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Influence of Lactobacillus rhamnosus Isolated from “Amabere Amaruranu” Cultured Milk on Adipogenesis

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ABSTRACT

Influence of *Lactobacillus rhamnosus* Isolated from “Amabere Amaruranu” Cultured Milk on Adipogenesis

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This study was performed to test the *in vitro* effects of a *Lactobacillus rhamnosus* isolate from “amabere amaruranu”, a traditional Kenyan cultured milk, on 3T3-L1 and Caco-2 cell lines. Cultures of fully mature 3T3-L1 adipocytes were treated with bacterial isolate cell extract (CE), filtered spent broth (FSB) from overnight bacterial culture, or with a PBS control. Expression levels of PPAR³ 1 and 2, C/EBP±, and ATGL proteins in 3T3-L1 cells were upregulated by FSB treatment. CE treatment did not affect protein expression levels. Expression of MTTP and SREBP-1c proteins in Caco-2 cells showed no change with either treatment. Optical density measurements from Oil-Red-O stained 3T3-L1 adipocytes increased from PBS control cells to 25µl/ml FSB treated cells; measurements were reduced by treatments above 25µl/ml FSB. In conclusion, filtered spent broth prepared from a culture of *Lactobacillus rhamnosus*, isolated from “amabere amaruranu” cultured milk showed PPAR³ 1 and 2, C/EBP±, and ATGL agonistic properties.

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CHAPTER 1

INTRODUCTION

Probiotic Definition

Prior to 2001, no one definition for the term probiotic was agreed upon. The problem was clear that as the market for probiotics grew, the science behind probiotics was lagging behind. In 2002, a joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) working group was assembled to draft effective guidelines for the evaluation of probiotics in food. One of the main recommendations of this group was to adopt the definition of a probiotic as “Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Joint FAO/WHO Working Group 2002). Other recommendations of this group were to adopt guidelines put forth in the report for testing bacterial strains for probiotic qualities, follow good manufacturing practices in the manufacturing of probiotic foods, as well as clear labelling of probiotic foods including bacterial strains present and any health claims (Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food 2002).

In 2008, Dr. Mary Ellen Sanders addressed the lack of oversight that still plagued the growing industry. She acknowledged that even though the FDA is the regulatory authority over probiotic products, it does not require a manufacturer of a probiotic product to obtain premarket approval of any claims of efficacy or safety of that product as long as the product is not marketed as a drug (Sanders 2008). She concluded that the improper use of the term probiotic within the industry could seriously undermine consumer confidence, and that further controlled human studies are needed to validate

health claims made by probiotic manufacturers (Sanders 2008). Her paper brought up many of the same problems that the joint FAO/WHO working group tried to address which could point towards the need for more oversight if the industry cannot regulate itself.

In the past few years, the probiotic industry has experienced unprecedented global market growth. According to a market report by BCC Research, formerly Business Communications Company, published in December 2011, the global probiotic market which includes sales of probiotic supplements, ingredients, and foods reached approximately $21.6 Billion (US) in 2010 and was expected to have a compound annual growth rate of 7.6% over the five year period (The probiotics market: Ingredients, supplements, foods 2011). In 2013, a panel of experts from the International Scientific Association for Probiotics and Prebiotics (ISAPP) was convened to revisit the definitions and guidelines put forth 12 years earlier by the Joint Working Group and provide a consensus statement for the appropriate scope and use of the term probiotic. This convention was prompted by the market boom, widespread use of the term probiotic on many products that did not meet minimum criteria, and scientific advances within the field. Ultimately, the committee retained the FAO/WHO definition from 2001 and called for more scientific evidence to be obtained before labelling a product probiotic (Hill et al. 2013).

Role of Probiotics in General Health

The probiotic industry has exploded over the last five to ten years with many probiotic products on the market. The problem arises that science has not been able to keep up with the pace of the market expansion and many of the product claims remain

scientifically unverified (Sanders 2008). With that being said, much work has been done to elucidate the beneficial effects of probiotics on the body.

With the gut microbiome being implicated in many disease states, probiotics have been increasingly studied because of their ability to change the microbial community within the gut (Ji et al. 2012). The idea of modulating the microbiome from a disease state to a healthy state to treat gastro-intestinal diseases that currently have very limited treatments is enticing. Much of the early probiotic work was focused around alleviating gastrointestinal (GI) diseases. Meta-analysis of clinical trials involving the treatment of infectious diarrhea with rehydration therapy and probiotics showed that probiotics significantly reduce the time period of diarrhea in patients treated with probiotics (Allen et al. 2004). When treating symptoms of Irritable Bowel Syndrome with probiotics, patients reported higher relief of symptoms than placebo group (Kajander et al. 2008).

Studies done on the effectiveness of probiotics to treat food allergies and atopic dermatitis have shown that probiotics do have an immuno-modulatory role. Many of these studies have been focused around newborns since it is believed that the maturation of the immune system is dependent on colonization of the newborn GI tract by normal newborn microbiota. Differences in the microbiome were observed in newborns delivered by vaginal delivery as opposed to Cesarean section (Dominguez-Bello et al. 2010). Also, antibiotics prescribed to infants alters the microbiome and can be linked with higher prevalence for immune mediated diseases such as Irritable Bowel Disease (IBD), asthma, and obesity (Zeissig and Blumberg 2014). Infants with cow's milk allergy, treated with Lactobacillus GG, showed significant decrease in the incidence

of atopic dermatitis (Majamaa and Isolauri 1997). Lactic acid bacteria have been shown to reduce cytokine production specific to allergic sensitivity, showing that probiotics can have a direct impact on immune cells (Pochard et al. 2002). It would, therefore, seem that probiotics can have a beneficial effect on the overall health of the host.

Lactobacillus rhamnosus Isolate

The potential probiotic used for this study was isolated from "amabere amaruranu", a traditional Kenyan cultured milk. This isolate was chosen for continued testing because Lactobacillus species are generally considered safe for food and supplementation (Joint FAO/WHO Working Group 2002). This isolate was shown to be stable in digestive tract conditions, have no ability to degrade mucin, and was sensitive to antibiotics. The isolate was identified as *L. rhamnosus* by both 16s rRNA and biochemical assay (Boyiri et al. 2014). These tests are recommended by the joint FAO/WHO working group to establish a new bacterial isolate as a potential probiotic.

Human Gut Microbiome in General Health and Disease

The gastro-intestinal tract is home to a great diversity of microbiota. There are approximately ten times more bacteria in the human gut than eukaryotic cells in the human body. The compilation of this microbiota is still being discovered, but recent studies have shown that the gastrointestinal tract is home to over 1200 distinct microorganisms (Rajilic-Stojanovic et al. 2007). Although the exact bacterial composition of the gut microbiome is unique to each individual, sequencing of the human gut microbiome has shown that the majority of the organisms living in the GI tract are members of either the *Firmicute* or *Bacteroidetes* phylum (Eckburg et al. 2005). The proportions of these two phyla are stable in healthy populations but have been

shown to differ significantly in disease states such as obesity (Ley et al. 2006). This diversity begins at birth. At birth, the neonatal intestine is sterile but immediately colonized by bacteria from maternal and environmental sources, and progress to an adult-like microbiome as the infant transitions to solid food (Palmer et al. 2007).

Ilya Metchnikoff was one of the first scientists to suggest that intestinal microorganisms might play a role in human health by benefitting the host (O'Toole and Cooney 2008). His ideas were mostly ignored until the 1990's, when research into the gut microbiome and its role in human health gained acceptance. The human gut microbiome has become a topic of focus for research since very little was known about it until recently. New high throughput sequencing techniques have allowed researchers to develop new insight into the composition and functionality of the microbiome. As the relationship between the gut microbiome and its human host is better analyzed, researchers are coming to realize that the gut microbiome has more effects than just in aiding digestion.

Gut Microbiome in Gastrointestinal Health

The gut microbiome could be looked at as another organ. In healthy individuals, the microbiome works symbiotically with the host to help facilitate digestion and uptake of nutrients from food. One important function to humans is that it allows for the processing and digestion of foods that would be otherwise indigestible, like plant polysaccharides (Bergman 1990; Samuel and Gordon 2006) and gluten (Caminero et al. 2014). Mutualism exists within the microbiome as organisms function to catabolize nutrients in the GI tract, which support a healthy diverse microbiome (Samuel and Gordon 2006). This mutualistic role that the gut microbiome plays has allowed for the

digestion and metabolism of foods without humans needing to evolve the ability to produce specific enzymes for this task. It has been determined for some time that long term lifestyle habits alter the make-up of the gastro-intestinal microorganisms, but now research shows that short term shifts in diet can alter the gut microbiome within days of such a shift (David et al. 2014). This same study showed a correlation between diet composition, be it plant based or animal based, and changes in bacterial diversity in the GI tract.

Being that the gut microbiome is integral for proper digestion, it should come as no surprise that altered gut microbiomes have been associated with gastrointestinal disorders. Studies done with Celiac Disease (CD) patients show that they have elevated levels of both *Bacteroides* and *Clostridium leptum*, along with decreased levels of Bifidobacterium as compared to healthy control groups (Collado et al. 2009). Another study showed that microbial diversity of fecal samples from untreated CD patients cluster together, while fecal samples from CD patients treated with a gluten free diet for at least two years show a microbial diversity that reflects that of healthy control subjects (Nistal et al. 2012). These studies point to dysbiosis within the gut microbiota. It is not clear, however, if these changes in the microbiome are caused by the diseases or cause the disease state, and more studies are needed to link specific microbial signatures to specific diseases (De Palma et al. 2014).

Gut Microbiome in Immunity and Gut-Brain Axis

The gut microbiome plays an important role in the development of a mature immune system. Infants born by Caesarian section are at a higher risk of developing allergies and asthma (Bager et al. 2008). Such studies reinforce the idea that

mutualism exists between the gut microbiome and the host immune system (Maynard et al. 2012). A recent study suggests that Clostridia, present in normal gut microbiome, may play a role in helping to reduce food allergen sensitization through an immune-regulatory role (Stefka et al. 2014). This could prove useful in finding a way to prevent food allergies or desensitize patients with existing food allergies.

It has been known for some time that there is a bidirectional flow of information between the brain and the gut. This has been termed the gut-brain axis. The gut microbiome has been shown to play a role in the communication between the brain and gut. This relationship was made apparent with studies of patients with hepatic encephalopathy. Hepatic encephalopathy patients showed signs of improvement after administration of antibiotics as well as probiotics (Victor and Quigley 2014). More evidence of a brain-gut microbiome link came from studies showing differences in the composition of the gut microbiome of autistic and control siblings (Finegold et al. 2010). Additionally, autistic patients reported improved symptoms with antibiotic use (Finegold 2011). Stress and gut microbiota dysbiosis has been reported as well. Stress introduced into an animal model was shown to induce changes within the diversity of the microbiome as well as innate immune response (Bailey et al. 2011). The innate immune response was not seen when the same experiment was performed with mice that had received an antibiotic treatment that reduced the gut microbiota. This suggests that the gut microbiome plays a role in innate immune responses to stress.

Human Gut Microbiota in Obesity and Type 2 Diabetes

As obesity continues to be a major health concern around the world, researchers look for ways to therapeutically target the disease. In the search for a solution to the

obesity epidemic, attention has been paid to the gut microbiota and its involvement in obesity. The intestinal microbiota plays a major role in digestion and energy harvest as shown through the study of germ free and conventionally raised mice (Backhed et al. 2004). Germ free mice have significantly lower total body fat than their wild type counterparts, even though the germ free mice were consuming more chow than the wild type mice. Also, the same study showed that the gut microbiota was able to regulate gene expression linked to energy harvest. The link between obesity and gut microbiota was revealed when metagenomic sequencing of obese and lean mice showed a difference in the makeup of the microbiome. Obese mice had a higher proportion of bacteria of the division Firmicutes, and less of the division Bacteroidetes than lean mice (Ley et al. 2005). This proportional difference of the gut microbiome was also observed in obese and lean humans (Ley et al. 2006).

To further tease out the contributing factors of obesity, metagenomic and biochemical aspects of the obese and lean mouse distal gut microbiome have been examined. The obese microbiome was found to be more capable of harvesting energy from the mouse's diet (Turnbaugh et al. 2006). This same study showed that this increased energy harvest ability was transmissible from obese mice to germ free mice by inoculating the germ free mice with the obese microbiota. Although this does not prove causality, it shows that the gut microbiome plays a major role in energy harvest and obesity.

Gastric bypass surgery has been an effective surgical option for severely obese individuals to rapidly lose weight. Recently, it was proposed that gastric bypass surgery works to reduce weight and improve glucose tolerance through a shift in the gut

microbiome instead of reducing weight by calorie restriction alone. In sequencing the gut microbiome of patients and mouse models of gastric bypass surgery, a shift in the microbiota that altered the energy balance in the host was shown (Liou et al. 2013). This study also showed that by transplanting the gut microbiota from mice that had just had gastric bypass into germ free obese mice, they were able to induce weight loss in the germ free mice.

As the main focus of this study is being put on obesity, it is important to note that another very serious and related problem is Type 2 Diabetes (T2D). T2D is associated with insulin resistance. Although there is no clear causality between obesity and T2D, obesity can increase the risk of acquiring insulin resistance and subsequent T2D through low grade chronic inflammation (Das and Mukhopadhyay 2011).

Endocannabinoid System in Obesity and Insulin Resistance

A major question that arises when looking at a correlation between the gut microbiome and obesity is how does the microbiome interact with the host systems in the presence of the mucosal barrier? The answer may be in the endocannabinoid system (ECS). The ECS consists of bioactive lipids that are important in controlling many physiological functions. Evidence for a link between the ECS and obesity has been shown by knocking out the expression of the bioactive lipid synthesis gene in mouse adipocytes, and thereby show that the ECS is responsible for energy homeostasis throughout the body (Geurts et al. 2015). The knockout mice had greater total fat mass, body-weight gain and a higher insulin resistance index while maintaining the same food intake as control mice. This same study reinforced the idea of an association between adiposity, the ECS and gut microbiome when researchers found

significant changes in the gut microbiota of knockout mice. While a shift in gut microbiome was associated with this genetic knockout, long-term antibiotic treatment improved body-weight gain, fat mass, and reduced insulin resistance index in knockout mice. Researchers were able to partially replicate the knockout phenotype by transferring the gut microbiome from knockout mice into wild type germ free mice. This study strengthens the argument for a correlation between the gut microbiome, ECS and obesity.

Probiotics and Their Influence on Adipogenesis

The gut microbiome controls many more aspects of the host body than had ever been imagined. Since there are correlations between disease states and shifts in host gut microbiome, then disease states should be improved by manipulating the gut microbiome into a more normal state. Research to support this hypothesis showed that a lean gut microbiome transferred to an obese mouse will incur significant weight loss (Ridaura et al. 2013). Hence a manipulation of the gut microbiome is a viable method to fight diseases that contribute to a disease shifted microbiome. Probiotics can manipulate the gut microbiome and regulate various host systems to improve many aspects of a disease.

A number of probiotic strains have been shown to affect adipogenesis in cell culture. A probiotic isolated from Kimchi, a traditional fermented dish, has inhibitory effects on intracellular lipid accumulation in vitro (Moon et al. 2012). This study treated 3T3-L1 cell culture with either spent media or cytoplasmic fraction from the culturing of the isolated probiotic, *Weissella koreensis,* a lactic acid bacteria. They were able to show that the probiotic treatment lowered both lipid accumulation inside of the cells, as

well as mRNA expression levels of key adipogenic transcription factors. Another probiotic isolated from kimchi called *Lactobacillus plantarum* KY1032 was also shown to maintain probiotic activity and regulate lipid metabolism by testing the cytoplasmic fraction in 3T3-L1 cells (Park et al. 2011). This probiotic significantly downregulated adipogenic transcription factors and also lowered lipid content within the adipose cells.

Cell culture studies are a great way to show proof of concept, but it is important to understand how probiotics interact with host systems. Mouse models work as good indicators of how a certain probiotic will interact with a human host. Different species and strains of lactic acid bacteria have been used to elucidate anti-obesity effects. *L. gasseri* BNR17 was one such bacteria that was shown to reduce levels of leptin and insulin while increasing expression of fatty acid metabolism related genes (Kang et al. 2013). A similar study used *L. rhamnosus* PL60 to incur weight loss and reduce white adipose tissue deposits in diet induced obese mice (Lee et al. 2006).

All of these studies have looked at modelling the human obesity condition, but even the best models will differ from human physiology. Therefore it is important to also look at human trials to show that probiotics can carry over the anti-obesity effects shown in models to human hosts. In a human trial using adults with obese tendencies, *L. gasseri* SBT2055 was shown to significantly reduce abdominal adiposity as well as other markers of obesity (Kadooka et al. 2010). Another study tested the effects of *L. acidophilus* NCFM to improve insulin sensitivity in male subjects with Type 2 Diabetes. The study showed that the probiotic was able to preserve insulin sensitivity compared with a control group (Andreasen et al. 2010). Human trials continue to provide insight into new treatments for obesity and Type 2 Diabetes.

Probiotic research has improved the collective knowledge of both the human body systems as well as how bacteria interact with these systems. This research has shown that many beneficial effects of probiotics are strain specific. These studies have created a precedent for effectively testing novel probiotics and their efficacy in regulating adipogenesis.

Adipogenic Transcription Factors

When studying effects of a compound on a biological system, it is important to understand how the compound elicits the effect that it has. To do so, the system can be studied at the molecular level to see how the compound affects the protein and gene expression levels that drive the biological system. In this case, adipogenesis is a complex interplay between many different genes that regulate each other as illustrated in Figure 1. The present study will focus on four primary transcription factors that have been shown to be critical to the maturation of adipose cells and the storage of lipids: PPAR³, C/EBP±, SREBP-1c, and ATGL.

In order to study the effect of a novel probiotic on the adipogenic pathway, cell culture modelling is the most efficient way to begin. The 3T3-L1 cell line was chosen for use in this study. The 3T3-L1 cell line is an adipocyte lineage that was first established in 1975. This cell line has been used extensively to study the mechanisms of adipogenesis because of its similarity to human adipocytes. Once exposed to a nutrient cocktail which includes isobutylmethylxanthine (IBMX), dexamethasone and insulin, the preadipocytes will differentiate into mature adipocytes. This maturation process expresses multiple metabolic programs that are characteristic of mature fat cells. The

validity of this cell line as a model of adipocyte formation is supported by many studies performed in both mouse and human tissue (Farmer 2006).

PPAR-³

PPAR³ is considered the master regulator of adipogenesis. Originally thought to work co-dependently with C/EBP±, it was shown that PPAR³ alone can induce adipogenesis. This was elucidated by doing both gain of function, and loss of function studies on PPAR³. Gain of function studies showed that ectopic expression of PPAR³2 in multiple fibroblast cell lines caused them to differentiate into adipocytes (Tontonoz et al. 1994b). The loss of function study was done by Rosen and collegues, and involved using embryonic stem (ES) cells that lacked a normal PPAR³ gene. These cells were not able to differentiate into adipose cells in vitro (Rosen et al. 1999). Rosen's study also used a chimeric mouse model to show that mice not expressing PPAR³ could not produce adipose tissue.

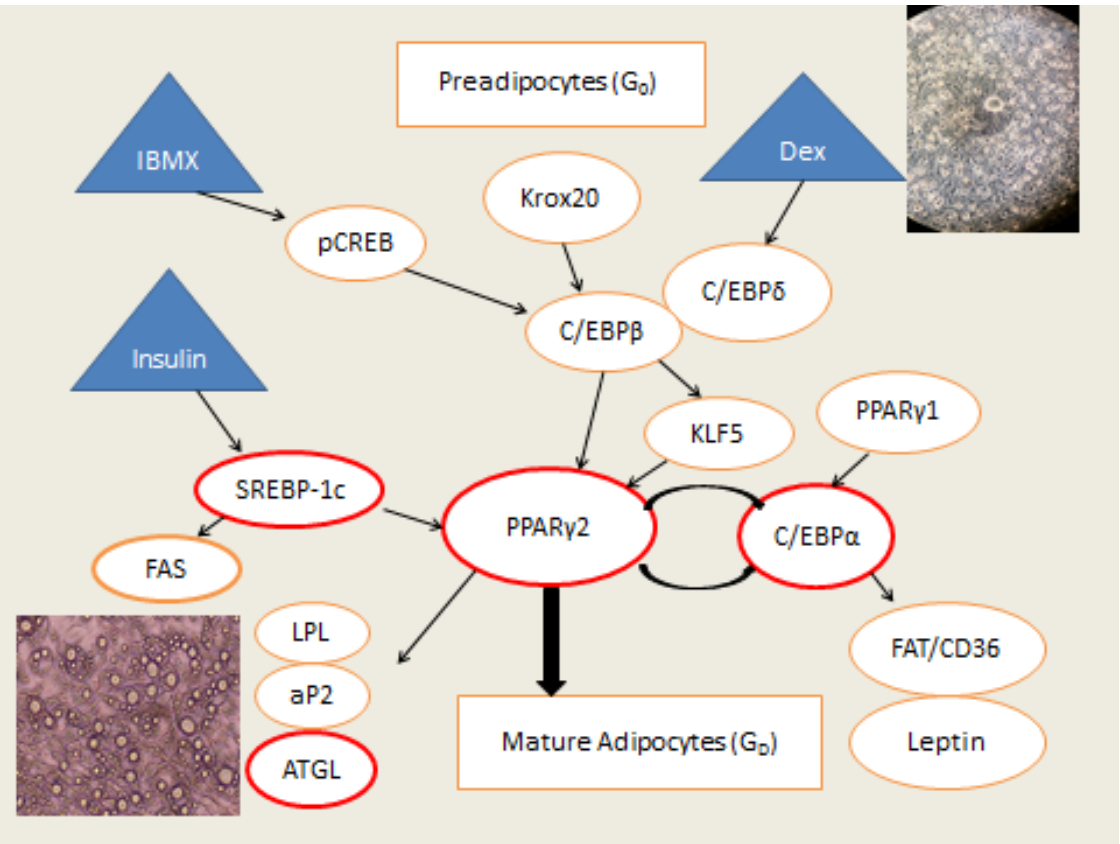


Figure 1. A pathway map of adipogenesis. Key points from this map are that PPAR³2 is the bottleneck point for the differentiation process. SREBP-1c is directly influenced by insulin. C/EBP± forms a positive feedback loop with PPAR³2 to maintain expression in mature adipocytes. The four transcription factors focused on in this project are outlined in bold.

PPAR³ is expressed in adipose cells as two isoforms, PPAR³1 and PPAR³2. Both isoforms are exactly the same except that the PPAR³2 isoform contains an additional 30 amino acids at the amino terminus (Tontonoz et al. 1994a). Tontonoz and colleagues showed that while PPAR³1 is expressed in several different types of tissue, PPAR³2 is specific to adipocytes. PPAR³2 was shown to be the critical transcription factor that regulated cellular differentiation and lipid storage during adipogenesis (Ren

et al. 2002). Ren and colleagues developed a knockout study that showed clearly that PPAR³1 expression without PPAR³2 expression could not trigger adipogenesis in 3T3-L1 cells, therefore showing conclusively that PPAR³2 is crucial for adipogenesis.

PPAR³ is the target for a class of antidiabetic drugs called thiazolidinediones. How this class of drugs worked was unclear until Lehmann and colleagues showed that Thiazolidinediones are direct agonists for PPAR³ (Lehmann et al. 1995). This class of drugs, which includes Rosiglitazone, is used to treat Type 2 Diabetes. Although these drugs are effective at controlling blood glucose levels in patients with Type 2 Diabetes, they are known for having many side effects. Other effective methods for treating Type 2 Diabetes with less side effects are needed.

C/EBP±

CCAAT/Enhancer-binding Protein ± is another critical transcriptional regulator of adipogenesis. Once assumed to be equal to PPAR³ in its role as a master regulator, it was shown to not be as important as PPAR³ in regulating the entire pathway. With that being said, C/EBP± has been shown to be critical in conveying insulin sensitivity to the mature adipocyte. Wu and colleagues were able to show that PPAR³ activates expression of C/EBP±, which then enters a positive feedback loop with PPAR³ ensuring terminal differentiation and high-level expression of both genes in differentiated cells (Wu et al. 1999). The differentiation pathway for creating mature adipocytes is not fully understood. What is now known is that as C/EBP² is needed to bind to the *C/EBP*± promoter site, but cannot induce transcription without the presence of PPAR³ (Zuo et al. 2006).

In addition to positively regulating PPAR³, C/EBP± is critically important in conferring insulin sensitivity to the mature adipocyte. During C/EBP± knockout studies, it was observed that the mature adipocytes had no insulin-stimulated glucose transport (Wu et al. 1999). C/EBP± is responsible for the expression of many insulin sensitivity factors such as GLUT4, as well as fatty acid transporter, FAT/CD36 (Qiao et al. 2008).

ATGL

Adipose Triglyceride Lipase (ATGL) was first identified in 2004 by three independent research groups. It is expressed predominantly in both white and brown adipose tissue and localizes on the lipid droplets (Zechner et al. 2005). This enzyme has high substrate specificity for triacylglycerol (TG) and is responsible for the first step in lipolysis, hydrolyzing TG into Diacylglycerol (DG) and fatty acid (FA) (Zimmermann et al. 2004). Before this discovery, the enzymes hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL) were thought to be the main enzymes that drive lipolysis. MGL performs the last step in the pathway of hydrolyzing monoglycerides into glycerol and FA. HSL was known to have broad substrate specificity to lipid esters. HSL knockout studies had shown that there were other enzymes involved in the pathway though. ATGL completed the pathway becoming the rate limiting enzyme for the process of TG mobilization (Chakrabarti and Kandror 2011).

ATGL levels vary during feeding and fasting. Both the mRNA and protein concentrations are affected by nutritional status. ATGL activity has been shown to be regulated through both gene expression and post-translational modification (Chakrabarti and Kandror 2011). The ATGL transcript was shown to be downregulated by insulin during refeeding periods, as well as downregulated due to treatment with TNF-± and is

a direct transcriptional target of PPAR^{3} (Kim et al. 2006). Apart from being regulated at the transcriptional level, ATGL is also regulated post-transcriptionally by the lipid protein Comparative Gene Identification 58 (CGI-58). CGI-58 binds to the lipid droplet by interacting with perilipin A when the cell is not stimulated. Once the adipocytes are stimulated, the CGI-58 dissassociates from the lipid surface and perilipin A to enter the cytosol where it interacts with ATGL protein to activate lipolysis (Zechner et al. 2009).

ATGL knockout studies showed that there was a significant reduction of FA released from adipose tissue and increased fat accumulation throughout all tissue in the knockout animal (Zechner et al. 2009). This was contrary to what was seen during HSL-knockout studies which showed a decreased fat mass in those animals. This indicates that ATGL is critical for maintaining FA levels within the body. Also contrary is that total cholesterol and HDL cholesterol concentrations are reduced in ATGL knockouts but raised in HSL knockouts. Human cases of ATGL deficiency have been reported. These cases arise from a mutation within the ATGL gene that leads to the expression of a truncated, but still enzymatically active, protein. Like ATGL knockout mice, these patients present with systemic tryglyceride accumulation along with cardiac myopathy but do not develop insulin resistance (Lass et al. 2011).

SREBP-1c

Sterol Regulatory Binding Protein-1c is a transcription factor that is primarily expressed in the liver, white adipose tissue, adrenal gland and brain (Shimomura et al. 1997). It is primarily responsible for expression of lipogenic genes. Genetic knockout studies showed that SREBP-1c regulates the expression of the lipogenic genes such as glucose-6-phosphate dehydrogenase and glycerol-3-phosphate acyltransferase as well

as cholesterol enzyme Fatty Acid Synthase (Liang et al. 2002). This makes SREBP-1c a key player in cholesterol and fatty acid metabolism, both important to patients with T2D. In obesity and T2D, glucose and free fatty acids are not stored properly in adipose tissue, allowing them to continue in high levels in the blood and store in other tissues.

The balance of metabolic energy is a complex, closely regulated balance between a fasting and fed state. Adipose tissue is the main site for excess energy storage, where in energy excess, triglycerides are built and stored. During energy depletion, these energy stores are broken down into free fatty acids and released into the bloodstream. The switch between fasting and fed state is primarily controlled by insulin levels, which is released in response to blood glucose levels. An increased insulin level signals a fed state whereas a low insulin level is indicative of a fasting state. Insulin was shown to be a direct agonist for SREBP-1c (Azzout-Marniche et al. 2000). The introduction of insulin into cultured cells caused the rapid synthesis of SREBP-1c thereby triggering increased expression of other lipogenic genes needed for energy storage.

Lipid Absorption and Transport by Intestinal Epithelial Cells

The gastrointestinal lumen is the gateway through which dietary fats are absorbed and a physical barrier to the gut microbiome. Because of the importance of the gastrointestinal lumen in nutrient and drug absorption, it is the target of many studies. Due to the fact that lipids are transported through the lymphatic system whereas water soluble compounds are transported through the hepatic system, lipid

transport is an enticing option to increase drug bioavailability by avoiding the first pass effect of the hepatic system (Nauli and Nauli 2013).

Lipids are first absorbed by enterocytes in the gut lumen as part of the nutrient absorption process. The enterocytes then package the lipids into chylomicrons and secrete them into the lymphatic system where they are hydrolyzed and absorbed by other tissues. For this packaging and transport to occur, a well-controlled pathway is triggered to transport the lipids to the Endoplasmic reticulum of the luminal cell where Microsomal Triglyceride transfer protein forms a chylomicron. Figure 2 illustrates the pathway for fatty acid absorption into the luminal cell and chylomicron synthesis in the Endoplasmic Reticulum. Chylomicrons are transported through the cell and exocytosed into the lamina propria and into the lymphatic system. They travel through the lymphatic system eventually entering the bloodstream and are broken down into components by endothelial cells (Dash et al. 2015).

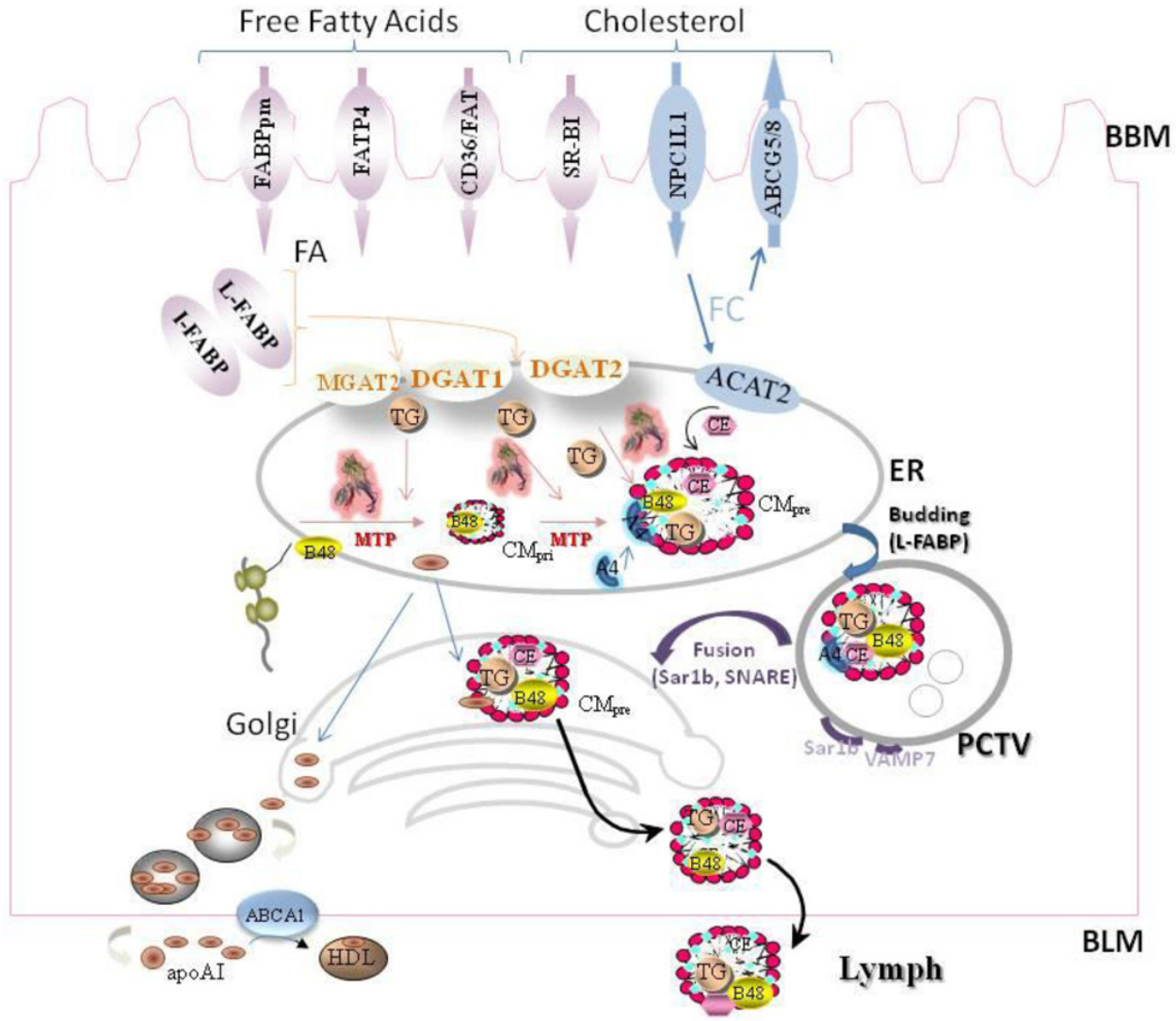


Figure 2: The pathway for fatty acid absorption and chylomicron production is a complex, closely regulated process. Fatty acids and cholesterol are absorbed through the brush border and transported to the ER where MTTP begins the process of synthesizing a chylomicron. The chylomicron is transported to the golgi where it matures and is exported to the lymphatic system. Image from (Pan and Hussain 2012). *Biochim. Biophys.Acta* 1821:727–35 (116)

The gastrointestinal tract has been the target of many studies to understand nutrient absorption and its role in obesity. An effective way to lower plasma triglyceride levels would be to reduce the uptake of them from the gastrointestinal lumen. One such study showed that polyphenol-rich black chokeberry extract has the ability to regulate gene expression, notably reducing SREBP-1c and other lipid metabolism genes in a dose dependent manner in enterocytes (Kim et al. 2013). This study indicates that lipid metabolism and chylomicron production could be altered by a natural extract. Another study was able to show that short chain fatty acids reduced the export of lipids and cholesterol out of the small intestine by reducing the expression of microsomal triglyceride transfer protein (MTTP) (Marcil et al. 2003). Even though it is not clear whether the probiotic extract being studied possesses hypolipidemic qualities, the presence of reduced expression of genes linked to lipid metabolism and chylomicron production would strengthen the argument that probiotics could be an effective alternative treatment for obesity and Type 2 Diabetes

CHAPTER 2

MATERIALS AND METHODS

Isolation of *L. rhamnosus* and preparation of filtered Spent Broth (FSB) and Cell Extract (CE)

L. rhamnosus was previously isolated and identified, using both 16S rRNA sequencing and biochemical testing, from “amabere amaruranu” a Kenyan cultured milk source (Boyiri et al. 2014). The *L. rhamnosus* isolate was cultured in deMan-Rogosa-Sharpe (MRS) broth and incubated at 37^0C for 24 hours until they reached an optical density of 2.0 at 550nm or approximately $1.0x10^9$ cfu/ml. At that time the bacterial broth culture was centrifuged at 1100g for 15 minutes at 4^0C to separate spent broth from live bacteria.

The supernatant was decanted to collect the spent broth. At this point, the spent broth was at a low pH due to the growth of lactic acid bacteria. The spent broth was raised to a pH of 7.4 using NaOH and filtered using a 0.22µm filter. FSB was then used to treat the differentiating adipocytes.

The bacterial pellet was resuspended in 50ml of PBS. The bacterial suspension was sonicated 5 times at 44% amplitude for 2 minutes with 6 minutes of rest. The sonicate was then centrifuged at 1100g for 15 minutes at 4^0C. The supernatant was collected, filtered through a 0.22µm filter, and labelled CE. The CE was used to treat the differentiating adipocytes.

To test for the presence of heat-labile proteins, FSB was heated to produce heat inactivated broth (HIB). To do this, FSB was heated in a water bath at 95ºC for 10 minutes.

Growth of 3T3-L1A Adipocyte Cells

To test the effects of the probiotic on adipogenesis, the bacteria and bacterial extracellular products were tested on an adipocyte 3T3-L1A cell line. The fibroblastic preadipocytes were grown to confluence in Dulbecco's Modified Eagles Medium (DMEM) containing high glucose supplemented with 10% Fetal Bovine Serum (FBS) and 100µg/ml Penicillin/Streptomycin at 37ºC and 5%CO_2. Two days after the cells reached full confluence, a differentiation cocktail of 1µg/ml Insulin, 1µM Dexamethasone, 0.5mM 3-isobutyl-1-methylxanthine (IBMX), and 2µM Rosiglitazone was introduced to the cell growth medium. The cells were incubated for 48 hours in this differentiation medium. After that time, the medium was changed to growth medium containing only 1µg/ml insulin and allowed to incubate 48 hours. After 48 hours in growth medium containing insulin, the cells were incubated for the remainder of the time in fresh growth medium. Media was changed every 48 hours until harvest on day 10.

The cells were harvested using a cell scraper 10 days after differentiation and were lysed using RadioImmuno-Precipitation assay (RIPA) buffer. The cell lysate was sonicated and centrifuged at 12,000 rpm for 20 minutes to separate any unwanted cell fragments from the proteins. The proteins were then collected as the lysate supernatant and stored at -80 for future assays.

Treatment of Differentiating Adipocytes

To test the dose dependent effects of the probiotic treatment, differentiating adipocytes were treated with different concentrations of FSB or CE (10, 25, 50, 75, or 100µl/ml of media) added to the growth media. The treatment was started at differentiation day 0 and continued for 10 days. New treatment was added at every media change. PBS was used as a negative control.

Growth of Caco-2 Cells

Caco-2 cells were grown in DMEM containing high glucose media supplemented with 10% FBS and 100µg/ml Pen/Strep until reaching confluence at 37ºC and 5% CO_2. Once confluent, the cell culture was allowed to continue growth until 14 days after confluence in which the cells had spontaneously differentiated to resemble the lining of the small intestine. At this time the cells were treated with different concentrations of FSB, CE, or PBS control for 24 hours and then harvested using a cell scraper and lysed using RIPA buffer. The cell lysate was sonicated and centrifuged at 12,000 rpm for 20 minutes to separate any unwanted cell fragments from the proteins. The proteins were then collected as the lysate supernatant and stored at -80 for future assays.

Quantification of Lipid Present in Adipocytes by Oil-Red-O Staining

Intracellular lipid accumulation was measured using Oil-Red-O staining. The 3T3-L1A cells were cultured as described above. At the time of differentiation, they were treated with differing concentrations of FSB or PBS as a control. After 10 days the cell plates were collected, growth medium removed and 10% formaldehyde added to the wells for 5 minutes. After 5 minutes, the old formaldehyde was removed and fresh formaldehyde added and allowed to sit for at least 1 hour. After this the formaldehyde

was removed and wells washed with 60% isopropanol and allowed to dry completely. At this point, Oil-Red-O working solution was added to the wells and left to sit for 10 minutes. The Oil-Red-O was removed and distilled H2O was immediately added to the wells. The wells were washed with water until no more red flakes were observed in the waste water and left to dry. At this point the cells were observed under microscopy for appearance of stained lipid droplets in the cells.

To quantify the lipid content in the wells, the Oil-Red-O was eluted by adding 100% isopropanol to the wells and letting it sit for 10 minutes. The isopropanol was then pipetted several times to remove all stain from the wells and transferred to a spectrophotometric cuvette. The stained isopropanol was measured by spectrophotometry for optical density at 500nm.

Western Blot Analysis

3T3-L1A cells were treated with differing concentrations of FSB, CE, or PBS control at time of differentiation and then continuously for 10 days. After 10 days they were harvested and lysed using RIPA buffer and protein content measured using bicinchoninic acid assay (BCA). To ensure uniform concentrations, all samples were diluted down to a protein concentration of 2.0 mg/ml as measured by BCA. Protein was separated by running through 10% SDS-PAGE gel and transferred to PVDF membranes. The membranes were blocked using 5% nonfat milk in Tris-Buffered Saline with Tween-20. They were then incubated with various antibodies against PPAR³, C/EBP±, ATGL, and SREBP-1c (Cell Signaling). Chemiluminescense was developed using the SuperSignal West Femto chemiluminescense kit (Thermo).

Analysis of the effects of FSB on Caco-2 cells was performed by measuring expression of Fatty Acid Synthase (FAS), Microsomal Triglyceride Transfer Protein (MTP), and SREBP-1c. Western blots were run as described above, and incubated against antibodies to these proteins.

Alphaview software was used to quantify all Western blot images. All data was reported in relative intensity compared to control bands.

Statistical Analysis

Statistical Analysis Systems (SAS) software was used to analyze quantitative data. One way ANOVA was run on the quantified western blot data. Individual data points were compiled and represented as Least Square means.

CHAPTER 3

RESULTS

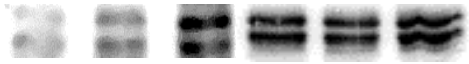
Effects of Probiotic Treatment on 3T3-L1A Cells

To measure the effect of various levels of the probiotic treatment, Western Blot analysis was performed on various transcription factors that are important in regulating adipogenesis and lipid storage in adipocytes. 3T3-L1A cells were treated with either Filtered Spent Broth or Cell Extract of *L. rhamnosus* cultures in varying doses (0, 10, 25, 50, 75, or 100µl/ml) at differentiation and the protein concentration was measured. The values for the Western Blot were quantified by measuring the band intensity relative to the control band. Therefore all values are presented as fold changes compared to the control band.

PPAR³1

PPAR³1 protein expression levels were increased in response to treatment with FSB roughly in a dose dependent manner. Expression levels showed 1.2 to 3.1 fold increase when compared to control levels as shown in Table 1. Table 2 shows the mean expression levels of PPAR³1 when treated with CE. As both Table 2 and Figure 4 show, there was no change in the expression levels of PPAR³1 or PPAR³2 after treatment with CE. The upregulation of protein expression following FSB treatment was a contrast to the no expression change when CE treatment was applied. The Western Blot shows that FSB treatment does induce increased expression of PPAR³1, while CE treatment had no effect.

Figure 3a



0 10 25 50 75 100 µl/ml FSB

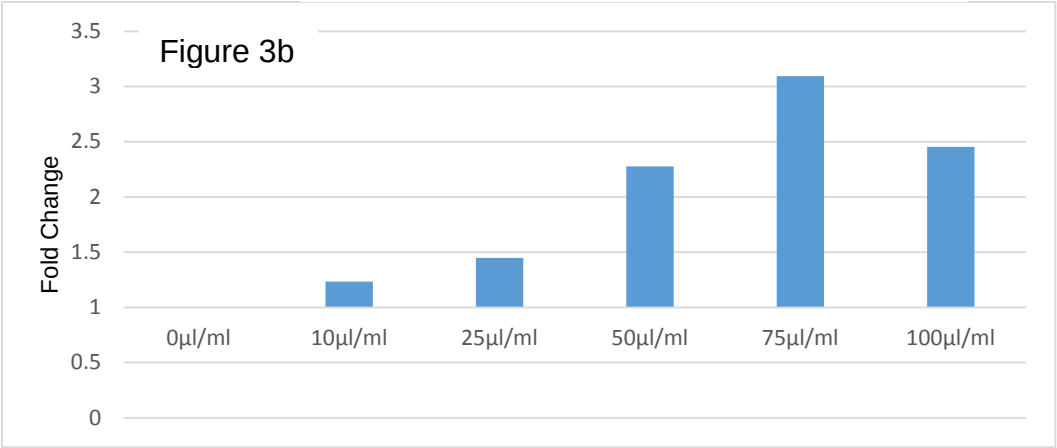


Figure 3: Effect of filtered spent broth (FSB) from a Lactobacillus rhamnosus overnight culture on the protein expression of PPAR³1 in 3T3-L1A adipocytes. Figure 3a is the western blot image. Figure 3b represents the quantification of the protein expression. Expression is presented as fold change relative to the control FSB concentration of 0 µl/ml. Each bar represents mean fold change values. The Y-axis has been set to 1 to normalize the graph to the control.

Table 1: Effect of filtered spent broth (FSB) from a *Lactobacillus rhamnosus* overnight culture on the protein expression of PPAR³1 in 3T3-L1A adipocytes

FSB concentration (µl/ml media)	Relative expression[a]
0µl/ml	1
10µl/ml	1.235
25µl/ml	1.449
50µl/ml	2.276
75µl/ml	3.094
100µl/ml	2.452

[a] Protein expression values are of mean fold change. n=3 to 7. Mean fold changes are of protein expression relative to the positive control.

Figure 4a

0 10 25 50 75 100 µl/ml CE

Figure 4b

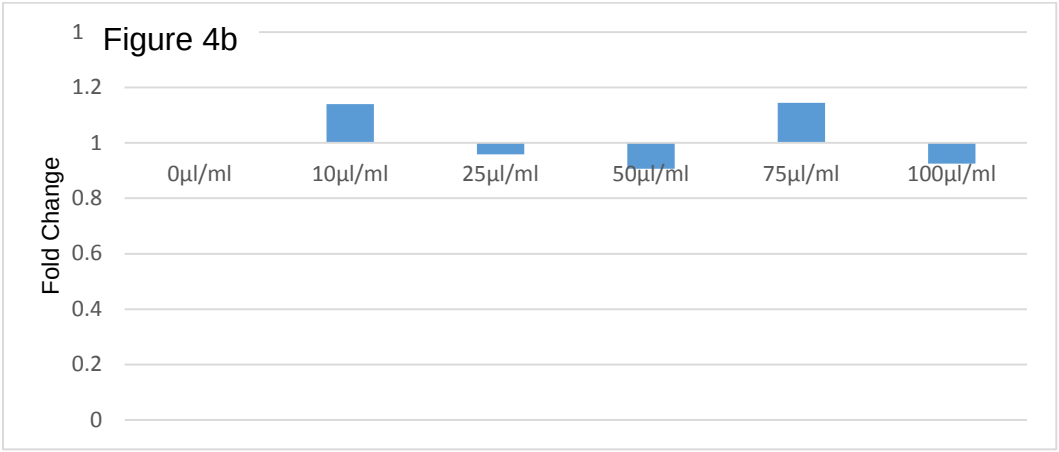


Figure 4: Effect of cell extract (CE) from a Lactobacillus rhamnosus overnight culture on the protein expression of PPAR³1 in 3T3-L1A adipocytes. Figure 4a is the western blot image. Figure 4b represents the quantification of the protein expression. Expression is presented as fold change relative to the control CE concentration of 0µl/ml. Each bar represents mean fold change. The Y-axis has been set to 1 to normalize the graph to the control.

Table 2: Effect of cell extract (CE) from a *Lactobacillus rhamnosus* overnight culture on the protein expression of PPAR³1 in 3T3-L1A adipocytes

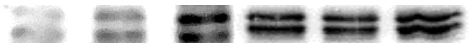
CE concentration (µl/ml media)	Relative expression[a]
0µl/ml	1
10µl/ml	1.14
25µl/ml	0.958
50µl/ml	0.907
75µl/ml	1.144
100µl/ml	0.925

[a] Expression values are mean fold change. n = 1 to 3. Mean fold changes are of protein expression relative to the positive control.

PPAR³2

PPAR³2 expression levels show a 1.2 to 3.0 fold increase in response to treatment with FSB (Table 3 and Figure 5). Table 3 shows that the mean values of the protein expression were almost identical to PPAR³1 levels. Table 4 and Figure 6 show that CE did not affect PPAR³2 expression levels as evident by no change in mean treated values from control. Like PPAR³1, ³2 expression was upregulated by the FSB but not CE which indicates that a component present in the FSB can interact with differentiating adipocytes to increase PPAR³ protein expression.

Figure 5a



0 10 25 50 75 100 µl/ml FSB

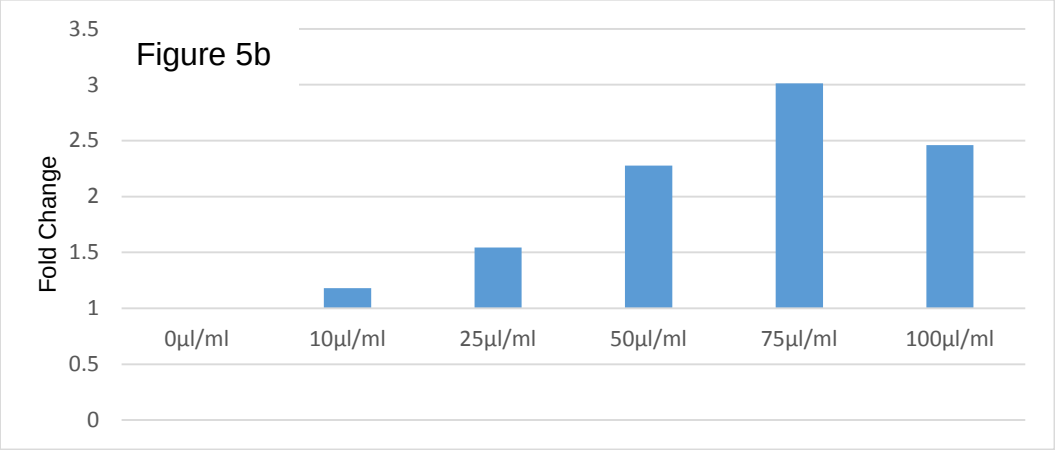


Figure 5: Effect of filtered spent broth (FSB) from a Lactobacillus rhamnosus overnight culture on the protein expression of PPAR³2 in 3T3-L1A adipocytes. Figure 5a is the western blot image. Figure 5b represents the quantification of the protein expression. Expression is presented as fold change relative to the control FSB concentration of 0µl/ml. Each bar represents mean fold changes. The Y-axis has been set to 1 to normalize the graph to the control.

Table 3: Effect of filtered spent broth (FSB) from a *Lactobacillus rhamnosus* overnight culture on the protein expression of PPAR³2 in 3T3-L1A adipocytes

FSB Concentration (µl/ml media)	Relative expression[a]
0µl/ml	1
10µl/ml	1.181
25µl/ml	1.545
50µl/ml	2.276
75µl/ml	3.013
100µl/ml	2.461

[a] Expression values are mean fold change. n = 3 to 7. Mean fold changes are of protein expression relative to the positive control.

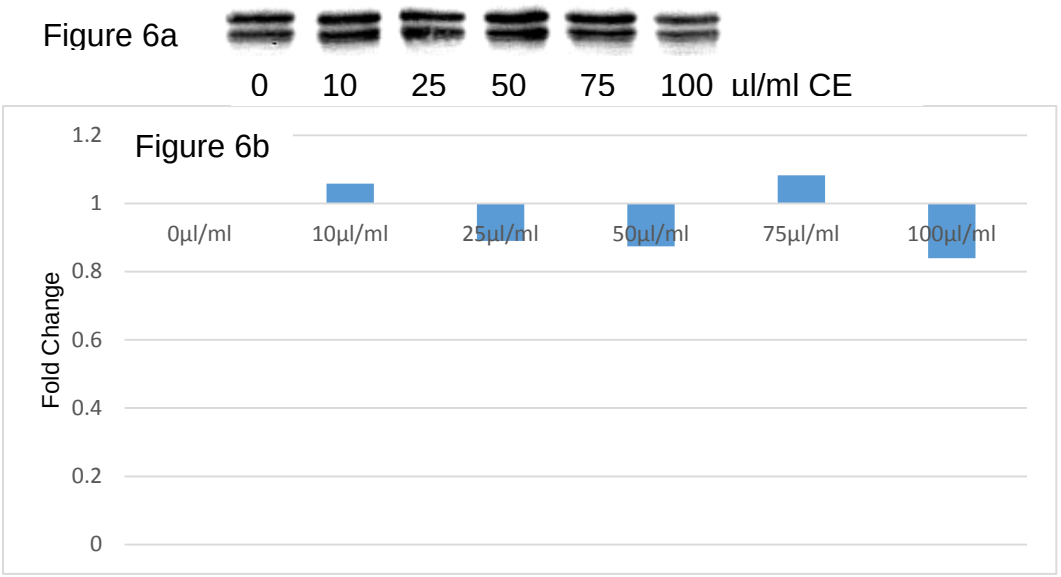


Figure 6: Effect of cell extract (CE) from a Lactobacillus rhamnosus overnight culture on the protein expression of PPAR³2 in 3T3-L1A adipocytes. Figure 6a is the western blot image. Figure 6b represents the quantification of the protein expression. Expression is presented as fold change relative to the control CE concentration of 0 µl/ml. Each bar represents mean fold changes. The Y-axis has been set to 1 to normalize the graph to the control.

Table 4: Effect of cell extract (CE) from a *Lactobacillus rhamnosus* overnight culture on the protein expression of PPAR³2 in 3T3-L1A adipocytes

CE concentration (µl/ml media)	Relative expression[a]
0µl/ml	1
10µl/ml	1.058
25µl/ml	0.89
50µl/ml	0.874
75µl/ml	1.082
100µl/ml	0.839

[a] Expression values are mean fold changes. n = 1 to 3. Mean fold changes are of protein expression relative to the positive control.

C/EBP±

C/EBP± is downstream of PPAR³ and is responsible for many aspects of insulin sensitivity within the adipocyte. The Western Blot data revealed that the FSB treatment did impart some upregulation of this protein expression but not to the extent of the PPAR³ expression. Protein expression was recorded at a 1.3 to 1.9 fold increase due to FSB treatment (Table 5, Figure 7). There was a slight increase in expression with higher doses of CE treatment, specifically a 1.3 fold increase at 100 µl/ml CE treatment (Table 6, Figure 8).

Figure 7a

0 10 25 50 75 100 µl/ml FSB

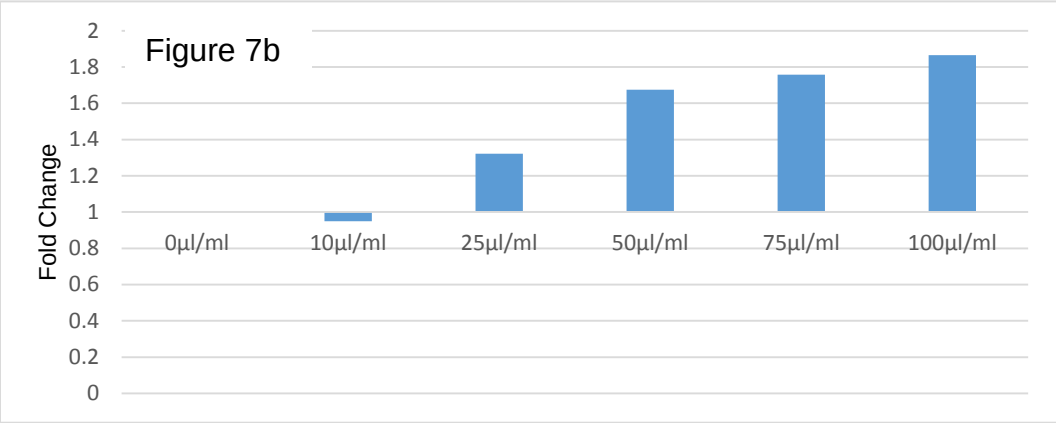


Figure 7: Effect of filtered spent broth (FSB) from a Lactobacillus rhamnosus overnight culture on the protein expression of C/EBP± in 3T3-L1A adipocytes. Figure 7a is the western blot image. Figure 7b represents the quantification of the protein expression. Expression is presented as fold change relative to the control FSB concentration of 0 µl/ml. Each bar represents mean fold change. The Y-axis has been set to 1 to normalize the graph to the control.

Table 5: Effect of filtered spent broth (FSB) from a *Lactobacillus rhamnosus* overnight culture on the protein expression of C/EBP± in 3T3-L1A adipocytes

FSB concentration (µl/ml media)	Relative expression[a]
0µl/ml	1
10µl/ml	0.949
25µl/ml	1.321
50µl/ml	1.674
75µl/ml	1.757
100µl/ml	1.866

[a] Expression values are mean fold changes. n = 1 to 6. Mean fold changes are of protein expression relative to positive control**.**

Figure 8a

0 10 25 50 75 100 µl/ml CE

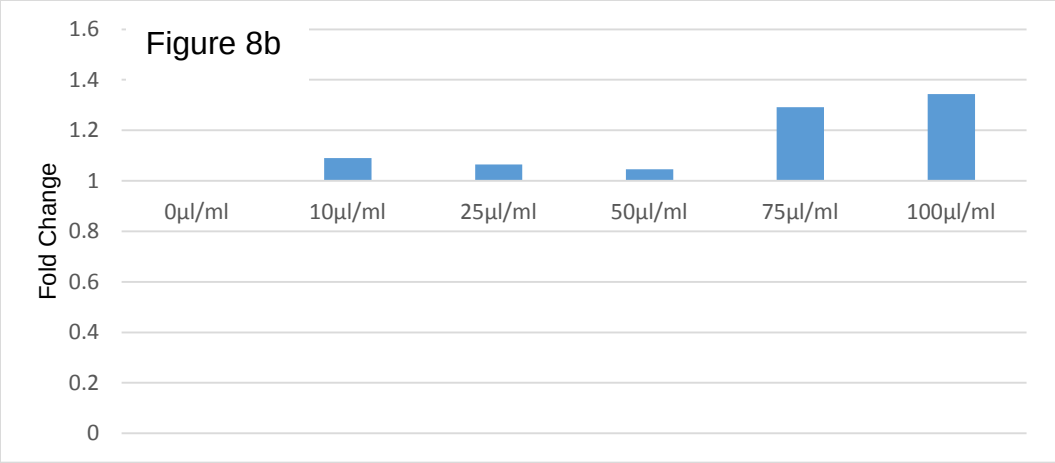


Figure 8: Effect of cell extract (CE) from a Lactobacillus rhamnosus overnight culture on the protein expression of C/EBP± in 3T3-L1A adipocytes. Figure 8a is the western blot image. Figure 8b represents the quantification of the protein expression. Expression is presented as fold change relative to the control CE concentration of 0 µl/ml. Each bar represents mean fold changes. The Y-axis has been set to 1 to normalize the graph to the control.

Table 6: Effect of cell extract (CE) from a *Lactobacillus rhamnosus* overnight culture on the protein expression of C/EBP± in 3T3-L1A adipocytes

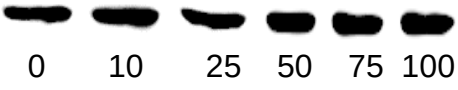
CE concentration (µl/ml media)	Relative expression[a]
0µl/ml	1
10µl/ml	1.09
25µl/ml	1.065
50µl/ml	1.046
75µl/ml	1.291
100µl/ml	1.344

[a] Expression values are mean fold changes. n = 1 to 2. Mean fold changes are of protein expression relative to the positive control.

ATGL

Western Blot analysis revealed that ATGL had the highest increase in protein expression due to FSB, with fold increases ranging from 1.2 to 3.2 times greater than control (Table 7, Figure 9). In contrast, ATGL also showed the lowest decrease in protein expression due to treatment with CE, with expression levels as much as 2 times lower than control values (Table 8, Figure 10). CE treatment was only able to decrease ATGL expression levels.

Figure 9a



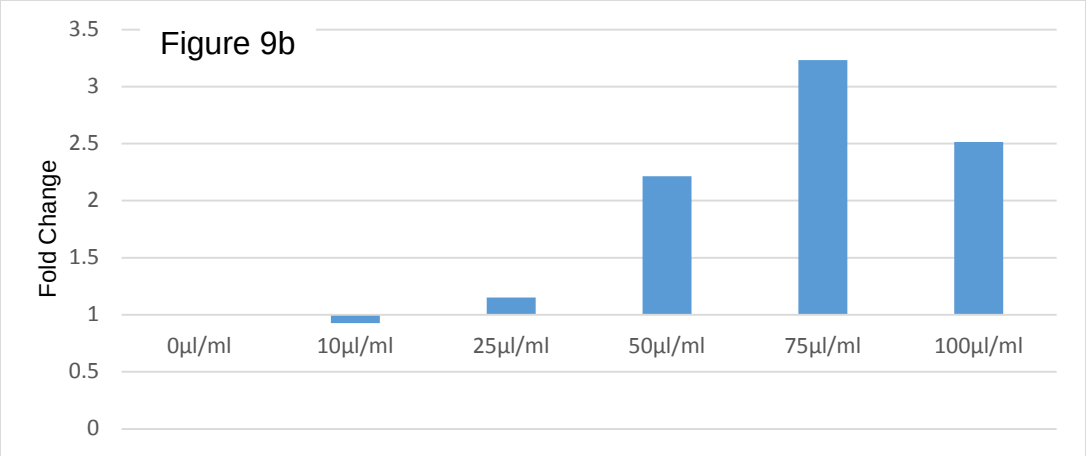


Figure 9: Effect of filtered spent broth (FSB) from a Lactobacillus rhamnosus overnight culture on the expression of ATGL in 3T3-L1A adipocytes. Figure 9a is the western blot image. Figure 9b represents the quantification of the protein expression. Expression is presented as fold change relative to the control FSB concentration of 0 µl/ml. Each bar represents mean fold changes. The Y-axis has been set to 1 to normalize the graph to the control.

Table 7: Effect of filtered spent broth (FSB) from a *Lactobacillus rhamnosus* overnight culture on the expression of ATGL in 3T3-L1A adipocytes

FSB concentration (µl/ml media)	Relative expression[a]
0µl/ml	1
10µl/ml	0.928
25µl/ml	1.151
50µl/ml	2.214
75µl/ml	3.234
100µl/ml	2.516

[a] Expression values are mean fold changes. n = 3 to 8. Mean fold changes are of protein expression relative to the positive control.

Figure 10a

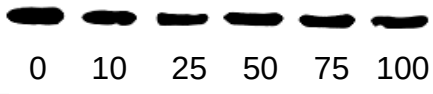


Figure 10b

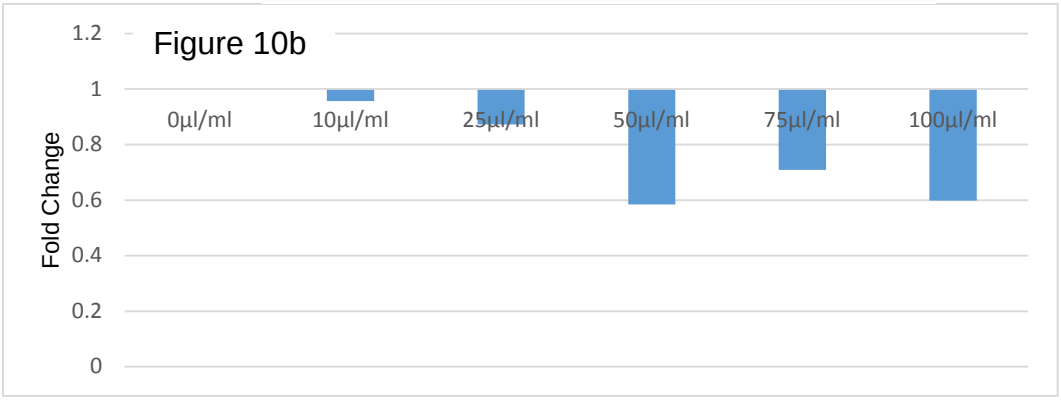


Figure 10: Effect of cell extract (CE) from a Lactobacillus rhamnosus overnight culture on the expression of ATGL in 3T3-L1A adipocytes. Figure 10a is the western blot image. Figure 10b represents the quantification of the protein expression. Expression is presented as fold change relative to the control CE concentration of 0 µl/ml. Each bar represents mean fold changes. The Y-axis has been set to 1 to normalize the graph to the control.

Table 8: Effect of cell extract (CE) from a *Lactobacillus rhamnosus* overnight culture on the expression of ATGL in 3T3-L1A adipocytes

CE concentration (µl/ml media)	Relative expression[a]
0µl/ml	1
10µl/ml	0.957
25µl/ml	0.872
50µl/ml	0.585
75µl/ml	0.709
100µl/ml	0.598

[a] Expression values are mean fold changes. n = 1 to 5. Mean fold changes are of protein expression relative to the positive control.

SREBP-1c

SREBP-1c expression levels were increased by FSB treatments to 1.2 to 1.4 times higher than seen with control treatment (Table 9, Figure 11). Unlike other protein expression levels which showed a dose response curve when plotted, SREBP-1c expression data after FSB treatments created a bell curve when plotted, with the highest expression level coming from 50 µl/ml of FSB treatment. CE treatment increased SREBP-1c expression levels 1.2 to 1.3 times control levels (Table 10, Figure 12). Dose response seemed to be absent with this treatment as all of the different doses except 50 µl/ml increased expression to roughly the same level.

Figure 11a

0 10 25 50 75 100 µl/ml FSB

Figure 11b

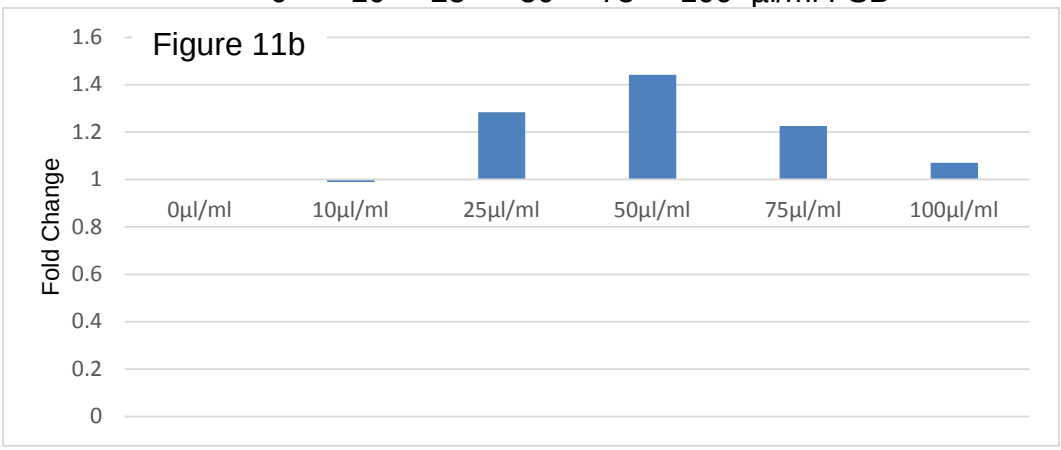


Figure 11: Effect of filtered spent broth (FSB) from a Lactobacillus rhamnosus overnight culture on the expression of SREBP-1c in 3T3-L1A adipocytes. Figure 11a is the western blot image. Figure 11b represents the quantification of the protein expression. Expression is presented as fold change relative to the control FSB concentration of 0 µl/ml. Each bar represents mean fold changes. The Y-axis has been set to 1 to normalize the graph to the control.

Table 9: Effect of filtered spent broth (FSB) from a *Lactobacillus rhamnosus* overnight culture on the expression of SREBP-1c in 3T3-L1A adipocytes

FSB concentration (µl/ml media)	Relative expression[a]
0µl/ml	1
10µl/ml	0.99
25µl/ml	1.284
50µl/ml	1.442
75µl/ml	1.225
100µl/ml	1.07

[a] Expression values are mean fold changes. n = 7 to 15. Mean fold changes are of protein expression relative to the positive control.

Figure 12a

0 10 25 50 75 100 µl/ml FSB

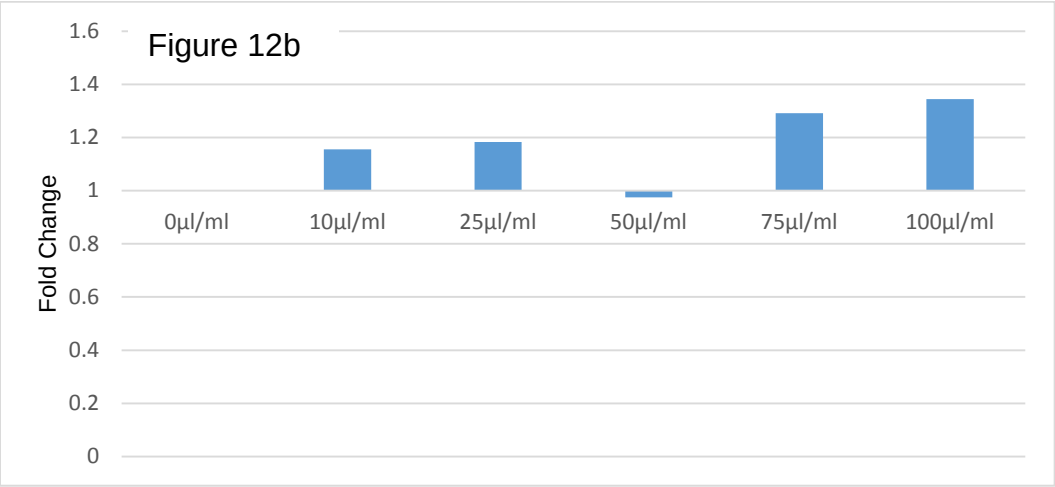


Figure12: Effect of cell extract (CE) from a Lactobacillus rhamnosus overnight culture on the expression of SREBP-1c in 3T3-L1A adipocytes. Figure 12a is the western blot image. Figure 12b represents the quantification of the protein expression. Expression is presented as fold change relative to the control CE concentration of 0µl/ml. Each bar represents mean fold changes. The Y-axis has been set to 1 to normalize the graph to the control.

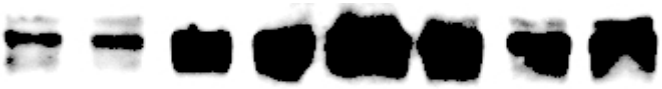
Table 10: Effect of cell extract (CE) from a *Lactobacillus rhamnosus* overnight culture on the expression of SREBP-1c in 3T3-L1A adipocytes

CE concentration (µl/ml media)	Relative expression[a]
0µl/ml	1
10µl/ml	1.155
25µl/ml	1.183
50µl/ml	0.975
75µl/ml	1.291
100µl/ml	1.344

[a] Expression values are mean fold changes. n = 1 to 7. Mean fold changes are of protein expression relative to the positive control.

Comparing PPAR³ Agonistic Ability of Probiotic Extract with Rosiglitazone

The initial Western Blot data suggested that PPAR³, and to a lesser degree C/EBP±, were upregulated by FSB treatment. The next step was to compare the agonistic ability of FSB treatment to a known PPAR agonist, rosiglitazone. Rosiglitazone was being used as a part of the cell culture differentiation treatment to produce high differentiation rates. For this part of the study, rosiglitazone was excluded from media of cells being treated with FSB, thus allowing the comparison of adipogenic protein expression in cells treated with FSB and cells treated with a thiazolidinedione. Table 11 shows the results of the Western blot.

Figure 13 

1 1 3 3 4 4 2 2

Figure 13. Western blot image of comparative FSB and rosiglitazone treatments. Treatment numbering is as follows: 1 = 0 FSB/No Rosiglitazone, 2 = 0 FSB/Rosiglitazone, 3 = 50 FSB/No Rosiglitazone, 4 = 100 FSB/No Rosiglitazone.

Table 11: Comparison of PPAR³ Expression levels from FSB treatment and Rosiglitazone

Treatment	PPARγ Expression
0 FSB/No Rosiglitazone	1
0 FSB/Rosiglitazone	1.75
50 FSB/No Rosiglitazone	2.01
100 FSB/No Rosiglitazone	2.04

Western blot analysis revealed the possible PPAR³ agonistic quality of FSB. FSB produced similar enhanced PPAR³ expression as Rosiglitazone, a known PPAR³ agonist.

Analysis of the cell culture treated with FSB without Rosiglitazone showed that the FSB treatment increased PPAR expression levels from control cells without Rosiglitazone. The analysis also showed that FSB treatment was able to upregulate PPAR expression to levels comparable to cells treated with Rosiglitazone. This indicates that the FSB treatment has comparable PPAR agonistic qualities to that of known PPAR agonists.

Testing Heat Inactivated Broth for Adipogenic Potential

To examine the possibility that the FSB contained a heat labile biomolecule, the FSB was boiled to denature any possible secreted proteins and then used as a cell culture treatment. Western blot analysis of PPAR^{3} and ATGL expression showed that heat inactivated the active component in the FSB treatment. Expression levels for both proteins were reduced to levels below control treatment (Tables 11 and 12).

Figure 14a

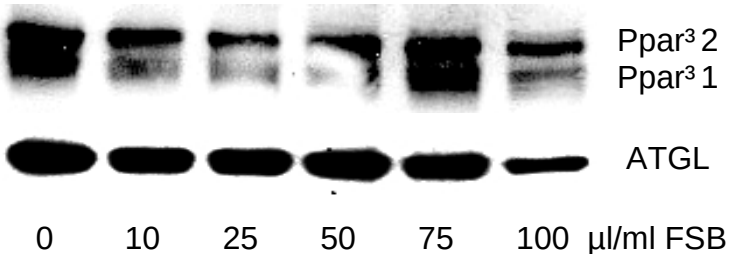


Figure 14a. Western blot image of PPAR^{3} 1 and 2 after HIB treatment.

14b. Western blot image of ATGL after HIB treatment.

Table 12: Effect of heat inactivated broth (HIB) on Expression of PPAR^{3}1, PPAR^{3}2, and ATGL

Treatment	^{3}1 Expression	^{3}2 Expression	ATGL Expression
0µl/ml	1	1	1
10µl/ml	-1.96	-1.68	-1.53
25µl/ml	-3.02	-2.92	-1.46
50µl/ml	-3.38	-2.06	-1.4
75µl/ml	-1.15	-1.52	-1.93
100µl/ml	-2.42	-2.06	-3.11

Table 12 shows the results of PPAR^{3} Western blot analysis on HIB treated cell culture. Expression levels for both ^{3}1 and ^{3}2 under HIB treatment were reduced from observed

values for FSB treatment. Expression of ATGL under HIB treatment was reduced from the observed values for FSB treatment.

Oil-Red-O Staining and Quantification of Lipid Content

An Oil Red O test was performed to measure the lipid content of each well. The data collected indicates that there was a slight rise in lipid content with the 10 and 25µl/ml FSB treatment. A gradual drop in lipid content was observed after 50µl/ml FSB treatment. Cell death due to FSB treatment over 25µl/ml FSB treatment was observed with this experiment. Lower cell density could contribute to the lower than expected lipid content of the cells.

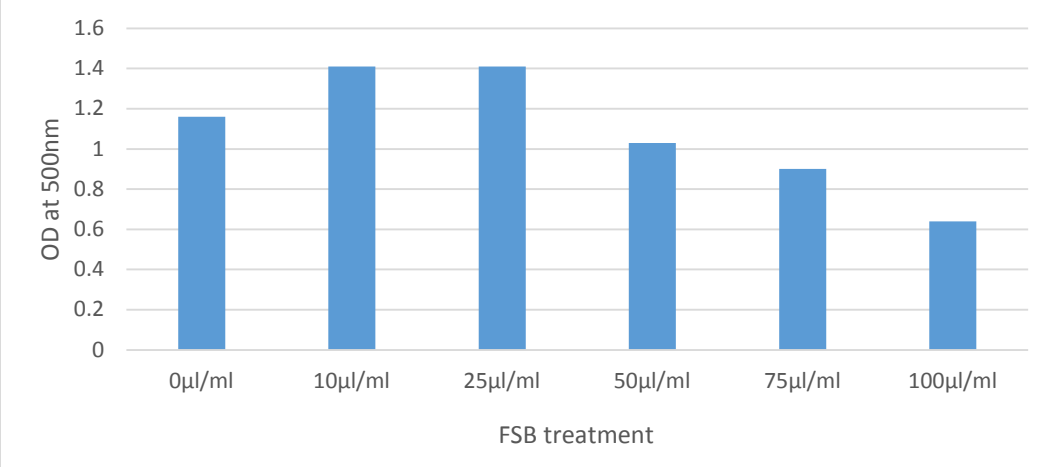


Figure 15: Oil-Red-O (ORO) staining and quantification by spectrophotometry of lipid content of mature 3T3-L1A cells treated with filtered spent broth (FSB) from a Lactobacillus rhamnosus overnight culture. Optical Density (OD) readings were taken at 500nm.

Table 13: Oil-Red-O (ORO) staining and quantification by spectrophotometry of lipid content of mature 3T3-L1A cells treated with filtered spent broth (FSB) from a *Lactobacillus rhamnosus* overnight culture

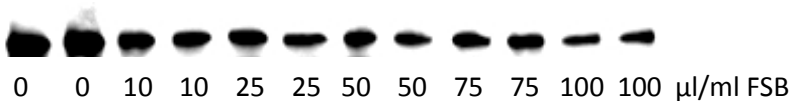
FSB concentration (µl/ml media)	Optical Density Measured at 500nm
0µl/ml	1.16
10µl/ml	1.41
25µl/ml	1.41
50µl/ml	1.03
75µl/ml	0.9
100µl/ml	0.64

ORO lipid content per well for FSB treatment of Cell culture. The optical density measurements were taken via spectrophotometry set to 500nm.

Effect of Filtered Spent Broth (FSB) from a *Lactobacillus rhamnosus* Overnight Culture in Enterocytes

FSB was used to treat Caco-2 cell culture to measure differences in protein expression for transcription factors important in lipid transport. Western blot analysis of SREBP-1c indicated that the FSB treatment did not affect protein expression (Table 14, Figure 16). MTTP protein showed an increased expression of 1.2 fold during the 100 µl/ml FSB treatment (Table 15, Figure 17).

Figure 16a



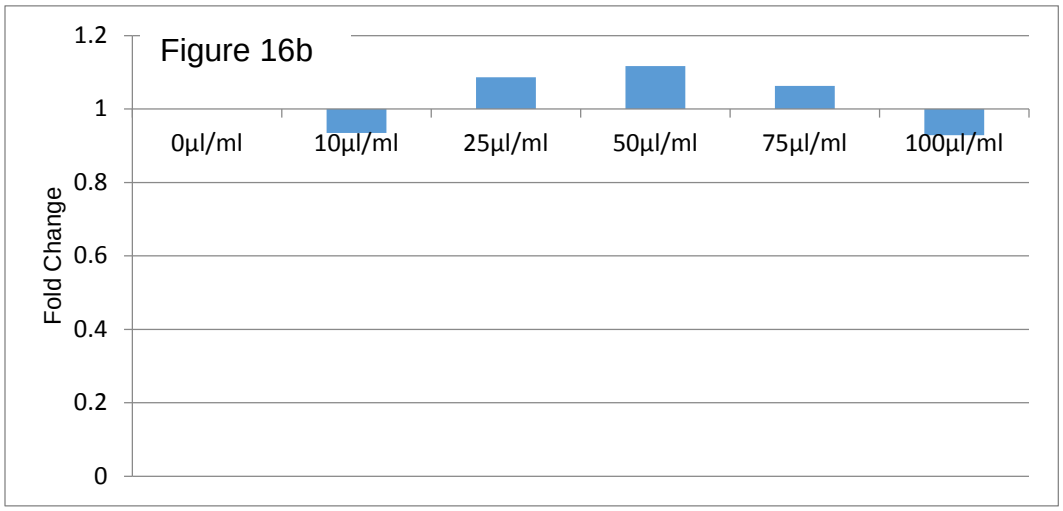


Figure 16: Effect of Filtered Spent Broth (FSB) from a Lactobacillus rhamnosus overnight culture on the expression of SREBP-1c in Caco-2 cells. Figure 16a is the western blot image. Figure 16b represents the quantification of the protein expression. Expression is presented as fold change relative to the control FSB concentration of 0 µl/ml. Each bar represents mean fold changes. The Y-axis has been set to 1 to normalize the graph to the control.

Table 14: Effect of Filtered Spent Broth (FSB) from a *Lactobacillus rhamnosus* overnight culture on the expression of SREBP-1c in Caco-2 cells

FSB concentration (µl/ml media)	Relative expression[a]
0µl/ml	1
10µl/ml	0.935
25µl/ml	1.086
50µl/ml	1.117
75µl/ml	1.063
100µl/ml	0.929

[a] Expression values are mean fold changes. n = 4. Mean fold changes are of protein expression relative to the positive control.

Figure 17a

0 10 25 50 75 100 µl/ml FSB

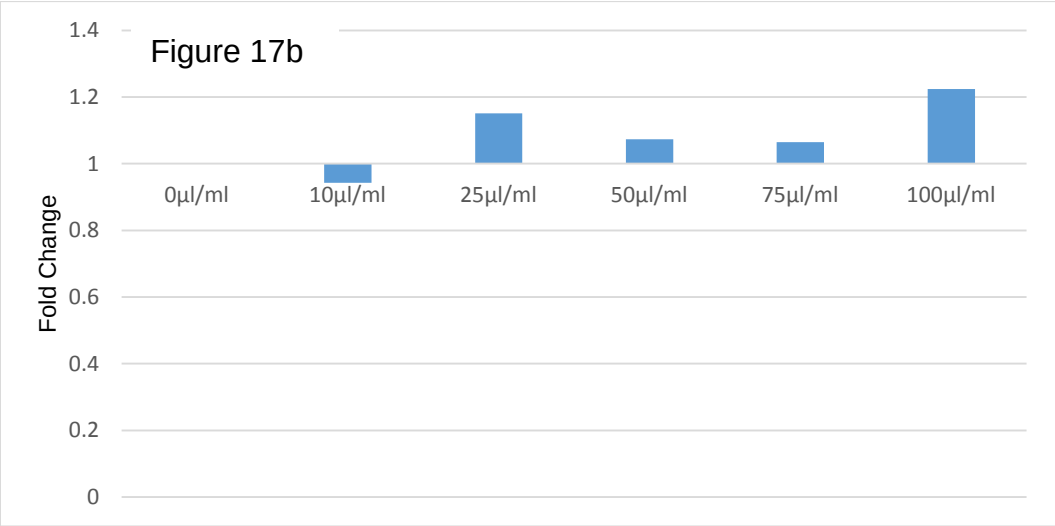


Figure 17: Effect of Filtered Spent Broth (FSB) from a Lactobacillus rhamnosus overnight culture on the expression of MTTP in Caco-2 cells. Figure 17a is the western blot image. Figure 17b represents the quantification of the protein expression. Expression is presented as fold change relative to the control FSB concentration of 0 µl/ml. Each bar represents mean +/- standard deviation. The Y-axis has been set to 1 to normalize the graph to the control.

Table 15: Effect of Filtered Spent Broth (FSB) from a *Lactobacillus rhamnosus* overnight culture on the expression of MTTP in Caco-2 cells

FSB concentration (µl/ml media)	Relative expression[a]
0µl/ml	1
10µl/ml	0.943
25µl/ml	1.151
50µl/ml	1.073
75µl/ml	1.065
100µl/ml	1.224

[a] Expression values are mean fold changes. n = 2. Mean fold changes are of protein expression relative to the positive control.

CHAPTER 4

DISCUSSION

With the obesity epidemic rising, more needs to be understood about the disease and possible new treatments. This study was performed to gain possible insight into the therapeutic uses of probiotics. The study tested a novel probiotic against a cell culture model of adipogenesis. Protein expression changes attributed to probiotic treatment of the cells. Western blot analysis showed that filtered spent broth (FSB) treatment upregulated key adipogenic transcription factors, PPAR³, C/EBP±, and ATGL expression. Contrasting findings have been reported where the adipogenic transcription factors were downregulated by probiotic treatment. Other probiotic anti-obesity studies have given contradictory findings to what was observed in the present study. Moon et al. (2012) showed that cytoplasmic fraction and cell free cultured medium of *Weisella koreensis* lowered lipid accumulation and mRNA expression of key transcription factors in differentiating adipocyte cell culture. Another study used cell extract of *Lactobacillus brevis* OPK-3 to downregulate expression of PPAR³ and C/EBP± (Park et al. 2014). By comparing the findings of this study to previously reported findings, it becomes clearer that different probiotics elicit strain specific responses.

Once the Western blot analysis indicated that there could be PPAR³ agonistic qualities associated with the FSB treatment, the next step was to test the FSB treatment against known PPAR³ agonists. Since rosiglitazone was already being used to produce

high differentiation in the cell culture, it was chosen for the PPAR^{3} agonist. The results indicated that FSB treatment was able to upregulate PPAR^{3} to levels comparable to rosiglitazone. The factor in FSB was inactivated by heat and is likely to be a heat labile protein or biomolecule. This is important in the search for new anti-diabetic drugs. Rosiglitazone is a potent drug against insulin resistance associated with Type 2 Diabetes (Gerstein et al. 2006). The drawback is that this drug can cause severe side effects. It is possible that the compound in the FSB treatment could be used to replace rosiglitazone, or a probiotic regimen could be used in conjunction with rosiglitazone to improve effectiveness while lowering drug dosage.

The gastrointestinal lumen stands as the physical barrier between the body and the gut microbiota. Studies by Kim (Kim et al. 2013) and Marcil (Marcil et al. 2003) have shown that the microbiome and biomolecules are able to interact with the gastric lumen to alter protein expression. This interaction is not well understood, but it could have an impact on how lipids are transported and stored. In the present study, FSB did not affect the expression of proteins SREBP-1c and MTTP that are known to play important roles in lipid transport. However, some probiotics have been shown to elicit strain specific responses of the gastrointestinal epithelial cells. In the study by Putaala et al. (2010), cell to cell adhesion, superoxide metabolism, and MAPK pathway were all regulated differently by the various probiotic bacteria.

The Oil-Red-O (ORO) staining in the present study showed that lipid content rose with the 10 and 25 µl/ml and then dropped with higher FSB doses. 50 µl/ml and above FSB treatments were observed to cause cell death during the ORO study. One drawback with ORO is that it only measures the amount of lipid content in each well

instead of measuring lipid content within each cell. Since there was cell death involved with some batches of the FSB treatment, this could have led to lower total lipid content readings.

An interesting observation during this study was that certain FSB treatments seemed to cause increased cell culture detachment and death. This was not observed with the first batch of FSB treatment made, but in some subsequent batches cell death was observed. Fresh MRS broth treatment was explored as a possible control treatment for FSB, but it was determined that MRS caused high cell death among the cells being cultured. It is possible that during some of the FSB preparations, the spectrophotometer that was used to measure optical density (OD) reading was not set to the same wavelength as previous batches. This could have given a premature reading of 2.0 OD leading to shortened growth times for some FSB treatments. Fresh MRS broth and FSB that had a shortened bacterial growth time seemed to produce high cell death when used as cell culture treatment. This observation led to the examination of the ingredients that make up MRS broth. When looking at the ingredients listed in MRS broth, one ingredient stood out as being a possible culprit for the cell death. Sodium acetate is included in MRS broth as both an energy source to the bacteria and a selective agent to prevent certain other organisms from growing in the broth. Sodium acetate has been shown to have cytotoxic effects in high doses (Sun et al. 2005). Normally the *L. rhamnosus* isolate would use up the sodium acetate as an energy source, but due to shorter incubation times for some of the FSB treatment batches, the bacteria did not have the time to break down the sodium acetate present in the broth. This could explain the decrease in cell viability associated with some of the FSB

batches with shorter bacterial incubation periods, as well as explain the high cytotoxicity of fresh MRS broth. It would be beneficial to mix an MRS broth without sodium acetate to see if the probiotic bacteria will grow, and if the broth causes high cell culture death.

Cell death caused by possible sodium acetate cytotoxicity could explain irregularities in the Oil-Red-O (ORO) results. Quantification of ORO actually measures lipid content per cell culture well instead of lipid content per cell culture cell. As the FSB treatment levels increased higher cell death was observed. This means that the 75 and 100µl/ml treatments induced higher cell death than the control. The resultant difference in cell numbers would mean that the high treatment wells had lower density of cells contributing to lower lipid content readings. Another possibility is that the cells were stressed by the cytotoxic compound. This stress could have hampered the adipocyte's ability to store lipids. The higher FSB treatments would have put higher stress on the cells, thereby giving the effect of lower lipid content with higher FSB treatment.

A recent study showed that *Lactobacillus rhamnosus* secretes proteins into surrounding media as the bacteria grows (Sanchez et al. 2009). The protein expression changes caused by FSB treatment with no changes seen from the CE treatment suggest that this isolate secretes a possible biomolecule into the surrounding environment that are cause these changes. Inactivation of the FSB by heat suggests that this biomolecule is heat labile. The source that was used to isolate the potential probiotic tested in this study was by all accounts, novel, and this specific *L. rhamnosus* strain could have peculiar characteristics, perhaps contradictory to other previously isolated strains.

At first thought, the idea of reducing adipogenesis may seem like the correct way to battle the continuing obesity epidemic. The problem with this idea is that obesity is considered a state of energy excess, whereby caloric intake is higher than energy expenditure. If adipogenesis is reduced without fixing the energy imbalance then the excess glucose and fatty acids will continue to build in the blood and be forced to be stored in the liver and other tissues. This can cause hepatomegaly, fatty liver disease, hyperlipidemia and other diseases characterized by increased circulating or improperly stored lipids (Rosen et al. 2000). The more appropriate approach is to increase adipogenesis to allow for proper clearance of circulating lipids, while at the same time addressing the energy imbalance. This study was designed to test the ability of a novel probiotic milk isolate *L. rhamnosus* to influence adipogenesis in vitro. Indeed, biomolecules present in spent culture from this isolate were able to increase adipogenesis when administered to differentiating adipocytes.

In summary, an overnight filtered spent broth (FSB) culture of a Lactobacillus rhamnosus isolate from “amabere amaruranu”, Kenyan traditional cultured milk preparation upregulated , PPAR³ , C/EBP±, and ATGL adipogenic transcription factors. The active ingredient in the FSB seemed to be heat-labile in nature. Finally, because the FSB shared similar PPAR³ agonistic ability as rosiglitazone, a diabetic drug, there might be a possibility of use of this biomolecule in development of new therapies.

Future research to define the limits and potential of any treatment with this probiotic needs to be conducted. The biomolecule that causes the observed changes in protein expression level and differentiation needs to be isolated and identified. This biomolecule could be very important in finding novel treatments for type 2 diabetes by

its potential to upregulate PPAR^{3} similar to current treatments. The probiotic itself should be tested in an animal model of obesity to further investigate the efficacy of a probiotic approach to obesity treatment. If able to be isolated, the biomolecule will have to go through cell culture testing to show that this compound causes the same effects as observed in this study, proving that it is the active agent in the FSB treatment shown in the present study.

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