reject or leave certain food sources to find others, possibly to seek greater growth potential (Zhang et al., 2005). It is remarkable that an animal as simple as *C. elegans* has the ability to exhibit dietary choice. Indeed, several experiments on dietary choices of *C. elegans* were performed by Shtonda & Avery, (2006) which resulted in worms choosing high-quality foods, which translated to foods that better support growth. They were also able to show that, like mammals, this choice of higher-

growth food requires sampling the food first. Lastly, *C. elegans* exhibit leaving behavior. Sometimes leaving a food occurs when food is hard to eat, but often can occur when the nematodes are exposed to volatile compounds (Shtonda & Avery, 2006).

A successful investigation into the ability of *C. elegans* to consume a probiotic-diet would thus prove very valuable as it would enable harnessing the potential of this simple model in studying probiotic-host interactions relevant to lipid-metabolism and obesity.

Preliminary data

We have previously examined the ability of probiotic strains *L. casei* and *B. longum* to influence triglyceride deposition in adipocytes *in vitro*, potentially mediated by their impact on the protein ANGPTL4, an inhibitor of the lipid-hydrolyzing enzyme LPL. Cell free supernatants from *B. longum* were able to enhance the levels of enterocytic FIAF in vitro (Figure 2), and decrease LPL activity (Figure 3) and triglyceride levels (Figure 4) in 3T3-L1 adipocytes, presumably due to decreased lipid uptake.

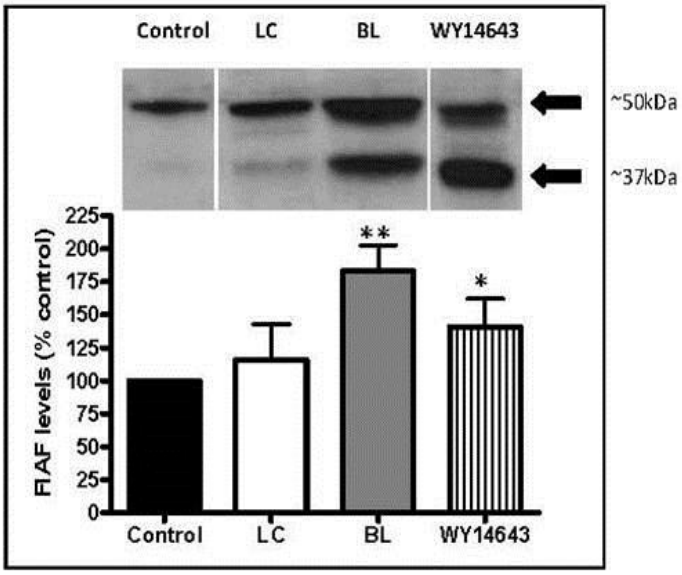


Figure 2. ***Bifidobacterium longum*** **increases the levels of secreted intestinal Fasting Induced Adipocyte Factor (FIAF)** ***in vitro*****.** HT-29 cells were treated with bacterial cell-free supernatants from *Lactobacillus casei (LC)* or *B. longum* (BL) for 24 hours. Secreted FIAF was then detected by immunoblotting. Treatment with PPAR-α agonist WY14643 was used as positive control for induction of FIAF expression. Results are presented as % control, relative to treatment with inoculated bacterial growth medium MRS. * and ** indicate a significant difference from control at $p<0.05$ and $p=0.001$, respectively.

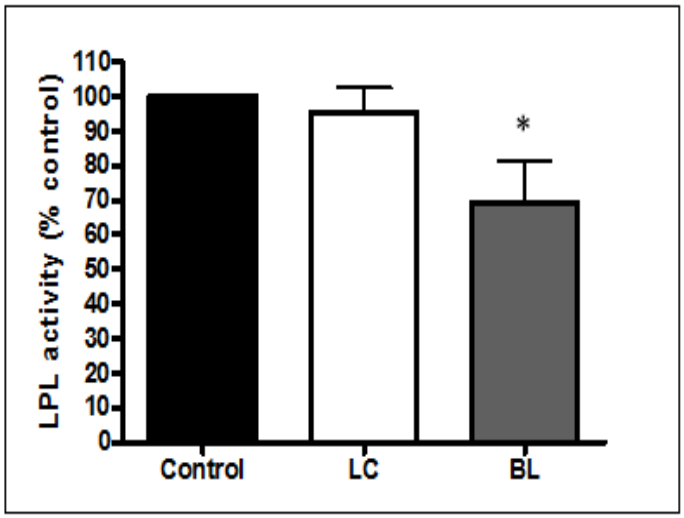
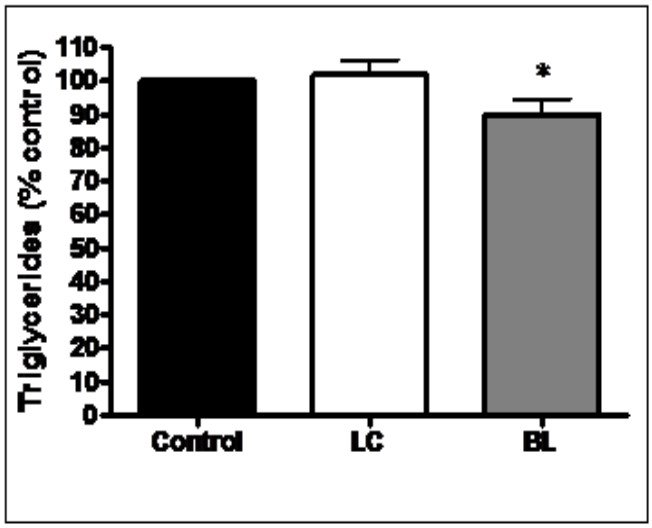


Figure 3. ***Bifidobacterium longum*** **decreases LPL activity in 3T3-L1 adipocytes**. Differentiated 3T3-L1 cells were treated with bacterial cell-free supernatants from *Lactobacillus casei* (LC) or *Bifidobacterium longum* (BL) for 48 hours. LPL was released from the cell-surface by heparin treatment and LPL activity was assayed by measuring p-nitrophenol released from p-nitrophenyl butyrate substrate. Results are presented as % control, relative to treatment with uninoculated bacterial growth medium MRS.

Figure 4. ***Bifidobacterium longum*** **decreases triglyceride accumulation in 3T3-L1 adipocytes.** 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. Results are presented as % control, relative to treatment with uninoculated bacterial growth medium MRS.
* indicates a significant difference with respect to control.



Based on the above data, we are interested in investigating if *B. longum* can exert similar effects *in vivo*, and investigate the related mechanisms of action. Considering the

attributes of *C. elegans* discussed earlier and the fact that we do not have access to rodent animal models at our institution, we have endeavoured to utilize the *C. elegans* model for the study of probiotic-host interactions. Specifically, we intend to devise a method to adapt *C. elegans* to a diet of a selected probiotic bacterium (e.g. *B. longum*) instead of its habitual diet of *E. coli* OP50, followed by investigating how this dietary adaptation impacts lipid storage and underlying mechanisms in this model system.

B. longum was chosen as our probiotic strain for investigation as it is a commonly found *Bifidobacterium* sp. in the adult human gut, and also utilized as a commercial strain in probiotic-containing foods and supplements.

Our initial attempts at feeding probiotic *B. longum* to *C. elegans* were met with several challenges, such as contamination with *E. coli* OP50, unwillingness to consume *B. longum* by the worms (crawling away from the *B. longum* lawn on solid medium), or stunted growth and/or reproduction. Through a series of iterations involving antibiotics (for preventing *E. coli* OP50 contamination), use of liquid medium instead of solid medium (for promoting probiotic-intake) and optimizing the probiotic concentration (to support adequate growth and reproduction), we were able to overcome the challenges outlined above, and devised an initial working protocol for successfully growing *C. elegans* on a diet of *B. longum*. The goal of the present work is to further refine this protocol, make detailed observations to aid its routine application, and use it to study the impact of *B. longum* on lipid storage in *C. elegans*.

We chose *C. elegans* as our model due to its short generation time, high reproductive rate, and similarities in metabolic regulation to mammalian models. Also,

the genome of *C. elegans* has been completely mapped which means that we can extend our research to elucidate regulatory mechanisms using transgenic or knockout strains in future. The ability to easily stain *C. elegans* is yet another added benefit. A variety of lipid stains such as Sudan black, Oil Red O, Fixed Nile Red, and Fed Nile Red stains can all be used to quantify fat deposition (Yen et al., 2010). The data derived from this research will add to the body of knowledge regarding probiotics and their potential role in obesity-prevention.

Based on the preliminary data from *in vitro* studies conducted previously in our lab (Pham et al., 2012), as well as the initial protocol we have developed for feeding *B. longum* to *C. elegans* (Shay et al., 2014), the aims of the present thesis are:

1. *Refine the B. longum-feeding protocol in C. elegans and make systematic qualitative and quantitative assessments to aid in the routine use of the model for investigating the impact of B. longum on lipid storage.* These include studying the growth pattern of *C. elegans* on a diet containing *B. longum* when compared to *E. coli* OP50, investigating the requirement for viable vs. unviable bacteria, enumerating the bacterial numbers in the *C. elegans* gut, as well as conducting a 30-day survival assay. In addition, we will screen a variety of other probiotic strains for their ability to serve as an alternative food source to *E. coli* OP50 and support *C. elegans* growth.
2. *Investigate the influence of B. longum-feeding on lipid stores and some related mechanisms in C. elegans.*

   Assessments will include measurement of triglyceride content and lipase activity in *C. elegans* that had been fed *B. longum* or OP50, as well as an exploration into the

possible mechanisms behind any differences noted, such as differences in the macronutrient composition of these two bacterial diets.

This research will expand on our earlier efforts to study the effects of *B. longum* on host adiposity, using an *in vivo* model.

CHAPTER II: MATERIALS AND METHODS

C. elegans maintenance

i. Solid growth media

C. elegans strain Bristol-N2 were propagated on 35 or 60 mm culture dishes filled with nematode growth medium (NGM) containing 1.7% agar, 0.3% NaCl, 0.25% Peptone, 1M $CaCl_2$, 1M MgSO4, 5 mg/ml Cholesterol (in ethanol), and 1M KH_2PO_4. NGM containing the peptone, NaCl, and agar was autoclaved and sterile $CaCl_2$, $MgSO_4$, cholesterol, and KH_2PO_4 were added after the solution cooled to 60° C. The solution was aseptically added to sterile petri dishes, where it was allowed to solidify at room temperature for 36-48 hours. Typically, 35mm plates were used for maintenance, while 60mm plates were used for propagation of worms for functional assays.

ii. Solid media feeding protocol

After solidification, the NGM was spread with a bacterial lawn of 25 µL or 50 µL of an overnight culture of *E. coli* OP50 (OD_{600}=0.6-0.8) for the 35 mm and 60 mm agar plates, respectively. After aseptically spreading the NGM with the OP50 lawn, plates were wrapped in parafilm and incubated for 9 hours at 37°C. Plates were then stored for use at 20°C. *C. elegans* were maintained on the plates by picking 15 adult worms and transferring them to the new plates every 4-7 days. All worm transfers were performed under aseptic conditions, to ensure that contamination did not occur. Plates were checked for contamination every day, and any plates that displayed symptoms of contamination were not used.

iii. Bacteria used

E. coli OP50 was inoculated at a ratio of 1/100 v/v into sterile Luria-Bertani (LB) medium. Inoculated media were incubated aerobically overnight at 37°C until the optical density reached 0.6-0.8 corresponding to bacteria in the log phase. This culture was then ready to be spread upon NGM agar.

Liquid culture protocol

i. Liquid media

Modified Nematode Growth Media (mNGM), a liquid, agar- and peptone-free medium containing 0.3% NaCl, 1M $CaCl_2$, 1M MgSO4, 5 mg/ml Cholesterol in ethanol, and 1M KH_2PO4 was used for liquid culture. The 0.3% NaCl was autoclaved, and filter-sterilized cholesterol and other salts were added after the solution cooled to 60°C.

ii. Bacterial diet preparation

Bifidobacterium longum (ATCC 15707) was grown anaerobically at 37°C in pre-reduced de Man, Rogosa, Sharpe (MRS) medium. OP50 was grown aerobically at 37°C in LB medium. Both bacterial strains were grown to an optical density of 0.6-0.8 at 600nm, measured using a BioMate3S Thermo Spectrophotometer. Once the bacteria reached the required optical density, cultures were pelleted and washed four times in sterile M9 buffer by resuspension. Washed pellets were collected by centrifuging for 5 minutes at 5000 RPM. The wash-buffer was then discarded, and concentrated bacterial pellets were suspended in M9 buffer. Typically, we used 20 mg of bacteria (wet weight) in 25 uL of M9 buffer. However, for certain experiments, concentrations of 10 mg/25 uL or 30 mg/25 uL were used. Once re-suspended in M9, the bacteria were stored at 4°C until use.

iii. Propagation of C. elegans

10 L4-stage *C. elegans* worms were transferred to 60 mm NGM plates and allowed to propagate for four days, resulting in a heterogeneous mixture of younger and older worms, as well as numerous eggs.

iv. Synchronization of C. elegans

Synchronization of the worms was performed with the purpose of ensuring that all worms are at a single life stage. On the fifth day of growth, *C. elegans*-containing NGM plates were soaked three times with 5 mL of sterile, deionized water, which was then removed quickly after addition. The water promotes the abstraction of *C. elegans* larvae from the agar, leaving only the eggs. The benefit of this soaking/rinsing method is that it allows for bulk growth and synchronization of *C. elegans*, rather than removing one worm at a time using a picking rod. This method saves a considerable amount of time, and is useful for growing a larger quantity of worms. The eggs hatch 18-24 hours later, at which point any *C. elegans* that had reached the L2 stage or older were removed using a platinum picking rod. The L1 larvae were then collected from the plate using an M9/Gentamicin wash and aseptically transferred into a sterile falcon tube.

v. Washing and decontamination of C. elegans

The collected L1 larvae were then washed three times in M9 buffer containing gentamicin (0.3% (w/v) KH_2PO_4, 0.6% (w/v) Na_2HPO_4, 0.025% (w/v) $MgSO_4.7H_2O$, 0.5% (w/v) NaCl, and 0.1% (v/v) of 0.1 g/ml Gentamicin). Worms were collected between washes by centrifugation (4000 RPM, 20°C, 5 minutes) and then re-suspended in fresh wash buffer.

vi. Transfer of C. elegans into liquid media

After the final wash, while still submerged in the wash buffer, the L1-stage *C. elegans* were placed on ice and allowed to settle to the bottom of the falcon tube. The wash buffer was then removed, and worms were suspended in 1900 µL of mNGM. The worms in mNGM were transferred to a well of a sterile untreated 6-well tissue-culture plate, and 100 µL of bacterial diet, containing either 40 mg or 80 mg of bacteria was added, bringing the total volume to 2 ml/well. One 60 mm plate of worms was used to fill two wells of a 6-well plate. Treatments were conducted in triplicates. The quantity of food applied to the worms depended on the specific experiment, but unless otherwise mentioned, a concentration of 80 mg/100 µl (20mg/25µl) was found to be optimal and used routinely. A lid was then applied to the plates, and plates were placed in an incubator at 25°C until the worms reached the L4 stage. At this point, the worms were ready to be harvested for several of the assays we performed.

Investigation of the impact of bacterial cell-free supernatants on C. elegans

i. Bacterial cell-free supernatant (CFS) preparation

The CFS of *B. longum* and *L. casei* were collected by centrifugation of log-phase cultures to remove the bacterial cells. The cultures were grown either aerobically for *L. casei*, or anaerobically for *B. longum*, at 37°C until they reach the log phase (OD~0.7-0.8), which was measured using a BioMate3 Thermo Spectronic at 600 nm. After removal of the bacterial pellet, the pH of the supernatant was adjusted to 7.0 using a 2 M NaOH. The CFS was then filter sterilized through a 0.2µm Corning Sterile Syringe Filter, and stored at -20^{o}C until use.

ii. *Size-fractionation of bacterial CFS*

The filtered, pH adjusted CFS collected in the previous step was fractionated through Vivaspin columns to generate fractions with molecular weight ranges >5 KDa and<5 KDa. Fractions were frozen until used.

iii. Preparation of MRS lacking carbohydrate or protein medium components

Different versions of MRS medium were prepared, lacking either the protein or carbohydrate ingredients. One version consisting of the protein-components (10 G peptone, 8 G Lab Lemco Powder, 4 G Yeast extract, 1 mL tween 80, 5 G Dipotassium Hydrogen Phosphate, 5 G Sodium Acetate Trihydrate, 2 G Triammonium citrate, 0.2 G magnesium sulphate $7H_2O$, and 0.05 G Manganese Sulphate $4H_2O$ in 1 liter of H_2O), and a carbohydrate-containing portion (20 G glucose, 1 mL tween 80, 5 G Dipotassium Hydrogen Phosphate, 5 G Sodium Acetate Trihydrate, 2 G Triammonium citrate, 0.2 G magnesium sulphate $7H_2O$, and 0.05 G Manganese Sulphate $4H_2O$ in 1 liter of H_2O). Both versions were pH adjusted to 7.0 using 1 M NaOH, and sterilized by autoclaving.

iv. Treatment with CFS fractions

C. elegans were propagated and washed according to the liquid culture protocol. After the wash, 10 L1 *C. elegans* were transferred to the wells of a 96-well plate containing 95µl of liquid mNGM, and 5 uL of 20 mg/25uL of OP50 as a food source. Different fractions (>5 Kda <5 Kda) of the CFS were tested on these *C. elegans.* The same fractions of MRS broth were tested as a control. Treatments ranged from 1%-11% (v/v).

Survival assay

i. Preparation of worms

C. elegans were grown, synchronized, and washed according to the liquid culture assay protocol. Worms were synchronized 24 hours before they were washed and prepared to seed into 96-well plates. Three 60-mm petri dishes of *C. elegans* were used, and the L1 larvae washed three times with M9 buffer containing gentamicin for 2 minutes at 2500 rpm, and then once in liquid NGM for 2 minutes at 2500 rpm. The ratio of worms/volume of media was adjusted to contain 20 worms/10 μL of M9. This adjustment was performed by aliquoting 10 μL drops onto a clear petri dish lid, and deriving the average number of worms per drop. The averages were taking using at least 10 total drops. Total volume of media was adjusted afterwards.

ii. Seeding the worms into plates

20 L1 worms were aseptically transferred into wells of a 96-well plate using 10 μL drops. When necessary, the number of worms per well was adjusted to 20 worms/well using a platinum picking rod. 5 uL of the bacterial diet were then added. Diets consisted of either *B. longum* or *E. coli* OP50, at a concentration of 20 mg/25 uL, or 10 mg/25 uL. After the introduction of food, 89 uL of liquid NGM or NGM/Gn was added to the wells. A lid was placed on the 96-well plates, and they were stored at 20°C until the L4 stage was reached (~35 hours). Experiment was performed in triplicate.

iii. Sterilization of C. elegans using fluorodeoxyuridine (FUDR)

To sterilize the animals, 26 μL of a 0.6 mM FUDR solution was added to each well containing L4 *C. elegans*. After the addition of FUDR, the volume of each well totaled 130 uL. Lids were applied to the 96-well plates and they were stored at 20°C.

iv. Scoring of C. elegans lifespan

C. elegans population in each well was first recorded when FUDR was added to the media. Afterwards, survival data was recorded every three days using a microscope and a platinum picking rod.

C. elegans' tolerance of various bacterial strains

Using our liquid-culture method of feeding, we investigated the survivability of *C. elegans* when fed several different probiotic bacterial strains. Worms were synchronized and decontaminated using the liquid culture protocol listed above. Experiments were performed in duplicate. Only survivability during the first life cycle of *C. elegans*, up to the adult stage, was noted. OP50 was used as a control, and all probiotic bacteria were used in quantities of 20 mg/25 uL. Bacterial strains used were: *Lactobacillus casei* (ATCC 334), *Lactobacillus reuteri* (ATCC 23272), *Lactobacillus gasseri* (ATCC 33323), *Lactobacillus plantarum* (ATCC 14917), *Lactobacillus fermentum* (ATCC 23271), *Lactobacillus rhamnosis* (ATCC 53103), and *Lactobacillus acidophilus* (ATCC 43121).

C. elegans' tolerance of live and heat-inactivated bacteria

i. Growth and heat-inactivation of bacteria

B. longum was grown anaerobically at 37°C in pre-reduced de Man, Rogosa, Sharpe (MRS) medium. OP50 was grown aerobically at 37°C in LB medium. Both bacterial strains were grown to an optical density of 0.6-0.8 at 600nm, measured using a BioMate3S Thermo Spectrophotometer. Both bacteria were heat-inactivated by heating in a water bath for 60 minutes at 95°C.

ii. Confirmation of bacterial inactivation

Bacterial Inactivation was confirmed by performing spread-plate counts after serial dilutions of both the live and inactivated bacteria, adding 100 uL of each dilution to an agar plate, and allowing the bacteria to grow either aerobically on LB agar for OP50, or anaerobically on MRS agar for *B. longum*. Confirmation was also obtained by inoculating 100 uL of M9, which contained 80 mg of either bacteria into 10 mL of their respective liquid growth media and measuring the O.D. after 72 hours for *B. longum*, or 7 hours for OP50, the time it took for the live bacteria to reach log-phase of growth. Both experiments were performed in duplicates.

iii. Assessment of C. elegans' viability with live and heat-inactivated bacteria

10 L1 *C. elegans* worms were decontaminated by washing four times in M9 containing tetracycline, and transferred to wells of a 96–well plate. Wells contained 5 uL of a 20 mg/25 uL M9 solution of either live or heat-inactivated *B. longum* or OP50, and 95 uL of mNGM. Wells with *B. longum* contained mNGM/Gn. Life stage and number of live worms was counted for four days, until worms reached the adult stage.

Bacterial enumeration

i. Decontamination

To enumerate *B. longum* and *E.coli* OP50 from the gut of the *C. elegans*, the worms fed either of these bacteria were disrupted, and the homogenate was spread upon the respective bacterial growth media for plate-counting. 50 L4 worms were used in each case; worms were paralyzed by suspending them in 100 uL of 25 mM levamisole in M9 buffer (LM buffer). The worms were then washed 4 times in M9 buffer containing either 15 µg/mL tetracycline for worms fed *B. longum*, 0.1 g/mL gentamicin for worms fed

OP50. Worms were pelleted at 2500 rpm for 3 minutes. Worms were then washed once more, in 100 µL of LM buffer at 2500 rpm for 3 minutes.

ii. Disruption and plating

The worms were then disrupted in 200 uL of PBS containing 1% Triton-X, using a battery-powered homogenizer, for 15 minutes/sample. 100 µL of the homogenate was used for serial dilution and plate-counts, on either pre-reduced MRS agar (5.2% MRS, 1.5% agar) for worms fed *B. longum*, or LB agar (2.5% LB, 1.5% agar) for worms that were fed OP50. To ensure that there was no contamination of *E.* coli in worms that were fed *B. longum*, homogenates of *B. longum*-fed worms were also plated on LB media. These plates were incubated (aerobically for OP50 on LB, anaerobically for *B. longum* on MRS) at 37°C for three days, and colonies were counted.

Triglyceride assay

i. Preparation and treatment of worms

Worms were grown to the L4 stage according to the liquid growth media protocol. Their diets consisted of B. *longum* or OP50 at a concentration of 10 or 20 mg of bacteria/ 25 µL of M9. Worms were grown in 6-well plates, with 100 µL of bacterial diet, and 1900 µL of liquid mNGM. Wells containing *B. longum* also contained 0.1 g/mL Gentamicin to prevent contamination of OP50.

ii. Sample preparation

Once the worms had reached L4, they were harvested and pelleted by centrifuging at 2500 RPM for 3 minutes. Worms were then washed in 500 µL of PBS 3 times. After each wash, worms were pelleted at 2500 RPM for 3 minutes, and the media was changed for new washing media. After the last wash, media was reduced to 200 µL, and 50 µL of

a 1% Triton-X solution was added. Worms were then ground using a motorized pestle for 15 minutes to ensure that lipids are solubilized. According to the manufacturer's protocol (Biovision, California, U.S.A) the solution was heated twice to 80°C for three minutes and chilled on ice. Lastly, the solution was filter sterilized to remove any solid particles that could interfere with the assay.

iii. Triglyceride quantification

To assay the triglyceride content of *C. elegans*, we used the Biovision Triglyceride Quantification Colorimetric/Fluorometric Kit (Biovision, California, U.S.A). For increased sensitivity, we utilized the fluorometric assay. The assay was performed according to the manufacturer's protocol. Briefly, trigylcerides are converted to glycerol and free fatty acids via the addition of lipase to the samples. The free glycerol in the sample is then oxidized using an enzyme mix, which generates a product which reacts with a provided probe to generate color. Results were recorded using spectrofluorometry at Ex/Em 535/590 using a Cary Eclipse Fluoresence Spectrophotometer. A standard curve was prepared using the 1 mM triglyceride standard provided in the kit. Results were standardized by protein content, which was determined using the Biorad protein assay.

Lipase assay

i. Sample preparation

Samples were prepared in a manner similar to the Triglyceride Assay. Worms are propagated in liquid culture, and washed as above. However, before disruption, no triton-x was added. This is due to the potential of Triton-x to interfere with the results of the assay. Worms were disrupted in 200 µL of M9 buffer for 15 minutes on ice, and then

filter-sterilized. The disruption was performed on ice to prevent protein degradation and enzyme inactivation.

ii. Lipase assay

To assay for lipase, we used the QuantiChrom Lipase Assay Kit (Bioassay Systems, California). After the samples were prepared, the manufacturer's protocol was followed. Briefly, this was a kinetic assay based on an improved dimercaptoproponol tributyrate method. In this method, SH groups formed by lipase cleavage react with 5,5'-dithiobis(2-nitrobenzoic acid) to form a yellow colored product. A color reagent and BALB reagent are combined with the sample, and readings are taken at 10 and 20 minutes. Results were recorded through spectrophotometry at 420 nm, using a BioMate3S Thermo Spectrophotometer. A standard curve was constructed using the calibrator (735 U/L lipase equivalents) which was provided with the assay kit. Results were standardized by protein content, which was determined using the Biorad protein assay.

Analysis of bacterial macronutrient composition

i. Growth of bacteria

B. longum was grown anaerobically at 37°C in pre-reduced de Man, Rogosa, Sharpe (MRS) medium. OP50 was grown aerobically at 37°C in LB medium. Both bacterial strains were grown to an optical density of 0.6-0.8 at 600nm, measured using a BioMate3S Thermo Spectrophotometer. Once the bacteria reached the required optical density, they were washed four times at 5000 RPM for 5 minutes in sterile M9 buffer. The wash-buffer was then discarded, and a concentrated amount of the bacteria were suspended in 25 µL of M9 buffer.

ii. Carbohydrate assay

Carbohydrate content was measured using the Phenol-Sulfuric Assay for total carbohydrates (Dubois et al., 1951). Serial dilutions of 20mg/25µL of both *B. longum* and OP50 were created, with a total volume of 1 mL. 1.1 mL of water was added to dilute the samples, as per the protocol. 50 µL of 80% phenol was added to each dilution, followed by 5 mL of concentrated H_2SO_4. Tubes were vortexed and allowed to rest for 20 minutes. Absorbance was read at 490 nm using a BioMate3S Thermo Spectrophotometer. A standard curve was created using a 1mg/2mL solution of glucose in water.

iii. Lipid assay

B. longum and OP50 were grown as discussed above. 40 mL of a 20mg/25µL solution were pelleted via centrifuge (4000 RPM for 5 minutes). The supernatant was discarded, and the bacteria (~30 mg) were then frozen using liquid nitrogen and ground a mortar and pestle. 10 mg of ground bacteria were quickly weighed, and submerged in 200 µL of a 2:1 Chloroform/Methanol solution to solubilize lipids (Bliah & Dyer, 1959). Samples were then decanted to remove solid particulate, and 200 µL of 2:1 Chloroform/Methanol solution was added, along with 200 µL of a 0.9% NaCl solution. Samples were centrifuged for 20 seconds at 8000 RPM to induce phase separation. Lipid phase was acquired and left on a tube-heater to evaporate the solvent. 0.25 mL of concentrated H_2SO_4 was added. Sample was vortexed, and heated for 10 mins on 100°C dry bath heater. 5 mL of a colorimetric reagent (Consisting of 3 G vanillin, 0.5 L H_2O, and 2 L conc. phosphoric acid) was added (Frings et al., 1972). Samples were incubated in dark for 30 minutes, and absorbance was read at 525 nm using a BioMate3S Thermo

Spectrophotometer. A standard curve was developed using various dilutions of 10 mg of olive oil.

iv. Protein assay

B. longum and OP50 were grown as discussed above. Serial dilutions of both bacteria were prepared, and the diluted sample was added into a 96-well plate with bacterial quantity ranging from 1 mg to 25 mg, while total volume remained at a constant 50 µL/well. Samples were incubated with 200 µL of a diluted (1 dye: 4 H_2O) Bio-Rad assay dye for 5 minutes, and absorbance was read at 595 nm using a BioMate3S Thermo Spectrophotometer. A standard curve was prepared using a 1 mg/mL stand of Bovine Serum Albumin.

CHAPTER III: RESULTS

Treatment with cell-free supernatant fractions

Considering the influence of CFS from *B. longum* on fat storage we observed in vitro using adipocytes, our goal was to investigate if this CFS would produce similar effects in *C. elegans*. However, MRS, its >/< 5Kda fractions, and its protein- or carbohydrate-containing fractions were not well-tolerated by *C. elegans*, nor were the bacterial CFS and their fractions. This may be due to toxicity related to high concentrations of glucose or amino-acids in MRS or CFS, that *C. elegans* do not typically encounter in their habitual growth medium NGM. When testing the different fractions, we found that in general, the *C. elegans* could not survive in solutions containing greater than 2-3% MRS or CFS, and the results even at these concentrations were inconsistent. On occasion, there were worms that survived above the 3% threshold, but these findings were assumed to be unreliable. It was very apparent the worms at any concentration of MRS or bacterial CFS were not healthy, and were markedly more lethargic. Results are displayed in table 1.

Fraction	1%	2%	3%
>5 MRS	NH	NH	DNS
>5 MRS	NH	NH	DNS
<5 MRS	DNS	NH	DNS
<5 MRS	DNS	DNS	DNS
>5 CFS	DNS	NH	DNS
>5 CFS	Y	NH	DNS
<5 CFS	NH	NH	DNS
<5 CFS	NH	NH	DNS
Protein-fraction of MRS	NH	NH	NH
Glucose-fraction of MRS	NH	NH	NH

Table 1. Treatment of *C. elegans* with fractions of CFS and MRS. Y= survived, healthy worms; NH=some worms survived, not healthy; DNS = Did not survive.

Survival assay

As our goal was to investigate the influence of our chosen probiotic strain *B. longum* on fat-storage in *C. elegans*, we first intended to ensure that the worms grew similarly when fed a diet of *B. longum* or OP50. To this end, we performed two different experiments: 1) we observed the survival and growth of the worms on *B. longum* or OP50 over the duration of a typical experiment (~40 hours, worm growth from L1-L4, harvested at L4 for experiments) and 2) we conducted a 30-day survival assay after sterilizing the worms at L4 stage (noted time to death). Table 2 shows the results from the first experiment, including the number of worms used for each treatment, and their survival and growth, for the first two days of the experiment. FUDR was added at the L4 stage, and then the second part of the experiment (the 30 day survival assay) was conducted. The table shows that the number of worms and the rate of growth of the worms in case of each treatment were comparable, and no significant differences were observed.

	0h	**13h**	**19h**	**24h**	**34h**	**38h**
OP50-10	20.00±0.00**(L1)**	20.00±0.00**(L2)**	20.00±0.00**(L3)**	20.00±0.00**(L3)**	20.00±0.00**(L4)**	20.00±0.00**(L4)**
BL10	20.00±0.00**(L1)**	20.00±0.00**(L2)**	20.00±0.00**(L3)**	19.80±0.20**(L3)**	19.80±0.20**(L3/L4)**	19.80±0.20**(L4)**
OP50-20	20.00±0.00**(L1)**	20.00±0.00**(L2)**	20.00±0.00**(L3)**	20.00±0.00**(L3)**	20.00±0.00**(L4)**	20.00±0.00**(L4)**
BL20	20.00±0.00**(L1)**	19.80±0.20**(L2)**	19.80±0.20**(L3)**	19.60±0.25**(L3)**	19.60±0.25**(L3/L4)**	19.60±0.25**(L4)**

Table 2. Number of worms per treatment, and rate of growth, before FUDR was added.
10=10mg/25µl, 20=20mg/25µl bacterial concentration; No significant difference in the number of worms was detected over the assay period in any of the treatments using one-way repeated measures ANOVA with Dunnett's multiple comparison test ($p>0.05$).

Over the 30 day survival assay period, a concentration-dependent effect was observed when comparing the worm survival and lifespan on an OP50 vs. *B. longum* diet. Worms fed the higher dose of *B. longum* (20mg/25µl) showed no significant ($P>0.05$) difference

in median survival when compared to worms fed the same concentration of *E. coli* OP50. Worms fed the lower concentration of *B. longum* (10mg/25µl) displayed a significantly ($P<0.05$) lower survival compared to worms fed the same concentration of *E. coli* OP50. The survival curve for worms fed the higher dose of bacteria is displayed in figure 5, while that for the lower concentration-fed worms is displayed in figure 6.

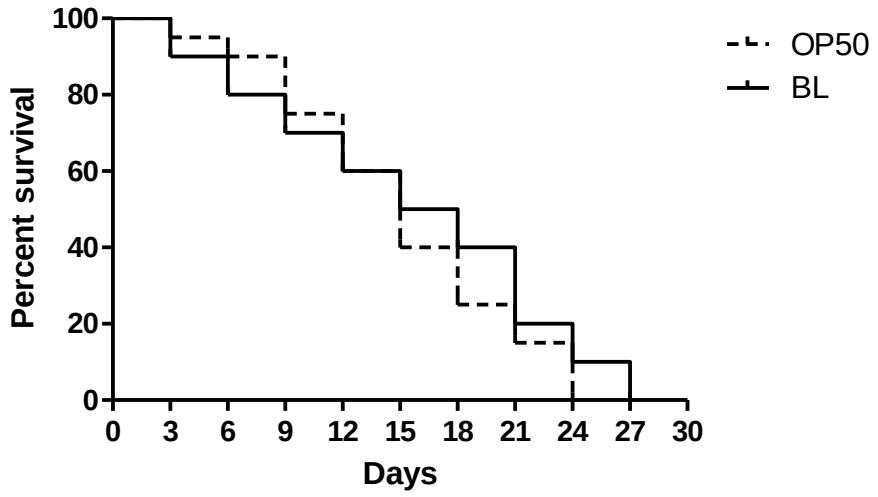


Figure 5. Survival curve of *C. elegans* fed a diet of *E. coli* OP50 or *B. longum* at a concentration of 20mg/25µL.Worms fed either *B. longum* or *E. coli* OP50 were allowed to grow to their adult stage in wells containing FUDR. Inactive worms were scored as dead. Median survival for *B. longum* and OP50-fed worms was 16.5 days, and 15.0 days, respectively. Comparison of the means using a log-rank test revealed a P value of 0.435, showing no significant difference in survival on the two diets.

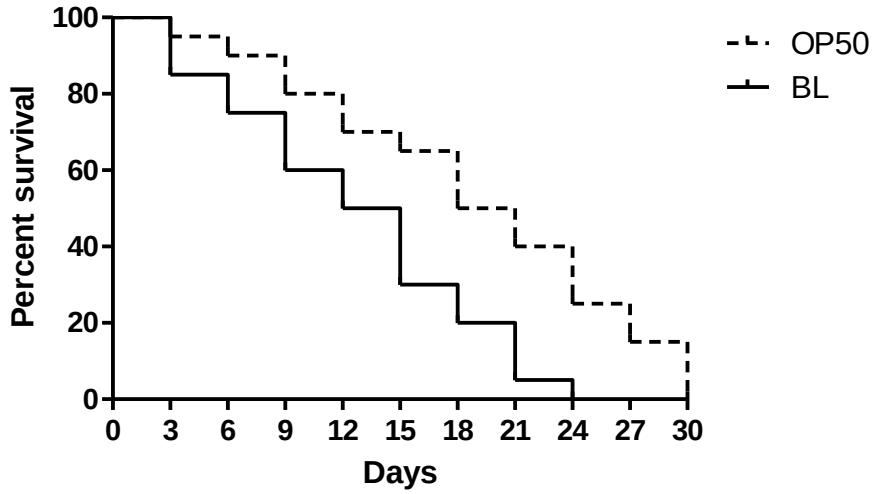


Figure 6. Survival curve of *C. elegans* fed a diet of *E. coli* OP50 or *B. longum* at a concentration of 10mg/25µL.Worms fed either *B. longum* or *E. coli* OP50 were allowed to grow to their adult stage in wells containing FUDR. Inactive worms were scored as dead. Median survival for *B. longum* and OP50-fed worms was 13.5 days, and 19.5 days, respectively. Comparison of the means using a log-rank test revealed a P value of 0.0052, showing a significant difference in survival on the two diets.

C. elegans are able to survive in liquid culture when treated with several different probiotic bacteria

The effect of several different probiotic bacteria on *C. elegans* survival in liquid culture was observed over the course of one life cycle of *C. elegans.* Results of survivability are summarized in table 3. All the probiotic bacteria tested were able to support growth of the worms to the L5 stage, at a similar rate (50-60 hours). This rate was however slightly lower than worms fed OP50 (44-48 hours) or *B. longum* (44-50 hours). The worms also lay eggs which hatched, except in the case of *L. casei* and *L. rhamnosus,* which were not able to support reproduction in the worms. The experiments were performed in duplicate, and time to L5 stage was relatively consistent, as shown in table 1.

Bacterium	Reached the L5 stage	Time to reach L5 stage	Reproduced?
L. casei	Y	50-60 hrs	N
L. reuteri	Y	50-60 hrs	Y
L. gasseri	Y	50-60 hrs	Y
L. plantarum	Y	50-60 hrs	Y
L. fermentum	Y	50-60 hrs	Y
L. rhamnosus	Y	50-60 hrs	N
L. acidophilus	Y	50-60 hrs	Y
B. longum	Y	44-50 hrs	Y
E. coli OP50	Y	44-48 hrs	Y

Table 3. *C. elegans* ability to survive and replicate when treated with several different probiotic bacteria. Experiment was performed in duplicate.

C. elegans' tolerance of live and heat-inactivated bacteria

C. elegans survivability and growth rate was observed over 60 hours to examine the effect of live and heat-inactivated bacteria on the survival and growth rate of *C. elegans*. Results are summarized in Table 2.

Results show that *C. elegans* fed either live OP50 or live *B. longum* reached the L4 life stage at similar times. In contrast, *C. elegans* that were fed OP50 or *B. longum* that were inactivated by heat did not progress past the L2 stage and reverted to dauer stage, suggesting that dead bacteria did not provide adequate nutrition and that worms require some metabolites generated by live bacteria in order to support their growth. Notably, there was not a significant difference in the number of worms between either of the live bacteria-fed sets. However, there was a stark difference in the number of worms that survived in each well of the heat inactivated bacteria-fed *C. elegans.* The difference became apparent within hours of beginning the experiment, and widened as time went on. Worms grew to the adult stage by 60 hours in case of both live OP50 and *B. longum*, with close to 100% survival rate, while they were growth arrested at the L2/Dauer stage when they were fed either of the heat-inactivated bacteria. Additionally, only 20-60% of the

worms were found to survive to 60 hours on the heat-inactivated bacteria, with survivability being higher in the case of OP50.

	0h	15h	19h	25h	33h	39h	60h
OP50-Live	10.00±0.00 **(L1)**	10.00±0.00 **(L2)**	10.00±0.00 **(L3)**	10.00±0.00 **(L3)**	10.00±0.00 **(L4)**	10.00±0.00 **(Young Adult)**	10.00±0.00 **(Adult)**
OP50-Dead	10.00±0.00 **(L1)**	9.33±0.33 **(L1)**	8.67±0.33 **(L2)**	7.67±0.33 **(L2/Dauer)**	7.00±0.58 **(L2/Dauer)**	6.33±0.33 **(L2/Dauer)**	6.00±0.00 **(L2/Dauer)**
BL-Live	10.00±0.00 **(L1)**	10.00±0.00 **(L2)**	10.00±0.00 **(L3)**	9.67±0.33 **(L3)**	9.33±0.33 **(L4)**	9.33±0.33 **(L4/Young Adult)**	9.33±0.33 **(Adult)**
BL-Dead	10.00±0.00 **(L1)**	6.67±0.33 **(L2)**	3.33±0.33 **(L2)**	2.67±0.33 **(L2/Dauer)**	2.00±0.58 **(L2/Dauer)**	2.00±0.58 **(L2/Dauer)**	2.00±0.58 **(L2/Dauer)**

Table 4. *C. elegans* fed either live or heat-inactivated B. longum and OP50 in liquid culture alters their ability to survive, as well as their growth rate. Growth was slowed in both treatments of heat-inactivated bacteria compared to live bacteria. Worms were monitered at the times specified, and life stage was recorded (L1-Adult). No significant reduction in worm numbers was noted over the course of the experiment in case of worms fed live OP50 or live B. longum, while a significant decrease in the number of surviving worms was noted 19h-onwards in case of OP50, and 15h-onwards in the case of B. longum. Changes in worm numbers were assessed by two-way ANOVA with Bonferroni multiple comparisons to number of worms at 0h. Experiment was performed in triplicate.

Confirmation of heat-inactivation of bacteria

The viability of the bacteria following heat inactivation was assessed by spread-plate counting on solid media, and also monitoring OD600 after their inoculation into liquid media. Results from the spread-plate method are summarized in tables 5 and 6. Results from liquid media are displayed in tables 7 and 8.

The results from the spread plate counts of live and heat-inactivated bacteria demonstrate that the heat-treatment employed was effective in achieving bacterial inactivation and that these bacteria were indeed non-viable or minimally-viable when fed to *C. elegans*. The plates that were spread with either of the live bacteria had colonies that

were too numerous to count (>300 colonies), while all of the plates that were spread with the heat-inactivated bacteria had too few colonies to count (<30 colonies).

Similar to the results from the plate counts, results from inoculation of the heat-inactivated bacteria into liquid growth media also demonstrated a very low number of viable bacteria, with OD600 values that were 15.6% and 17.2% of the OD600 values of the live bacterial cultures at the same time point for *B. longum* and OP50, respectively.

Live	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
B. longum	TNTC	TNTC	TNTC	TNTC	TNTC
OP50	TNTC	TNTC	TNTC	TNTC	TNTC

Table 5. Spread-plate counts of live bacteria (serially diluted from 20mg/25 µL). TNTC=Too numerous to count.

Heat-Inactivated	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
B. longum	TFTC	TFTC	No Growth	No Growth	No Growth
OP50	TFTC	TFTC	TFTC	No Growth	No Growth

Table 6. Spread-plate counts of heat-inactivated bacteria (serially diluted from 20mg/25µL). TFTC= Too Few to count.

	Live	**Heat-inactivated**
Optical Density (600nm)	**0.758 +/- 0.037**	**0.118 +/- 0.014**

Table 7. OD600 of live and heat-inactivated *B. longum* after 72 hours (100 µL of 20mg/25uL inoculated into 10 mL of MRS). Data presented as mean +/- SEM.

	Live	**Heat-inactivated**
Optical Density (600nm)	**0.885 +/- 0.029**	**0.153 +/- 0.004**

Table 8. OD600 of live and heat-inactivated *OP50* after 7 hours (100 µL of 20 mg/25µL inoculated into 10 mL of LB). Data presented as mean +/- SEM.

Enumeration and positive decontamination of bacteria

Having confirmed that *C. elegans* were able to survive and grow similarly on the control OP50 diet, as well as our test *B. longum* diet in the preceding sections, we now wanted to ensure that we were able to detect these bacteria from the gut of the worms, quantify the numbers of intestinal bacteria, and check for any bacterial cross-contamination. We were able to confirm that worms had been consuming similar amounts of both *B. longum* and OP50, by enumerating the numbers of ingested bacteria after disrupting the worms by homogenization. To eliminate the possibility of contamination of *B. longum*-fed worms with OP50, homogenates from these worms were also plated on LB agar and incubated aerobically, under which conditions no bacterial growth was observed. The lack of contamination from bacteria in different treatments is shown in table 9.

The bacterial counts from *C. elegans* that had been fed OP50 were 2.1×10^4 CFU/mL, and those from worms that had been fed *B. longum* were 1.8×10^4 CFU/mL, as enumerated by spread-plate counting on their respective growth media (LB or MRS). The differences in bacterial numbers were not statistically significant ($P>0.05$). These results are displayed in Figure 7.

Diet of *C. elegans*	**Tetracycline wash**	**Gentamycin wash**	**Growth of colonies on MRS?**	**Growth of colonies on LB?**
B. longum	Yes	No	Yes	No
***E. coli* OP50**	No	Yes	No	Yes

Table 9. Checking for absence of bacterial cross-contamination in the gut of *C. elegans.*

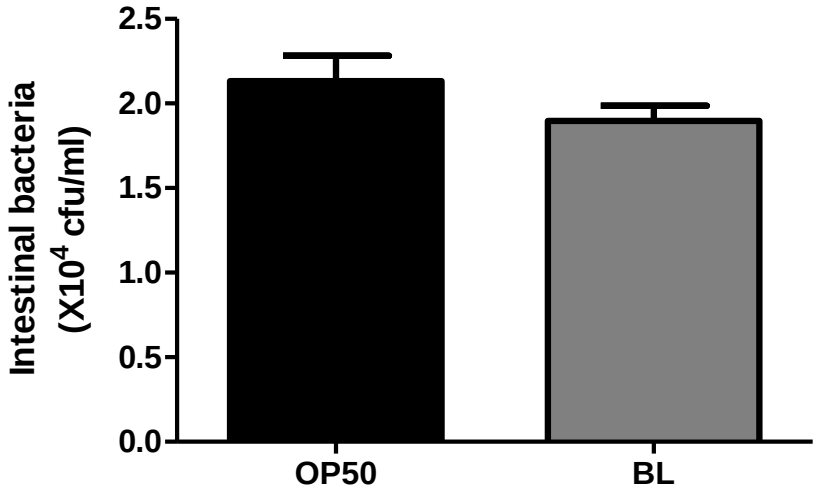


Figure 7. Bacterial quantification from the gut of *C. elegans* fed either *E. coli* OP50 or *B. longum*. Data were plotted as means +/- SEMs. *C. elegans* grown in liquid mNGM were fed either *B. longum* or *E. coli* OP50. Disruption of 50 worms and sunsequent plating on pre-reduced MRS agar or LB agar revealed no significant (P>0.05) difference in the amount of these bacteria *C. elegans* were consuming, based on the Mann-Whitney Test.

Triglyceride Assay

Further to preliminary *in vitro* data from our laboratory demonstrating that CFS from *B. longum* were able to decrease triglycerides in mouse adipocytes (3T3-L1 cells), we wanted to investigate if feeding live *B. longum* impacted triglyceride levels *in vivo*, using our feeding protocol developed above, in *C. elegans*. Homogenates from *C. elegans* that had been fed either *B. longum* or *E. coli* OP50 revealed different levels of triglycerides. *C. elegans* that consumed a diet of *B. longum* contained significantly (P<0.05) less triglycerides than worms that were fed *E. coli* OP50. As displayed in figure 8, *B. longum*-fed worms had total triglyceride levels of 82.04 +/- 7.94% relative to those of OP50-fed worms.

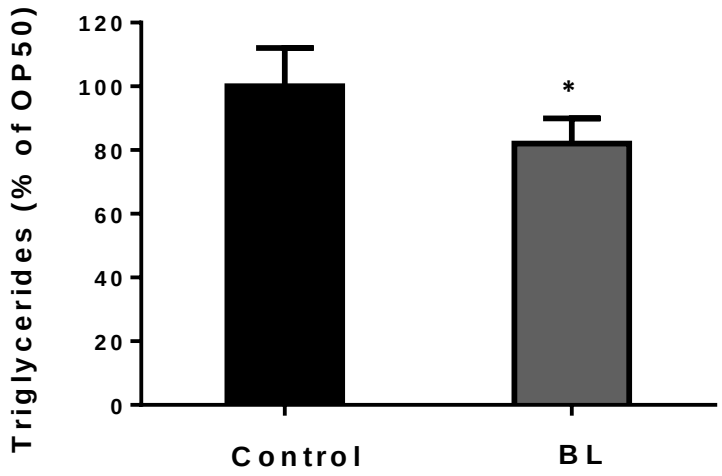


Figure 8. Differences in triglyceride content of *C. elegans* fed *B. longum* or OP50. Data plotted as % control +/- SEMs. *C. elegans* grown in liquid mNGM were fed either *B. longum* or *E. coli* OP50 control. Significantly ($p<0.05$) less amount of triglyceride was detected in the worms fed *B. longum* (82.04 +/- 7.94%) as compared to worms fed *E. coli* OP50, based on the Mann-Whitney test. Triglyceride content (mM) was normalized to total protein (mg/mL).

Lipase assay

In previous *in vitro* experiments conducted with mouse adipocytes (3T3-L1 cells), we have found that *B. longum* supernatants were able to reduce the activity of LPL. As *C. elegans* triglyceride droplet stores are partly regulated by a variety of lipases, we evaluated if *B. longum*- and OP50-fed worms had detectable differences in lipase activity. The lipase assay revealed significantly ($P<0.05$) lower amount of lipase activity in worms that were fed *B. longum* (83.53 +/- 4.71%) compared to worms that were fed a diet of OP50. Results are shown in Figure 9.

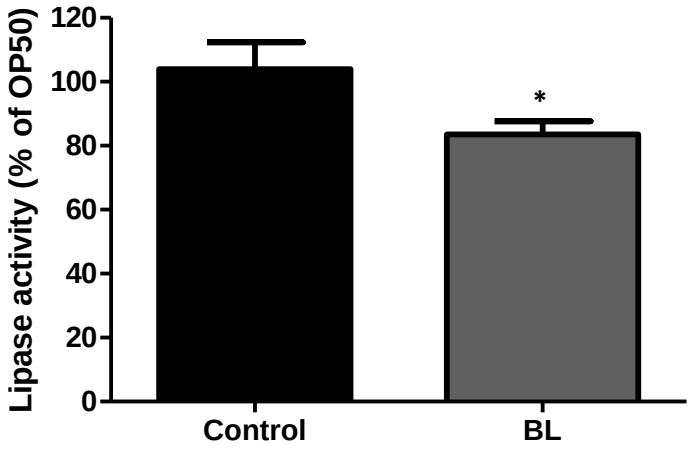


Figure 9. Lipase activity of *C. elegans* fed *B. longum* or *E. coli* OP50. Data plotted % control +/- SEMs. Lipase assay revealed differences in lipase activity in worms fed OP50 or *B. longum*. Worms fed *B. longum* had significantly ($P<0.05$) less lipase activity (83.53 +/- 4.71%) than the *E. coli* OP50 control, based on the Mann-Whitney test.

Analysis of bacterial macronutrient composition

To investigate if OP50 and *B. longum* impacted the growth and lipid content of *C. elegans* as a function of differences in the nutritional composition of these two diets, we analyzed the macronutrient composition of equivalent quantities of *B. longum* and OP50.

We found that there are statistically significant differences in the carbohydrate, lipid, and protein content of the two bacteria. *B. longum* contains significantly less carbohydrates (93.15 +/- 0.38%) and lipids (90.12 +/- 2.20%), and significantly more protein (182.38 +/- 1.28%) compared to OP50. This data is displayed in figure 10.

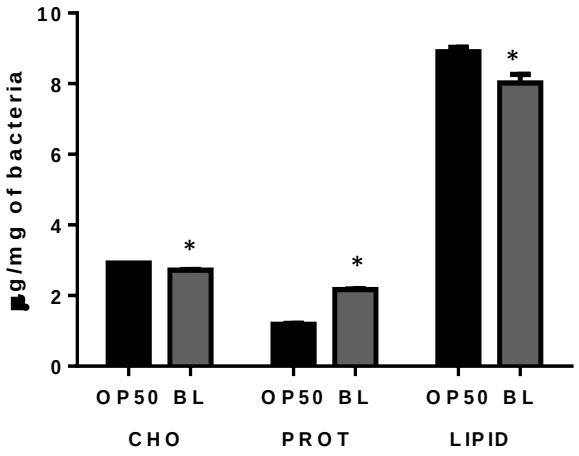


Figure 10. Macronutrient Composition of *B. longum* and *E. coli OP50*. Data plotted % control +/- SEMs. Macronutrient analysis revealed different nutrient composition of the bacterial diets. *B. longum* contained a significantly ($P<0.05$) less amount of CHO (93.15 +/- 0.36%) and lipids (90.12 +/- 2.20%), and significantly greater ($P<0.05$) amount of PRO (182.38 +/- 1.28%) than the *E. coli* OP50 control, based on the Student's T-Test.

CHAPTER IV: DISCUSSION AND FURURE DIRECTIONS

Obesity is an energy homeostasis disorder which develops when energy intake exceeds energy expenditure. Gut microbiota via an ability to influence fat synthesis and storage has emerged as an important factor in host adiposity, and a target for its dietary modulation (Machato & Cortez-pinto, 2012). In order to develop a deeper understanding of the effects of probiotic bacteria on obesity, we performed several *in* vitro studies on the metabolites of *B. longum.* To examine the role of specific gut bacteria in modulating fat stores and associated mechanisms, we need simple, inexpensive, and powerful animal models. An attractive choice of such a model organism is the nematode *C. elegans*, in which many pathways of fat storage and energy use are conserved.

In light of our preliminary data, which examined both the effect of *L. casei* and *B. longum* CFS on lipase activity and triglyceride levels in 3T3-L1 adipocytes, and FIAF expression in HT-29 intestinal epithelial cells, we endeavored to utilize *C. elegans* as a model for further investigating such probiotic-host interactions. Our initial attempts at growing *C. elegans* on *B. longum* as a food source rather than *E. coli* OP50 were met with several challenges: reluctance of *C. elegans* to consume *B. longum* and preference for OP50, carry-over of OP50 on the worm surface and in the gut, and non-comparable growth of *C. elegans* on *B. longum* and OP50. By varying the type of medium (solid/liquid), its composition, increasing *B. longum* concentration, and inhibiting growth of OP50 growth by using an antibiotic, we devised a successful method to grow *C. elegans* on *B. longum* for over one generation, detect *B. longum* in the *C. elegans* gut at numbers comparable to the human small intestine (with no detectable *E. coli* OP50), and study its impact on triglyceride storage. The results of our method development support

the idea that *C. elegans* can indeed be utilized as a model to study the influence of our chosen probiotic *B. longum* on host metabolism, and that it may be possible to further investigate host-microbe interactions in *C. elegans* using other types of probiotic bacteria as well.

To examine the effect of our bacteria of choice on *C. elegans,* in addition to feeding the live probiotic bacteria to the worms, we also attempted to treat the worms with only the CFS of *B. longum* or *L. casei,* while feeding them their regular diet of OP50. This was done in an effort to mimic the *in vitro* experiments, and to observe the effect of the probiotic secreted factors, without having to account for differences in the macronutrient composition within the diet of the *C. elegans*. However, as reported in the results section, the worms did not tolerate the CFS well, appeared unhealthy and exhibited poor survival in the presence of the CFS or the medium (MRS)-control, even at much lower concentrations of 1-2% (v/v), compared to concentrations of 20% (v/v) used in our *in vitro* experiments. This is likely due to the high concentration of glucose in the MRS and CFS, which adversely affects *C. elegans* (their growth medium NGM does not contain any glucose), as well as much higher concentration of protein components compared to the low concentration of peptone present in NGM (Schlotterer et al., 2009; Laminita et al., 2004). In fact, except for maintenance, all experiments in this thesis utilized mNGM that lacked peptone, as it promoted the intake of the alternate bacterial diet of probiotics. The adverse impact could also be attributed to volatile short-chain fatty acids in the CFS, as *C. elegans* have been reported to dislike volatiles (Bargmann 2006) In fact, previous experiments with *C. elegans* that were fed *L. casei* in our lab have

shown that *C. elegans* do not grow well on *L. casei* and do not reproduce (this thesis), presumably due to lactic acid accumulation.

With the inability to treat our worms exclusively with the metabolites of our probiotic bacteria, we focused our attention towards promotion of the consumption of the whole live probiotic bacteria. *C. elegans*' preference towards certain types of bacteria has been well documented. Several articles have demonstrated this food-preference of *C. elegans*, and reported that the worms will actively leave certain foods to hunt for other bacterial sources, suggesting that *C. elegans* search for food that best supports their growth (Shtonda & Avery, 2006). We also observed this as part of previous research related to this project (Al-Tameemi, 2013), where *C. elegans* crawled away from *B. longum* lawns on solid media to find OP50, and continued to feed on any carry over OP50 transferred to the plates from the worm-surface, if they were not decontaminated with gentamicin. It is presumable this differentiation in food preference occurs in liquid media as well, however, we were able to overcome this issue by ensuring that no carry-over of OP50 occurs in *B. longum*-treatments, by decontaminating the worms using tetracycline and gentamicin before introduction into the *B. longum*-containing liquid medium, and incorporating gentamicin into the medium to prevent OP50 growth. The consumption of *B. longum* in liquid culture is also promoted by the fact that the worms are unable to crawl away from the food.

We considered some important factors during our method-development for studying *B. longum*'s impact on metabolic processes in *C. elegans*. Firstly, it was important to ensure that the *C. elegans* are actually consuming similar quantities of the test bacteria and its traditional diet, OP50. Secondly, it was important to ensure that the

longevity and health of the worms is not compromised when changing the diet. Lastly, it is important to ensure that there is no contamination of OP50 in the *B. longum*-treated worms, as this can lead to misleading or incorrect results. We feel that by systematically addressing these concerns, as discussed below, we have validated our method for reliable use in functional assays intended to study bacterial impact on host metabolism.

Before conducting any comparative experiments examining growth or metabolism as a function of different bacterial diets, we confirmed the consumption of our probiotic bacteria by *C. elegans*, and lack of cross-contamination between OP50 and *B. longum* treatments. To address these contamination matters, we washed the L1 worms several times with M9-containing 0.01% Gentamycin sulfate as we transferred them into liquid culture. Gentamycin sulfate targets gram-negative bacteria (notably, *E. coli*), while leaving most gram-positive bacteria unharmed. This washing technique is appropriate when promoting a non-contaminated transfer of worms into new, sterile media, as was reported by the previous graduate student on this project (Al-Tameemi, 2013).

Initially, our enumeration was performed by washing the *B. longum* or OP50-fed L4 worms several times in a gentamycin and levamisole-containing buffer, disrupting the worms, and plating the serially-diluted homogenates on LB media or pre-reduced MRS agar for worms fed OP50 or *B. longum*, respectively. However, when we attempted enumerate the worms from the gut of *C. elegans* after they had reached the L4 stage, we suspected that bacteria, regardless of type, could adhere to the outside of *C. elegans* and skew our results (as these surface bacteria would wash into the homogenates). To address this concern, we increased the number of times the worms were washed, and changed the antibiotic for worms fed *B. longum*. We washed worms that were fed OP50 with

gentamycin, and worms that were fed *B. longum* with tetracycline, which target gram-positive bacteria, such as *B. longum* (Masco et al., 2006). We also ensured that contamination of OP50 was not occurring in wells of *B. longum*-fed worms by plating the disruptates of *B. longum*-fed worms on LB agar. The proper antibiotics, combined with the use of levamisole to paralyze the worms and ensure that the intestinal bacteria were not expelled from the gut due to pharyngeal pumping, assured us that our bacterial enumeration results were accurate.

After addressing the contamination problem and ensuring that *C. elegans* would consume *B. longum* as a food source in liquid media if no other bacterial food source was present, our next concern was ensuring that the growth rate and longevity of *C. elegans* was not greatly altered by the dietary switch to *B. longum*. To address this, we performed a 30-day survival assay, comparing worms on different diets. For our treatments, we used our typical dose of 20 mg/25µL bacteria in M9 (4mg/well), and a lower dose of 10 mg/25 µL of bacteria in M9 (2 mg/well). These doses applied to the both of the bacterial diets of *E. coli* OP50 and *B. longum*. The bacterial concentration of 20 mg/25 µL was experimentally optimized as suitable to support *C. elegans* growth by a prior graduate student (Al-tameemi 2013). In this thesis, we compared the growth and survival of *C. elegans* on this concentration of bacteria over the duration of a typical experiment (growth from L1 to L4) and also up to a 30 day period until all worms were dead. Our findings indeed indicate that the 20 mg/25µL dose is most suitable for growth of *C. elegans* and for functional assays, as there was no significant difference between the growth rate and lifespan of worms fed this dose of OP50 or *B. longum*.

We did make an interesting observation in worms fed the lower concentration of bacterial diet, at 10mg/25µl. When worms were fed this lower dose, we noticed a significant decrease in median survival in worms fed *B. longum* when compared to worms fed OP50. Although this is consistent with the protocol formulated by the student who initiated this method-development in our lab (Al-Tameemi 2013), it is a somewhat curious finding. It raises the question of why the growth of *C. elegans* would be significantly different to the OP50 control when given lower concentration of *B. longum*, even when their growth is not statistically different when fed higher amounts of *B. longum*. These findings are consistent with literature, which states that caloric restriction increases the lifespan of *C. elegans* (Lakowski & Hekimi, 1998). The concentration dependence may also be attributed to a specific bacterial component or metabolite that is not present or produced in sufficient concentrations to support growth at this lower treatment dose.

After refining our protocol for *B. longum*, and addressing any concerns that could impact the validity of the model, we decided to conduct a preliminary screen to investigate if *C. elegans* could survive and reproduce on other strains of probiotic bacteria. Using the same liquid media procedure as *B. longum*, we examined seven other strains as the diet for *C. elegans.* These seven strains were *L. casei, L. reuteri, L. gasseri, L. plantarum, L. fermentum, L. rhamnosis*, and *L. acidophilus*. Given the ability of Lactobacillus sp. to markedly lower media pH, the success of this screening in liquid culture was somewhat surprising (Narendranath & Power, 2005). However, several other studies have examined the effect of various Lactobacillus strains on longevity, immune-modulation and anti-pathogenicity in *C. elegans* using solid media (Ikeda et al., 2007;

Lee et al., 2011; Kim et al., 2012). Our screening experiments saw *C. elegans* on all seven strains of bacteria survive and reach the adult stage in a reasonable amount of time (~50-60 hours), which was slightly slower than the OP50 control (~44-48 hours). Of the seven strains, only two (*L. casei* and *L. rhamnosus*) did not reproduce. These results warrant further investigation, towards use of *C. elegans* as a host model for other probiotic bacteria.

Another exploratory experiment we performed on our *C. elegans* model was examining their ability to flourish on bacteria that had been inactivated via heating. The goal of this experiment was to determine if *C. elegans* simply utilize the structural components of bacteria as a source of nutrition, or whether generation of active metabolites by live viable bacteria is necessary for promoting worm growth. Worms fed live OP50 flourished, as expected, and worms fed live *B. longum* also survived and grew at a similar pace. Worms fed heat-inactivated OP50 reverted to their dauer stage, and their numbers slowly tapered as the worms either died or became unresponsive. This is expected, as research often shows that live OP50 is necessary for larval development, however, worms fed heat-inactivated OP50 after they reach their L4 stage have been shown to have a longer lifespan (Amrit et al., 2014; Gems & Riddle, 2000). Worms fed heat-inactivated *B. longum* also did not grow into their adult stage, and their brood size dwindled somewhat faster than worms fed heat-inactivated OP50. It would be interesting to see if similar to the reported observations for OP50, worms can survive on heat-inactivated *B. longum* once they have already reached L4 stage on a diet of live bacteria.

After refining and validating the protocol for feeding *B. longum* to *C. elegans*, including ensuring that there was no contamination, that *C. elegans* were consuming *B.*

longum, and that the rate of growth, survival and lifespan of the worms was similar when fed *B. longum* or OP50, we focused on utilizing the protocol for examining the influence of *B. longum* on lipid stores in *C. elegans*. Specifically, we examined the effect of the probiotic bacterial diet on the triglyceride content and lipase activity of the worms as one of the main objectives of our study. It is understood that *C. elegans* balance lipid storage and utilization pathways through many complex transcriptional, translational, and post-translational mechanisms (Ashrafi, 2007). As changes in diet, as well as food availability, elicit changes in expression of metabolic genes and fat deposition, we examined the effect of dietary changes on lipase and triglyceride levels (Van Gilst et al., 2005).

Our results show that worms fed *B. longum*, when compared to worms fed OP50, have significantly less triglyceride levels and lipase activity. There are several possible mechanisms through which this may be occurring, such as alterations in expression of genes involved in regulation of lipid metabolism, which remain to be investigated. Examples of these genes include *fat*-5, *fat*-6, and *fat*-7, which are homologs of Δ9-desaturases in humans and are under the control of nuclear hormone receptor-8 (Magner et al., 2013). A pathway involving the use of the genes is displayed in figure 11. These results support the previous data we obtained from mouse adipocytes 3T3-L1, and suggest that *B. longum* consumption may reduce fat-storage. In 3T3-L1 cells, our results have supported the possibility of *B. longum* regulating lipase activity via ANGPTL4; however, *C. elegans* lipid storage is dynamically regulated by a multitude of factors and there is no known homolog of ANGPTL4. It is therefore likely that some alternative genes in the fat synthesis/storage pathway are responsible for the observed reduction in

triglyceride levels. Changes in diet have been shown to dramatically provoke changes in fat composition in other studies with *C. elegans* (Brock et al., 2007).

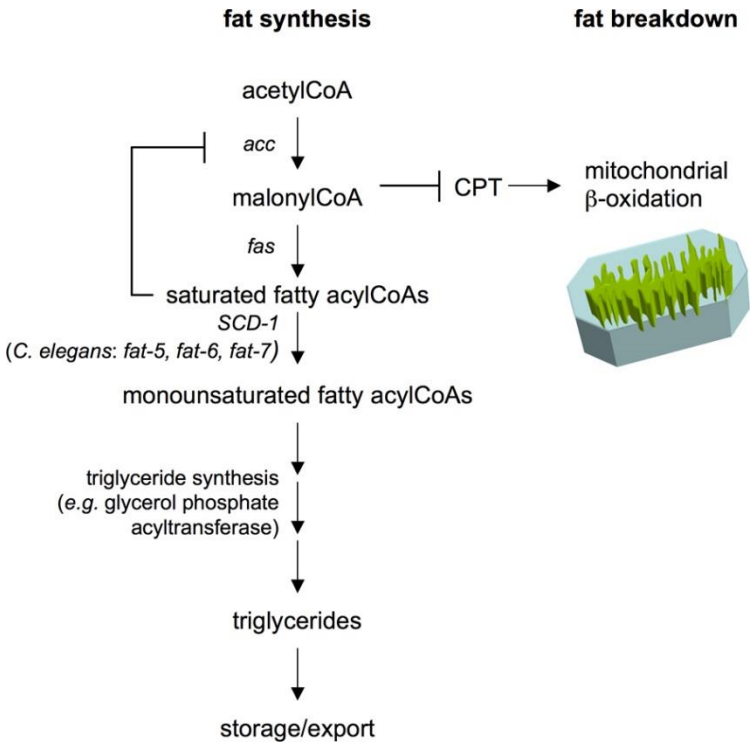


Figure 11. Lipid metabolism pathway in *C. elegans*. AcetylCoA is the building block of fatty acids that are assembled into storage triglycerides through step-wise enzymatic processes. Inhibiting delta-9 desaturase activity (SCD-1 in mammals, fat-5, fat-6, and fat-7 in C. elegans) causes accumulation of saturated fatty acids. Consequently, acetylCoA carboxylase (ACC) is inactivated through feedback inhibition. Reproduced from (http://www.wormbook.org/chapters/www_obesity/obesity.html)

To elucidate if the observed reduction in triglyceride levels with *B. longum* may be partly due to differences in the nutritional composition of the two bacterial diets, we analyzed their macronutrient composition. If there were a major difference in the lipid or carbohydrate composition of the two different strains of bacteria, then it is likely that they will differentially influence fat synthesis and storage pathways. Even though *C. elegans*

lipid biology is still very much in its infancy, it is known that *C. elegans* do convert excess energy into triglycerides (Mullaney & Ashrafi, 2009). We did find a higher amount of carbohydrates and lipids, and a lower amount of protein in OP50 when compared to *B. longum*, which does provide us with some possible reasoning for the shift in lipid deposition. Notably, our bacterial macronutrient composition results showing protein as the main constituent of *E. coli* and *B. longum* are consistent with literature examining bacterial macronutrient composition (Neidhardt & Umbarger, 1996).

It is however unlikely that these differences in carbohydrate and lipid content in our bacterial strains would be the primary determinant of the changes in lipid content of the worms. A study performed by Brooks et al., (2009) examined the effect of four different strains of *E. coli* on *C. elegans* lipid levels. These four strains had markedly different carbohydrate and lipid levels, and the study actually found an inverse correlation between carbohydrate levels in these bacterial strains, and the lipid stores in *C. elegans*. Such an inverse correlation was not observed in our study, which suggests that there may be another reason our *B. longum*-fed *C. elegans* displayed decreased lipid deposition, which could have to do not just with the bacterial nutrient composition, but with specific regulation of fat-regulatory genes in *C. elegans* by *B. longum* or its metabolites.

To further display the lack of effect of dietary macronutrient composition on *C. elegans*, Brooks et al., (2009) increased the glucose in the *C. elegans* growth medium by up to 5%, resulting in an increase in cellular glucose levels in some strains by up to 300%, but this had no effect on the triglyceride composition of the nematodes fed these bacteria. The only exception was OP50, where an increase in glucose content led to a

slight increase in the triglyceride content of the worms, which lends some support to our observations.

The same study also examined differences in fatty acid composition of the four bacterial strains, and found no correlation in the fatty acid composition of the diets and the lipid deposition in the worms. The results from this study therefore do not support the notion that the reduction in *C. elegans* lipid levels were solely due to differences in the macronutrient composition of the two bacterial diets. It is certainly a possibility, however, and something that can be investigated further along with changes in other relevant transcription factors and genes that regulate fat stores in *C. elegans*.

Future research directions

As part of this thesis, we have successfully developed, refined and validated a protocol that enables the use of *C. elegans* as a model to study the influence of a probiotic *B. longum* on fat-storage, and shown that feeding *B. longum* decreases triglyceride levels in *C. elegans*. To the best of our knowledge, this is the first reported use of the *C. elegans* model for studying the impact of probiotics on fat-storage. Our *B. longum*-feeding protocol is simple and reproducible, and eliminates the challenges we encountered while trying to adopt previously published methods for probiotic-feeding in *C. elegans*, such as non-consumption of probiotic strains, poor growth and contamination with OP50. While the thesis has produced some interesting and useful data, there are still many unanswered questions that need investigating in future, many of which can hopefully be answered by utilizing our method and model system. Some examples of future directions for research on this topic are discussed below.

When considering our 30-day survival assay, we noticed a difference in the survival and lifespan of worms fed *B. longum* at higher and lower amounts. This is a very curious finding, and leads one to wonder if there is some compound produced by *B. longum* that is beneficial for *C. elegans* at a high concentration, but not at a low concentration. It would be quite interesting to perform several survival assays on varying concentrations of *B. longum* to assess whether higher concentrations of *B. longum* than ones tested in our study could further promote the survival/longevity of *C. elegans.* Little work has been done on this topic due to the fairly recent adoption of *C. elegans* as a model for probiotic-host studies.

While much research has been performed on the dietary preferences of *C. elegans,* it is unknown why *C. elegans* do not prefer *B. longum*, and choose not to consume it unless it is the only available food. There are several variables which come into play when inspecting dietary preference of *C. elegans*, such as difference in bacterial scent, size, nutritional level, and the tendency of bacteria to clump together or remain isolated (Shtonda, 2006). Of the ~1000 cells in an adult *C. elegans*, ~300 are neurons, ~30 of which have a dedication to biochemical sensory (Ezcurra, 2009). Those neurons are integral in the ability of *C. elegans* to respond to toxins, scents, and foreign fauna. Further investigation into the molecular, morphological, and nutritional differences of *B. longum,* as well as other probiotic bacteria, are needed to detail reasons for the innate preference to not consume *B. longum*. It was also quite interesting to observe *C. elegans* growing to a mature state and even reproducing, albeit a slightly slower rate than the OP50 control, on most of the other seven strains of probiotic bacteria we examined (*Lactobacillus* sp.). It should thus be possible to translate our method to other probiotic

strains with some minor optimization, and use it as a general protocol for such studies, rather than specific only to *B. longum*.

To investigate further into the mechanisms underlying the influence of *B. longum* on lipid metabolism in *C. elegans*, a combination of proteomic and genomic approaches should be used. Genetic manipulation has been crucial in *C. elegans* research since its inception, and thanks to the complete genomic map of *C. elegans*, these techniques can be extremely informative. We are presently working towards utilizing the above approaches to elucidate the basis of *B. longum*'s impact on *C. elegans*' triglyceride stores. Gel electrophoresis and coomassie staining of the total-protein extracts from *C. elegans* fed either *B. longum* or OP50 will allow us to compare notable differences in protein expression between the two treatments. Any proteins of interest will then be identified via mass spectrometry. This approach will provide some insight into the proteins that are being differentially regulated by *B. longum*, and the results can be interpreted in light of related changes in gene-expression.

There is a wide selection of transgenic green fluorescent protein (GFP) reporter strains of *C. elegans* that are available through the CGC, including genes related to lipid metabolism. These strains will allow visualization of differentially-regulated genes in response to our two bacterial treatments. Transcriptional changes in genes which are relevant to *C. elegans* fat synthesis, such as lbp-1, nhr-49, sbp-1, atgl-1, mdt-15, acs-20, fat-6, and fat-7, will also be analyzed by real-time PCR to investigate their role in mediating *B. longum's* effect on the lipid composition of *C. elegans.*

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