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Effects of Lactobacillus rhamnosus MRS6AN on Intestinal GPAT3 pathway involved in the lipid transport in enterocytes

Ayodeji Oludare Ipinmoroti
East Tennessee State University

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by

Ayodeji Oludare Ipinmoroti

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Edward M. Onyango, PhD, Chair

Ranjan Chakraborty, PhD

Lu Yongke, PhD

Victoria Palau, PhD

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ABSTRACT

Effects of *Lactobacillus rhamnosus* MRS6AN on Intestinal GPAT3 Pathway Involved in the Lipid Transport in Enterocytes

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Ayodeji O. Ipinmoroti

Accumulation of triglyceride (TG) in enterocytes of the small intestine follows two specific absorption pathways: the monoacylglycerol acyltransferase pathway (MGAT) and glycerol acyltransferase pathway (GPAT), the latter (GPAT) is usually found in the small intestine. In this study, we investigated the effects of *Lactobacillus rhamnosus* MRS6AN, an isolate from "amabere amaruranu" a Kenyan traditional cultured milk, on triglyceride accumulation and expression of GPAT3, I-FABP, *MTP* and NPC1L1 in Caco-2 cell enterocyte model. Intracellular triglyceride level (TG) of Caco2 cells was significantly reduced by live *L. rhamnosus* (LB) compared with other bacterial products. *MTP* expression in Caco2 cells was minimally reduced in live *L. rhamnosus* (LB) treated Caco2 cells. However, the expression level GPAT3, I-FABP and NPC1L1 was reduced in Caco2 cells treated with live bacteria. Data from this study suggests that *Lactobacillus rhamnosus* MRS6AN may reduce lipid uptake and accumulation perhaps via modulation of GPAT3 pathway.

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DEDICATION

I dedicate this thesis to God Almighty who has given me the privilege to achieve this much and have also seen me thus far.

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CHAPTER 1

BACKGROUND

Human survival depends on its environment and organisms with which it interacts (Chivian 2010) and its adaptability is slower compared to that of other smaller organisms, such as viruses and bacteria that are characterized by faster and more efficient rate of metabolism (Resta 2009), therefore for a more contestable adaptability and metabolism, human and other complex organisms have established symbiotic relationship as a mutual way to overcome metabolic and genetic related deficiencies (Gerritsen 2011). This mutual relationship which has systematically become a lifestyle has proven to be of long-term advantage and its being said that the community constituted by these complex organisms have become thehuman's largest organ known as the gut microbiota (Resta, 2009) and (Gerritsen 2011). The concept of ingesting nonpathogenic and beneficial live microorganisms to improve the gastrointestinal health status and incur other health benefits has been traced to as far back as 20th century and ever since then until now it's been a tireless effort trying to obtain and select effective strains good enough to be 'probiotic' (Maheshwari et al. 2012). Before 2001, no definition was really given to describe the term probiotics (Matur and Eraslan 2012). The term "probiotics" was first introduced and defined in 1953 by Werner Kollath as microbial derived factors that stimulate the growth of other microorganisms (Maheshwari et al. 2012), in 1995 Roy fuller came up with his own definition of probiotics as a live feed supplement which beneficially affects the host, thereby improving its intestinal microbial homeostasis (Fuller et al. 1995). But as the quest towards the discovery of an alternative and more potent intestinal health breakthrough grew stronger, various governmental organizations agreed to work collectively in other to establish effective principles and guidelines for evaluation and selection of probiotics in food (Saran et al. 2011). Probiotics in its long-term definition are live microorganisms including bacteria and yeasts which when ingested in adequate dosage

confers health benefits to the digestive system as well as gastrointestinal tract (Kechagia et al. 2013). The most commonly used and predominant genera are the *Bifidobacterium* and *Lactobacillus* (Cani et al. 2007). Probiotic potentials of these beneficial microorganisms depend largely on its reproducibility and stability which are important factors for survival within the gastrointestinal tract (Boyiri and Onyango 2014). Their sources are many; they are abundant in vegetables, dairy products, meats, human body, and pasteurized milk products among others.

The GIT

The Human gastrointestinal tract known as the alimentary canal is a long tube of about 9 meters in length, with two opening ends at the mouth and the anus (Iqbal and Hussain 2009). The small intestine which is longest and the most complex part spanning about 7 meters in length and 2 to 3 meters in diameter is composed of various parts which include the mucosa, sub-mucosa, a single epithelium layer facing the lumen among others (Phan and Tso 2001). A large chunk of nutrient absorption takes place in the small intestine, this is due to its highly-differentiated structure (Iqbal and Hussain 2009). This structure could be understood by examining it in terms of crypt of Lieberkühn where constant regeneration and replacement of worn out intestinal cells occur (Iqbal and Hussain 2009). On the surface of intestinal wall are fingerlike protrusions known as villi, they are responsible for uptake of nutrients (Tso and Crissinger 2000). The villi extend from the proximal region (made up of the duodenum and jejunum and are responsible for chyme absorption) to distal ileum where gastrointestinal hormones are released and bile salts recycled (Tso and Crissinger 2000). Another important part of the GIT is the apical region of the intestinal wall, a membrane where nutrients taken up by absorptive cells are metabolized and secreted across the basolateral membrane into the lymphatic vessel (Phan and Tso 2001).

Physiology of Enterocytes

Enterocytes are type of cells that absorb water and nutrients from the digestive tract. It is a columnar cell (tall and narrow in shape), with a "brush" border made of tiny protrusions called 'microvillus' which project into the space inside the gut (Stanfield 2016). Enterocytes originate inside intestinal glands, or crypts, in the small intestine and colon (Tso and Crissinger 2000). In the small intestine, enterocytes produce and secrete digestive enzymes, which bind to their brush borders and help break down sugars and proteins, making them smaller and easier to absorb (Tso and Crissinger 2000). Enterocytes in the large intestine absorb water and electrolytes (Shen Q X et al 2005). Most absorption of nutrients and digestion take place in the large intestine, the surface complexity in addition to other characteristics account for its unique adaptive features (Stanfield 2016) and (Tso and Crissinger 2000).

Probiotics and Gastrointestinal Health

One of gastrointestinal motility or the kinetics of its content is also an important variation in the maintenance of gastrointestinal homeostasis, and abnormality in motility can bring about discomfort varying from constipation to diarrhea (Kleessen et al. 1997). The interest in probiotics with respect to their motility regulatory roles is "making waves" in the treatment GI disorder (Huang et al 2004). Probiotics have been demonstrated to play a critical role in influencing gastrointestinal motility (Kleessen et al. 1997). Probiotic therapy of *Lactobacillus acidophilus* appear to significantly improve and restore impairment of interstitial cells of cajal caused by traumatic brain injury (TBI) (Sun 2015). Administration of *L. acidophilus* also improved contractile activities such as enhancement of smooth intestinal muscle movement, and prevention of MLCK dependent ML_{20} phosphorylation and MYPTI/MLCP dependent ML_{20} phosphorylation inhibition by TBI (Sun 2015). The idea of making use of these beneficial microorganisms

in modulating the microbial ecosystem (microbiome) from an abnormal or disease condition to a healthy state despite the present limited measure available for the treatment of gastrointestinal diseases is enticing. Studies have demonstrated the influence of probiotic isolates in the treatment of gastrointestinal diseases (Kleessen et al. 1997). The study involving the use of probiotics therapy conducted by Verdu et al. (2009) demonstrated the effect of *L. rhamnosus* R0011 and *L. helveticus* R0052 on upper gastrointestinal tract dysfunction caused by protracted and chronic *Helicobacter pylori* infection. It was noted that mice infected with *H. pylori* for 4-6 months could not restore normal gastric functions even at 2 weeks-post eradication of *H. pylori,* but gastric functions soon became normal and regular after administration of *L. rhamnosus* and *L. helveticus* (Verdu et al. 2009). In addition, probiotics oral administration at 2-week post eradication of *H. pylori* restored normal feeding behavior (Verdu et al. 2009). In support of the various evidences showing the inhibitory role of probiotics in gastrointestinal diseases, Marshall-Jones in 2006 concluded that probiotic consumption by healthy cats altered their gastrointestinal microflora by inhibiting the growth of *Clostridium* sp and *Enterococcus faecalis* (Marshall-Jones 2006). Early research studies on probiotics were mainly based on the treatment of gastrointestinal disorders such as diarrhea (Kleessen et al. 1997). Probiotic inhibition of *E. coli* induced diarrhea has been shown to result when a combination of strains of *L. gasseri, L. acidophilus* and *L. fermentum* were isolated from healthy weaning pigs after 10^8cfu/ml of *E. coli* was orally administered. Huang and colleagues in 2004 concluded that diarrhea incidence was significantly decreased by reduction in *E. coli* count with a corresponding significant increase in the population of lactic acid bacteria (*Lactobacilli*) and anaerobes, indicating susceptibility of *E. coli* infection to presence of *Lactobacilli* sp (Huang et al. 2004). Meta-analysis of various clinical trials and procedures involving treatment of diarrhea using rehydration therapy has proven the use probiotics to be a potent measure for the treatment of diarrhea patients

(Oberhelman et al. 1999). For example, stool consistency can be improved through feeding on *Bifidobacterium* cultured milk, a laxative effect that requires large masses of the probiotic genera (Ishibashi et al. 1996). Some *Bifidobacteria* strains have been demonstrated to possess anticarcinogenic, antimutagenic and antitumorigenic properties (Nagpal 2012). Some studies involving the use of laboratory animals have buttressed the influence of probiotic genera like *Lactobacilli* and *Bifidobacterium* on the suppression of tumorigenesis. In a study conducted in 1993, it was shown that strains of *B. longnum* suppressed azomethane-induced colon carcinogenesis in rats (Reddy and Rivenson 1993). Another study carried out by Singh and colleagues documented that this same species of *Bifidobacterium* also reduced carcinogenesis caused by food mutagen, 2-amino-3-methylimidazo[4,5-*f*] quinoline (Singh et al. 1997).

Probiotic Role in Immune Functions

Certain strains of *Bifidobacterium* and *Lactobacilli* within the human GIT induce enhanced immune function by stimulating both innate and adaptive immune system as well as activation of specific antibodies to invading pathogens (Valeur et al. 2004). Although this has been demonstrated mainly in mice models, human studies are now been carried out to justify claims. Certain strain of Lactobacilli has been shown to increase secretory IgA level and activation of rotavirus specific antibodies when ingested (Phuapradit et al. 1999). These gut-associated microorganisms interact with a variety of cells involved in immune responses; T lymphocytes, T-helper and regulatory cells, dendritic cells among others where they modulate anti-inflammatory immune responses, gut development and integrity (Mercola 2008) and (Galdeano et al. 2008). According to Perdigon et al. (1995), probiotics selected for their ability to influence immune responses via immunomodulatory and immunoregulatory pathway could stimulate cytokine

expression and significantly reduce the level of serum C-reactive proteins (CRP) (Perdigon et al. 1995). Probiotic *Lactobacillus reuteri* orally administered could increase immune cell population such as CD4 positive cells (Valeur et al. 2004). Biopsy samples from stomach, duodenum and ileum obtained from 9 healthy human subjects has shown the colonization of the gastrointestinal tract by endogenous *L. reuteri* which led to an increase in the population of CD-positive T-Lymphocytes in the ileal epithelium (Valeur et al. 2004). *Invitro* study of the probiotic influence of *Enterococcus faecium* on immune cell recruitment and epithelia integrity revealed that porcine and human intestinal cells (Caco-2) exhibit low trans-epithelial resistance, increased mRNA level and protein expression of IL-8 when predisposed to enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC). However, reduced trans-epithelial resistance was inhibited and decreased proinflammatory stress cell responses were observed when EHEC and or ETEC were co-incubated with *E. faecium* (Shanti 2014).

Another immune system related function exerted by probiotics include ability to reverse the state of metabolic dysbiosis and restoration and maintenance of immune homeostasis (Schriffin & Blum 2002). Probiotic strains *Lactobacillus casei and Lactobacillus rhamnsosus* GG have been demonstrated to express certain surface exopolysaccharides and surface glycoproteins which interact with different immune sensors such as TLRs and NODs expressed on the surface of intestinal epithelia. These structures recognize certain molecular associated molecular patterns (MAMPs). This interaction may induce a signaling cascade that initiates innate immune responses, such as expression of anti-microbial factors, antigen presentation, pro and anti- inflammatory cytokine production (Claes et al. 2012) and (Baarlen et al. 2013).

Lipid Absorption in Enterocytes

Western diet lipids are primarily made up of phospholipids, free cholesterol, triglycerides which eventually constitute 90% of overall dietary lipid consumed. (Tso and Crissinger 2000).

An important phase of fat digestion and absorption is bile salt which emulsify the fats allowing them to form solution as micelles in chyme, and so increase the surface area on which the pancreatic lipases can operate (Figure 1). Micelles are small, and because they have the hydrophilic side externally, they effectively allow the fats to act like water soluble particles. This allows them to penetrate the unstirred water layer adjacent to the small intestine epithelium which represents an important barrier for lipid uptake by enterocytes (Tso and Crissinger 2000). In the absence of bile salts, a biological detergent that allows the formation of mixed micellar structure with lipolysis products, very few fatty acids make it through this unstirred water layer and much of the fat will pass through the gut undigested and unabsorbed causing steatorrhea (fatty stool) (Arthur and Guyton 1975). Bile salts significantly increase the resulting lipid product's concentration which is close to the brush border membrane for effective absorption (Tso and Crissinger 2000) and (Tso et al. 2004). The micelles enable the fatty acids and cholesterol to cross the unstirred layer and get in contact with the brush border, where they easily cross the fat-soluble cell membrane. A few smaller free fatty acids transfuse across the cell and out at the baso-lateral border, passing into the capillaries. However most fatty acids enter the smooth endoplasmic reticulum where they are repackaged into chylomicrons. These are excreted off the cell by exocytosis (Shen Q X et al. 2005).

Digestion and absorption of dietary lipid and other nutrients take place within the first 20% of the small intestinal region. Several enzymes associated with dietary lipid uptake and re- esterification are

dominantly expressed, especially, in the mid-region of the small intestine where lipid absorption takes place (Cao et al. 2004) and (Storch et al. 2010).

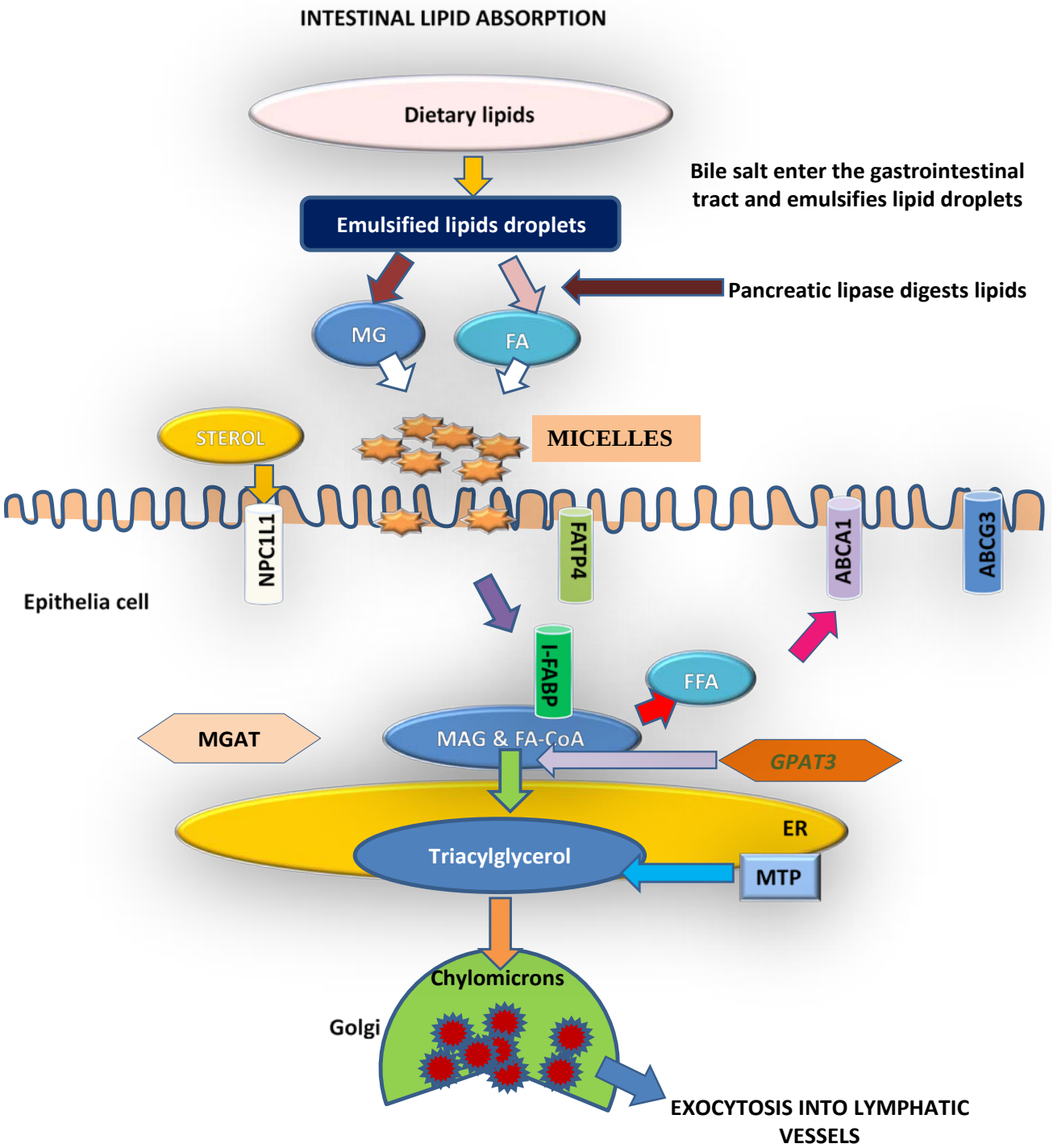

Figure 1. Dietary lipid absorption pathway showing lipid transport in enterocyte.

TAG Synthesis

Dietary lipids are either absorbed as free fatty acids (FA) and *sn*-2 monoacylglycerol (sn-2-MG) esterified by monoglycerol-acyltransferase (Ahn 2010) or as esterified *sn*-1 glycerol phosphate and fatty acyl-CoA by glycerol phosphate-acyltransferase (Shan et al. 2010). Two pathways had been reportedly involved in the re-esterification of triglycerides, the glycerol-3-phosphate acyltransferase pathway and the monoacylglycerol acyltransferase (MGAT) pathway (Figure 2). Nevertheless, synthesis of TG in the small intestine is primarily through the MGAT pathway (Tso and Crissinger 2000). The present study will focus mainly on the expression five predominant proteins and transporters that have been known to play a critical role in lipid uptake and accumulation by intestinal cells. A recently characterized GPAT3 has been also shown to be involved in maintaining lipid homeostasis in the gastrointestinal tract (Khatun et al. 2015).

Khatun et al. (2015) investigated the role of a novel intestinal glycerol-3-phoshate acyltransferase pathway in dietary lipid homeostasis within the small intestine. Previous studies have documented a vital role played by GPAT3 in the synthesis of TAG in adipocytes (Cao 2006). GPAT3 has also been found to be abundantly expressed and readily detectable in the small intestine especially in the jejunum (Khatun et al. 2015). When dietary triglycerides are absorbed by enterocytes they undergo luminal hydrolysis into monoglycerides and fatty acids after which they are secreted as chylomicrons which transport then through lymphatic vessels of the lamina propria of the basolateral surface of the enterocyte (Hussain 2014). This process is carried out through two main routes namely Glycerol-3-phosphate and Monoacylglycerol pathway, G3P is acylated by a member of G3P acyltransferase and is further acylated and dephosphorylated to produce diacylglyerol (Kennedy and Weiss 1956). Khatun et al. (2015) reported that GPAT3 was abundantly expressed in the apical surface of enterocytes of small intestine, and that

GPAT3 knockout mice exhibited lipid accumulation in enterocytes and they displayed modest elevation in the level of enteric FFA (Khatun et al. 2015). Although detail analysis of subcellular localization of GPAT3 expression was hypothesized, its targeted disruption was however found to result in dysregulation of TG secretion from the small intestine (Shan et al. 2010). Understanding the rudiments guiding the absorption of nutrients and handling of dietary lipids in the gastrointestinal tract is the target of much of the ongoing research. This present study is designed to investigate the effects of *Lactobacillus rhamnosus,* a probiotic isolate from a Kenyan locally cultured milk product on the expression of genes involved in lipid homeostasis in enterocytes.

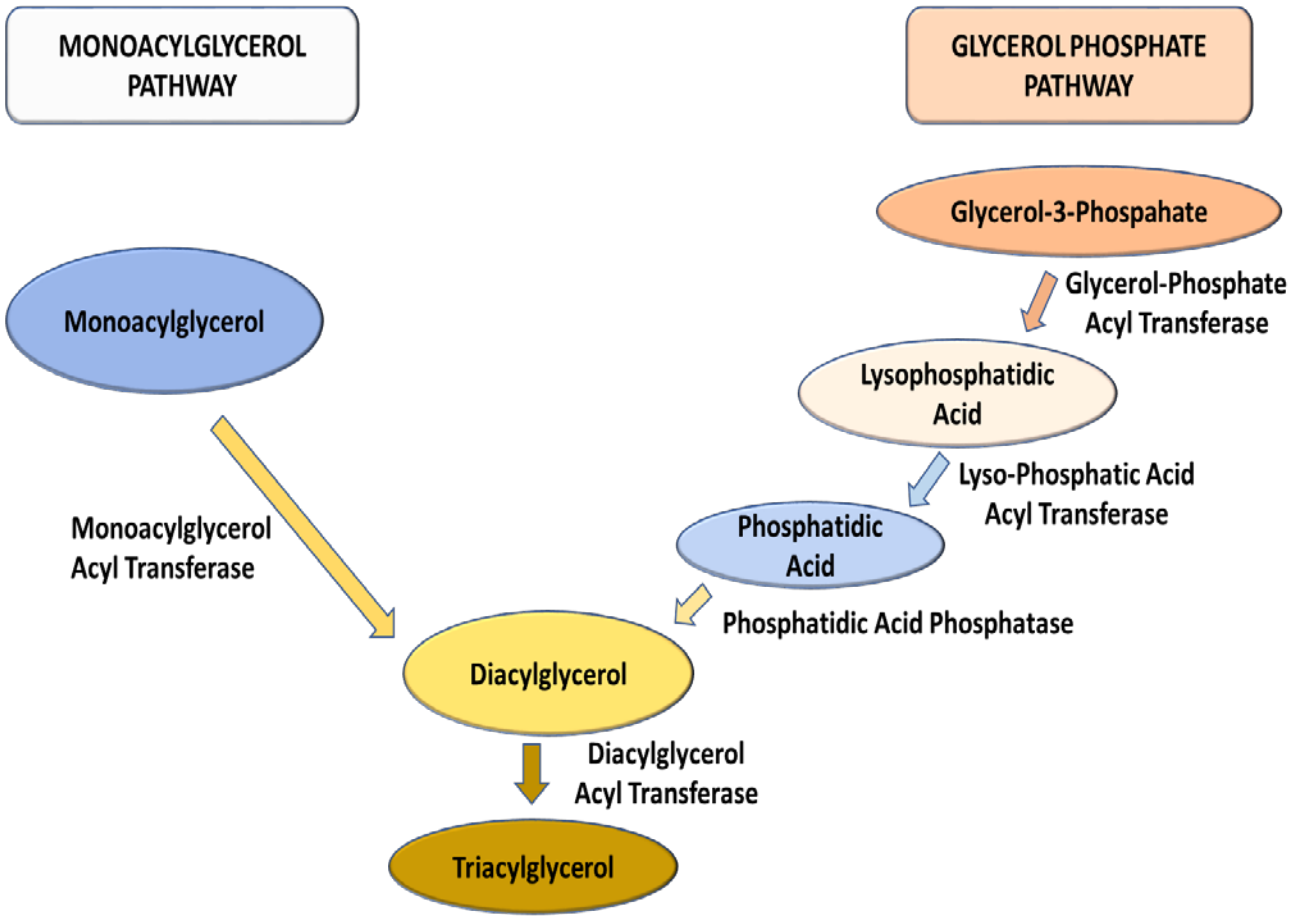

Figure 2. Biochemical pathway of lipid metabolism via Glycerol-3-phosphate (G3P) and Monoacylglycerol (MG) pathway

Glycerol-3-Phosphate Acyltransferase (GPAT3)

GPAT catalyzes the first step in the *de novo* synthesis of glycerolipids (Shan et al. 2010) involving the esterification of Glycerol-phosphate and Fatty acyl-CoA at the *sn*-1 position a reaction that is catalyzed by GPAT to produce lysophosphatidic acid, a precursor for the synthesis of triacylglycerol and phospholipids (Dircks and Sul 1999) and (Coleman and Mashek 2011). Esterified product of monoglyceride (*sn*-2 MG) act as an inhibitor to the synthesis of glycerol-phosphate (Phan and Tso 2001) and it is therefore

believed that 80% of TG re-esterified and re-synthesized occur through the MGAT pathway. Four mitochondrion associated isoforms of GPAT have been characterized in mammals based on their sensitivity and resistivity to sulfhydryl group modifying reagent N-ethylamide. The NEM resistant isoforms are commonly found in the mitochondria of liver cells while sensitive isoforms are ER-bound (Shan et al. 2010) which account for most GPAT activities in most tissues (Yet et al. 1995). The first two isomers GPAT1 and GPAT2 belong to the same subfamily on the phylogenetic classification and these mitochondria associated isoforms do not share significant homology with the microsomal GPAT3 and GPAT4 (Shan et al. 2010). Although GPAT3 has been reported to be relatively low in human and mouse liver, and that it is more expressed in the adipose tissue. Shan et al. (2010) concluded that more copies of GPAT3 are expressed in the small intestine of mouse than GPAT4 whose mRNA abundance is found in the brown adipose tissue. Murine *gpat3* gene had been found to be abundantly expressed in the heart, adipose tissue and the small intestine where it is majorly localized in the jejunum microsomal masses, a predominant site for TG absorption (Khatun et al. 2015). Khatun et al. (2015) experimental studies revealed that there was a significant decrease in GPAT activities in the jejunum of GPAT3 knock-out mice. This result indicates that GPAT3 is responsible for the abundant GPAT activities in the jejunum of mice.

Niemann Pick C1-Like1 (NPC1L1)

One of the most critical proteins involved in the uptake of lipids by enterocytes is the NPC1L1, it is a homolog of Niemann-Pick C1 protein which when mutated results in a genetic disorder expressed as accumulation of cholesterol in the lysosome (Carstea et al. 1997) and (Loftus et al. 1997). This protein is overly expressed in the small intestine, mostly on the surface of enterocytes (Altmann et al. 2004) and it's abundant at the apical surface of the absorptive enterocytes (Davies et al. 2005) and (Sane et al. 2006).

Niemann-Pick Cell 1 like 1 (NPC1L1) has been shown to be predominantly expressed in tissues such as the liver, small intestine, lung, kidney, heart and pancreas (Davies et al. 2005). Although NPC1L1 expression is abundant in both human liver and small intestine, studies using liver-derived HepG2 cells and Caco2 cells as models have revealed that human NPC1L1 is overly expressed in the human liver than in the small intestine respectively (Davies et al. 2005). In terms of localization in the small intestine, Christiaan and colleagues in 2010 concluded that NPC1L1 is highly expressed in the jejunal region of the small intestine than other regions. Its expression in the ileum is higher compared with that of the duodenum. NPC1L1 is primarily localized in the endocytic recycling compartment but it migrates to the plasma membrane when cells are deprived of cholesterol (Lin et al. 2011). Consequently, mutation or attenuation of NPC1L1 in mice led to significant reduction in intestinal cholesterol (Altmann et al. 2004). Cholesterol is incorporated into micelles containing bile acid, fatty acid and monoglyceride. This helps it to diffuse through the unstirred water layer which is the major barrier between the outer and the brush border membrane and is eventually transported by NPC1L1 into the cell. Evaluation of the plasma lipid level and cholesterol level in NPC1L1 knockout mice revealed observable reduction in the plasma lipid level as well as LDL cholesterol compared to the wild type (Davies et al. 2005), this result indicates the importance of NPC1L1 in the process of cholesterol absorption (Davies et al. 2005).

Intestinal Fatty Acid Binding Protein (I-FABP)

This is one of three subfamilies already discovered and characterized in enterocytes, they are the cytosolic proteins responsible for the processing of products derived from the hydrolysis of TG. Intestinal fatty acid binding protein (I-FABP) is predominantly expressed in enterocytes of mammals and has been suggested to be involved in the absorption of fatty acids from the lumen into the cell (Alpers et al. 2000). Three isoforms FABP1, FABP 2 and FABP 6 studied by Esteeve and his team in 2015 exhibits varying localizations in the small intestine. FABP1 and FABP 2 were highly expressed in about 70% of proximal small intestine and distal jejunum (Agellon et al. 2002). Studies have also confirmed that human FABP1 has high affinity for short-chain and medium-chain fatty acids (Esteeve et al. 2016) by participating in cellular absorption and transport of unesterified fatty acids and metabolism of dietary lipids (Montoudis et al. 2008), (Storch and Thumser 2010) and (Lagakos et al. 2011). Investigations have shown that other isoforms of FABP present in the small intestine also take part in dietary lipid uptake irrespective of I-FABP expression. Furuhashi and Gökhan in 2008 demonstrated that male I-FABP knock out mice gained more weight and acquired larger liver mass after their feeding period. They therefore suggested that fatty acid uptake might have been mediated by other members of FABP expressed in the small intestine and the liver. Overexpression of I-FABP results into a decrease in the incorporation of free cholesterol by causing a significant reduction in the gene expression level of cholesterol transporter NPC1L1 (Motoundis et al. 2008).

Fatty Acid Transfer Protein (FATP4)

This is a major type of fatty acid transport protein found in the small intestinal enterocytes, it is a functional fatty acid transporter that belongs to the family of VLC acyl-CoA synthetase and it responsible for the uptake of long-chain and VLC fatty acid (Gimeno et al. 2003) and acylation of VLCFA, and are localized in the subapical brush border membrane facing the lumen of the small intestine and in the endoplasmic reticulum. FATP4 is the only FABP expressed in the small intestine, with highest expression in the proximal duodenum and jejunum than in the distal part of the small intestine (Nassir et al. 2007). It also predominantly co-exists with FATP1 in the brain (Fitscher et al. 1998). However, FATP4 mRNA is not evident in other small intestinal cells like the smooth muscle, mesenchymal and endothelial cells (Trumpp et al. 1999) and the protein level are highly expressed in differentiated enterocytes but little or no expression is observable in the crypt where there is high rate of metabolism. FATP4 does not only function as a transporter, it may also play very critical role in the metabolism of very long chain fatty acid (VLCFA). *Invitro* studies have suggested that other transporters may have the same role as FATP4, rendering it dispensable. Shim and team in 2009 investigated the physiological role of FATP4 in lipid uptake using transgenic mice, there was reportedly no significant compensatory increase in expression of other members of FATP in FATP4 knockout mice, suggesting that it plays an expendable role in intestinal dietary lipid absorption (Shim et al. 2009). On the contrary, targeted deletion of FATP4 lead to 40% reduction of fatty acid absorption by intestinal enterocytes. *In vivo* analysis of consequential effect of low FATP4 expression level on lipid absorption revealed that mice carrying a deletion allele of FATP4 caused a decrease in LCFA uptake by enterocytes but did not affect the overall *in vivo* lipid absorption compared to the wild type (Gimeno et al. 2003).

Microsomal Transport Protein (MTP)

Microsomal transfer protein or microsomal triglyceride transfer protein is an endoplasmic reticulum lumenal protein that is responsible for the assembly and secretion of chylomicrons rich in triacylglycerol and VLDL lipoprotein in the liver and small intestine (Jamil et al. 1995) and (Iqbal et al. 2014). This evidence was first documented by Wetterau and Zilversmit in 1984, when they isolated and purified *MTP*. Secretion of chylomicrons depend on *MTP* and an indispensable Apolipoprotein-B (ApoB), and they physically engage in a protein-protein interaction during lipoprotein biogenesis (Hussain et al. 2003). Immunoprecipitation of HepG2 cells with anti-apoB and anti-*MTP* resulted in precipitation of apoB by anti-*MTP* and its subunit antibodies suggesting the interaction between the two proteins (Wu et al. 1996). *MTP* may harbor three functionally independent domains for binding, associating and transfer of lipid (Hussain et al. 2003). *MTP*'s ability to efficiently transport lipids depends on acylation of TAG products and the readiness of the lipid masses to bind *MTP* which supports it preferential specificity for binding and transporting nonpolar lipid (Jamil et al. 1995). *MTP* transfer of lipid was studied with a model membrane (Atzel et al. 1993). It was revealed in the study that *MTP* molecules use their binding domains to momentarily interact with the membrane by extracting the lipid, detaching and delivering the lipid to a second membrane by rapidly binding to it and continuing to a next cycle (Atzel et al. 1993).

Influence of Probiotics on Gastrointestinal Lipid Profile

There is evidence that the relationship that exists between alteration of intestinal microflora utility and/or composition and metabolic dysfunction may be related to the cause of some cardiovascular diseases such atherosclerosis and low-grade chronic inflammation, a principal factor in the onset of obesity related metabolic instabilities (Kobyliak et al. 2016).

Recent studies have established that one of the several roles played by probiotics include the protective effect against patho-physiological alteration associated with excess accumulation of fat within the body (Aronsson et al. 2010). Several underlying mechanisms have been attributed to the lipid lowering potential of several probiotic bacteria strains enabling them to modulate energy harvest, inflammation and lipid metabolism genes and their enzymatic activities (Delzenne et al. 2011). Yoo et al. (2013) demonstrated that some Lactobacilli strains; L. curvatus and L. plantarum reduced fat accumulation in both the liver and the adipose tissue. Fat accumulation within the GIT, liver and the heart has been known to be associated with dysregulation of lipid and glucose in a study that was carried out using germ free mice (Yoo et al. 2013). A link between obesity and gut microbiota was unveiled when metagenomic sequencing of obese and lean mice showed different gut microbiome (Ley et al. 2006).

Animal studies carried out by Yoo et al. in 2013 suggested that administration of probiotics could reduce in sizeable amount the weight gained because of excess high fat diet intake. Feed supplemented with *Lactobacillus curvatus* HY7601 or combination of *Lactobacillus curvatus* HY7601 and *L. plantarum* KY1032 effectively reduced total adipose tissue weight in mice fed with high fat diet for 9 weeks. Similarly, a recent study had reported that a 12-week high fat diet supplemented with either *L. paracasei* CNCMI-4270*, L. rhamnosus* I-3690 *or Bifidobacterium animalis subsp. lactis* I-2494 significantly suppressed HFD-induced overweight irrespective of reduction in food intake in mice. The use of three strains of *Lactobacillus plantarum* CECT 7527, 7528 and 7529 by Montserrat and his team in 2013 to reduce the level of cholesterol in the small intestine of pig demonstrated that the probiotic strains could reduce cholesterol level in the lower gastrointestinal tract by secretion of large quantity of propionic acid and butyric acid. Another mechanism of cholesterol reduction is by binding and adherence of cholesterol to bacterial cellular surface (Montserrat et al. 2014).

A double-blind, randomized and placebo-controlled trial conducted to evaluate the impact of perinatal probiotic administration and intervention on childhood gut and overweight development following a 10 year sequel, showed that administration of *Lactobacillus gasseri* SBT2055 containing fermented milk for a period of 48 days resulted in a significantly reduced abdominal visceral and subcutaneous fat accumulation by 4.6% and 3.3% respectively, leading to a 1.5% total reduction in body mass index (BMI) (Kobyliak et al. 2016). This demonstrates that *L. gasseri* has a beneficial impact on the GIT metabolic disorder.

Cardiovascular diseases (such as atherosclerosis and other health disorder like coronary heart disease), liver cirrhosis, dyslipidemia and type-2 diabetes, have been traced back to hypercholesterolemia (Das and Mukhopadhyay 2011). Although no clear relationship has been established between obesity and type-2 diabetes, nonetheless, obesity has been shown to increase the risk of acquiring insulin resistance which is a principal cause of type 2 diabetes (Das and Mukhopadhyay 2011). Probiotic isolates could be used in the improvement and control of insulin resistance and enhancement of the lipid profile leading to prevention of NAFLD (non-alcoholic fatty liver disease, a chronic liver disorder. Kim and his team in 2013 demonstrated this by conducting a 13-week study involving the use of *L. rhamnosus* GG to improve the insulin sensitivity and lipid accumulation in high fat diet (HFD) fed mice (Kim et al. 2013). This study was reiterated by Tabuchi and colleagues in 2003, when they showed that *Lactobacillus rhamnosus* GG could decrease glycated hemoglobin and reduced glucose intolerance streptozocotin-induced diabetic mice leading to a sizeable increase in insulin secretion (Tabuchi et al. 2003). Comparably, *Lactobacillus rhamnosus* GG administration to high fructose diet induced NAFLD prone mice resulted in gut barrier restoration and inhibition of liver steatosis and development of inflammation in mice (Ritze et al. 2014).

The absorption of dietary lipids and reabsorption of biliary cholesterol in the jejunum, lumen and duodenum contribute to the regulation of plasma cholesterol level. In other words, reduction in absorption of dietary lipid in the small intestine helps to decrease plasma cholesterol level (Liong and Shah 2005). Ever since Mann and Spoerry in 1974 discovered the hypocholesterolemic effects of fermented milk taken by the people of Massai tribes, the relationship between lactic acid bacteria and cholesterol absorption has become a study of great focus and interest. Several hypotheses have been proposed to demonstrate this relationship. It's believed that the consumption of dietary lipids in the intestine by some intestinal bacteria reduces the level of cholesterol available for absorption (Pigeon et al. 2002) and (Pereira et al. 2002). The role of a novel probiotic isolate on intestinal absorption of lipids by enterocytes is the subject of the present study. Caco-2 cell culture model will be used in this study. Caco-2 cell line is a continuous cell of heterogeneous human epithelial colorectal adenocarcinoma cells, developed by the Sloan Kettering Institute for Cancer Research through research conducted by Dr. Jorgen Fogh (Awortwe 2014). Though it was derived from a colon (large intestine) carcinoma, when cultured under specific conditions the cells become differentiated and polarized such that their phenotype, morphologically and functionally, resembles the enterocytes lining the small intestine.

Various protein and enzymatic activities have been investigated, some of which are known to be involved in the regulation of lipid metabolism in the GIT. Huang and Zheng in 2009 showed that NPC1L1 (Niemann-Pick C1-like 1) expression can be decreased by specific strains of *L. acidophilus* and *L. rhamnosus* GG. These strains were better inhibitors of NPC1L1 than other strains tested. Brown and Yu (2009) have also demonstrated the role of heterodimers of ATP-Binding Cassette transporters ABCG5 and ABCG8 in the intestinal disposal of sterols. Moreover, two probiotic strains of Lactobacilli which include *Lactobacillus plantarum* NR74 and *Lactobacillus rhamnosus* BFE5264 isolated from Massai fermented milk (Marthara et

al. 2008) and Korean Kimchi (Lee et al. 2011) and their probiotics potential have been studied *in vitro.* These bacteria stimulate the up-regulation of liver X receptor (LXR) also known as nuclear receptor (NR) which controls some transcriptional factors related to lipid metabolism and down-regulate a number of genes involved in cholesterol absorption, efflux, transport and eventually excretion (Yoon et al. 2012). This they do by enhancing the expression of heterodimer of LXR which are LXRα and LXRβ in Caco2 cells (Yoon et al. 2012).

Choice of Lactobacillus rhamnosus MRS6AN

Among the various isolates characterized by Boyiri and Onyango in 2015, *Lactobacillus rhamnosus* MRS6AN was found to exhibit greater probiotic potential, in that it does not degrade mucin (Boyiri and Onyango 2015), a glycoprotein of the mucus layer produced by epithelia cells (Boskey 2013). It was isolated from a traditionally cultured Kenyan milk called "Amabere Amaruranu" alongside other isolates belonging to the same genus and Acetobacter. *Lactobacillus. rhamnosus* MRS6AN was characterized using 16sRNA. *Lactobacillus rhamnosus* MRS6AN also demonstrated stability in extreme harsh and unfavorable conditions such as bile salt and acidic conditions (Boyiri and Onyango 2015). In terms of antimicrobial activities, they inhibited the growth of other bacterial test strains (Boyiri and Onyango 2015). Nevertheless, no data or evidence has been documented regarding the role of *L. rhamnosus* MRS6AN on intestinal lipid absorption and TG accumulation. Hence, this present study is focused on the influence of *L. rhamnosus* MRS6AN on intestinal lipid absorption and accumulation in enterocyte, and its consequential effect on GPAT3 mediated pathway, fatty acid binding protein (FABP) and microsomal transport protein (*MTP*).

Hypothesis

L. rhamnosus MRS6AN will modulate absorption of lipids by regulating the expression glycerol-3-phosphate acyltranferase (GPAT3) and the expression of genes involved in GPAT3 pathway.

Objectives

Firstly, to determine and quantify cellular lipid uptake by Caco2 cells when co-culture with *Lactobacillus rhamnosus* MRS6AN, heat inactivated MRS6AN, its cell extract or its filtered spent broth.

Secondly, to determine the expression level of GPAT3 enzyme, *MTP*, I-FABP involved in lipid absorption via GPAT3 pathway and a sterol transporter NPC1L1 in Caco2 cells when co-cultured with *L. rhamnosus* MRS6AN, heat inactivated MRS6AN, its cell extract or its filtered spent broth.

CHAPTER 2

MATERIALS AND METHODS

Isolation of Lactobacillus rhamnosus and Preparation of Probiotic Cell Extract

Identification and isolation of *L. rhamnosus* MRS6AN from a Kenyan locally cultured milk (amabere amaruranu) was previously carried out using biochemical testing and 16S rRNA sequencing and they demonstrated optimum probiotic characteristics compared to other isolates in its stability in the presence of acid and bile salt and its inability to degrade mucin. Other isolates characterized include *L. paracasei, L. casei, L. reuteri, L. acidophilus* and *Acetobacter* sp. The isolate was cryo-preserved at -80^{0}C with storage beads (Boyiri et al. 2015).

Growth Media for Bacteria

Oxoid DeMann Rogosa Sharpe Media (CAT No. CMO359) was supplied by Thermo scientific.

Human Adenocarcinoma Cell (Caco2 cells)

Human colon derived Caco2 cells are fast growing cells, they begin to exhibit characteristics similar to those of enterocytes lining the wall of the small intestine after they have attained 21 days post confluence demonstrating a differentiated monolayer (Olejnik 2003) and developing tight junctions for regulation of permeability of molecules and ions (Assimakopoulos et al. 2011).

Human Colon Derived Caco-2 Cell Culture Media

Dulbeco's Modified Eagle's Media (DMEM) supplemented with 10% fetal bovine serum (Hyclone, Cat No. SH30088.03, supplied by GE Lifecare Healthsciences, Logan Utah, 84321), 5% Penicillin-streptomycin (Corning cellgro, Meditech, Inc. Mannasas, VA 20109) and 5% Sodium-pyruvate was used to culture Caco2 cells (Hyclone, Cat No. SH30239.01, supplied by Thermo Fischer Scientific).

Primary and Secondary Antibodies

Mouse anti-human I-FABP (E-9, Cat No: sc-374482), *MTP* 110 (2G2, Cat No: sc-58807) and ACSVL4 (H-6, Cat No: sc-393309) were obtained from Santacruz Biotechnology and GPAT3 (Cat No: AP18013c) was obtained from VWR. The antibodies were used in western blot protein assay for FABP, *MTP*, FATP4 and GPAT3 respectively. Secondary anti-mouse M-IgG-kappa BP-HRP (Cruz marker, Cat No: sc-516102-CM) was obtained from Santacruz Biotechnology for the detection assay.

Lactobacillus rhamnosus MRS6AN Culture

Lactobacillus rhamnosus was cultured, until an early stationary phase of growth, in nutrient rich MRS broth media under anaerobic conditions at 37 ^{o}C overnight. The optical density was monitored at 600 nm (OD_{600}) until it attained an optical density of 2.0. Pure culture of bacteria was verified using MRS agar media. Bacterial cells were centrifuged at 3000 g for 5 mins at 6 ^{o}C. The supernatant was decanted. Bacteria pellets were washed in PBS pH 7.2 and re-suspended in DMEM media (10^7 cfu/ml) at the time set for co-culture.

Preparing Probiotic Cell Extracts

A seed culture was prepared by inoculating a storage bead stored at -80 ^{0}C in a test tube containing 5 ml DeMan-Rogosa-Sharpe broth (MRS) and incubated for 18hrs at 37^{o}C. Ten to fifteen drops of seed culture were subculture into a 250 ml flask containing 100 ml MRS broth and incubate on shaker for 24 hrs. at 37 ^{0}C. Optical density reading was monitored at 600 nm (OD_{600}) until it reached 2.0. Ten milliliters (10 ml) of bacteria culture was dispensed into 50 ml centrifuge tube and centrifuged at 14000 rpm or 1100 g for 15 minutes. The supernatant was collected as the spent broth from the live bacteria (LB) by decanting into a 50 ml cork screw flask and the resulting pellets were washed twice in PBS pH 7.4. (The pH of the spent broth was low due to production of lactic acid by bacteria, but was raised to 7.4 using NaOH). The spent broth was filtered with 0.22 µm Millipore filter to collect the Filtered spent broth (FSB). Bacteria pellets were re-suspended in 50 ml PBS, and the suspension was sonicated 5 times at 44 % amplitude for 2 minutes each at 6 minutes interval of rest. The sonicated suspension was centrifuged at 1100 g for 15 minutes at 4 ^{0}C. The resulting supernatant was collected and filtered through 0.22 µm Millipore filter to obtain the cytoplasmic fraction (CF). Live bacteria were subjected to heat treatment to examine the effect of heat inactivated bacteria. Live bacteria cells were incubated at 80 ^{0}C for 30 minutes to produce heat killed inactivated bacteria (HIB).

Passaging of Caco2 Cells

Cultured cells were grown until they attained 70 % confluence. To detach the cells, 3 ml of trypsin-EDTA was added to flask containing attached cells. Cells were incubated at 37 ^{o}C for 7 minutes for cells to entirely detach from the bottom of the flask. DMEM was immediately added to the flask after incubation to stop trypsin activity. Cells were spun at 200 g for 5 minutes and trypsin containing media was aspirated.

Caco2 cells were re-suspended in fresh complete growth media and dispensed into cell culture flasks, this procedure was repeated 6 times in order to prolong lives of cells in the culture by avoiding the senescence that is associated with prolonged high cell density.

Cell Culture of Human Adenocarcinoma Cells (Caco2 cells)

Caco-2 cell line was obtained and cultured in DMEM containing 100 µg/ml Penicillin-Streptomycin (Pen-Strep) supplemented with 10 % FBS and 5 % sodium pyruvate at 37 ^{0}C in a humidified atmosphere with 5 % CO_2 using 24-well plates until confluence. The cells were allowed to grow, attach and differentiate in culture flask until 16 days post confluence after which cells are believed to have undergone spontaneous differentiation to look like intestinal epithelia cells representing lining of the small intestine.

Preparing Lipid Mixture

A 100x lipid mixture was prepared in 1 ml micro-centrifuge tube. The Lipid mixture contained 48 mg of sodium taurocholate (NaTc), 50 mg oleic acid, 40 mg egg lecithin (lecithin is very sticky), 50 ml of PBS and the volume was made up to 900 ul to obtain 1x lipid mixture. The Lipid mixture was incubated at 37 ^{o}C and vortexed so that the concentrations (oleic acid, lecithin and sodium taurocholate (NaTc)) were 2.0 mM, 1.36 mM and 1.0 mM respectively. The final solution was added to 89.1ml of ready to use complete growth media and filtered using 0.22 millipore filter.

Cell Viability Assay

Differentiated Caco2 cells were treated with 100 ul or 200 ul or 300 ul of the lipid mixture and incubated for 2 hrs. at 37 ^{o}C. Treated cells were trypsinized with 200 ul of trypsin-EDTA per well and

incubated for 7 minutes at 37 ^{o}C until they were completely detached from the bottom of the plate. They were spun at 200 g for 5 minutes and resuspended in PBS pH 7.4. The cell suspension of approximately 1×10^{6} cells/mL was prepared in PBS pH 7.4 and the sample was thoroughly mixed. A 1:1 mixture of the cell suspension and 0.4 % trypan blue solution was prepared by adding 200 ul of cell suspension to an equal amount of trypan blue solution. The sample was gently mixed and allowed to sit for 5 min at room temperature. Prior to use, the hemocytometer and coverslip were washed with 70 % (v/v) ethanol and allowed to dry. Fifteen microliters (15 ml) of cell suspension was added to the chamber through the edge of the chamber between the cover slip and the V-shaped groove in the chamber. The cell suspension was drawn into the chamber by capillary action. It was allowed to sit for 1–2 min and then cells were viewed using phase contrast microscope and viable and non-viable cells were counted using ASIO SOFTWARE.

To evaluate the effect of oleic acid on cell viability, cell suspension of approximately 1×10^{6} cells/mL of Caco2 cells treated with 100 ul, 200 ul and 300 ul of lipid mixture containing oleic acid was prepared in PBS pH 7.4 and samples were thoroughly mixed. A 1:1 mixture of the cell suspension and 0.4 % trypan blue solution was prepared by adding 200 ul of cell suspension to an equal amount of trypan blue solution. Viable and non-viable cells were enumerated using hemocytometer.

Number of cells (viable or non-viable)

$$= \frac{(A+B+C+D)}{4} \times 10^{4} \times 2 \times \text{sample dilution}$$

$$\% \text{ Viable cells} = \frac{\text{Number of viable cells}}{\text{Total number of cells}}$$

Probiotic Co-culture with Caco2 Cells

Differentiated Caco2 cells were treated with 200 ul of the test strain's suspension containing approximately 10^7 CFU/ml of live *L. rhamnosus* or heat inactivated bacteria (HIB) or the cell free culture supernatant which is the filtered spent broth (FSB) or cytoplasmic fraction (CF) or growth media as control. Treated cells were incubated overnight (approximately 16hrs) at 37 ^{0}C and 5 % CO_2.

Treatment of Caco2 cells with Dietary Lipids

Caco2 cells co-cultured overnight with live bacteria or bacteria extracts were divided into three treatment Protocols. Protocol 1's overnight spent media was discarded and cells were directly overlaid without washing with 400 ul of fresh growth media containing 100 ul lipid mixture; Protocol 2 cells were washed three times in phosphate buffer saline (PBS), pH 7.4 and treated with lipid and fresh treatments; and Protocol 3 cells were washed three times in PBS and treated with 400 ul of complete growth media containing 100 ul lipid mixture in media without the treatments. Caco2 cells were incubated for 2 hrs as determined by the preliminary study at 37 ^{0}C, 5 % CO_2. Cells were washed in PBS three times and lipid content was determined using Oil-Red O staining method (ORO). Accumulated intracellular triglyceride was also quantified using triglyceride reagent.

Determination of Optimum Lipid Accumulation Time in Enterocytes

Caco2 cells were seeded into 24-well plates after 5-6 passages. Cells were grown for about 5-6 days until they attained almost 100 % confluence. Cells were allowed to continue growing until 21-day post confluence. Cells were treated with 400 ul of complete growth media containing 100 ul lipid mixture (oleic acid, lecithin and sodium taurocholate (NaTc) and incubated for 30 mins, 1 hr, 2 hrs, 3 hrs or 4 hrs. Caco2

cells were harvested after each incubation period and subjected to intracellular triglyceride accumulation analysis and Oil-Red O staining assay to quantify and compare the level of lipid uptake by cells at each time point.

Incorporated Lipid Content Quantification Assay (Oil-Read-O Staining)

Intracellular lipid accumulation was measured using Oil-Red-O staining (ORO). Caco2 cells were cultured as described above. When the cells had differentiated, they were treated with different concentrations of live bacteria (LB) or heat inactivated bacteria (HIB) or cytoplasmic fraction (CF) or filtered spent broth (FSB) or growth media (as a control) and were incubated overnight at 37 ^{o}C. After incubation, co-cultured cells were collected, and subjected to treatment with lipids as described above. At the end of the incubation, growth media was aspirated and 10 % formaldehyde was added to the wells and allowed to act for 5 minutes. Formaldehyde was removed and fresh formaldehyde was added and allowed to sit for at least 1 hour. The formaldehyde was removed and wells were washed with 60 % isopropanol and allowed to dry completely. At this point, Oil-Red-O working solution (To prepare Oil Red O stock solution, 60 mg of Oil Red O was dissolved in 20 ml of 100 % isopropyl-alcohol. Oil Red O working solution (prepared by adding 3 parts of Oil Red O stock solution to 2 parts of distilled water, the solution was allowed to sit for 10 minutes and then filtered using Whatman filter paper) was added to wells and incubated for 10 minutes. The solution was removed and the wells were immediately washed with distilled water (dH_2O) until no more red flakes were observed in the waste water. Wells were then allowed to dry. Caco2 cells were observed using phase contrast microscope for appearance of stained lipid droplets in the cells.
To quantify the lipid content in the wells, elution of Oil-Red-O stain was performed by adding 100 % isopropanol to the wells and allowed to sit for 10 minutes. The isopropyl-alcohol was pipetted several

times to remove all stain from the wells. The resulting solution was transferred into spectrophotometric cuvettes, lipid content in the isopropanol was determined using spectrophotometry method at an optical density of 492 nm.

Quantification of Intracellular Triglyceride Accumulation

Triglyceride reagent was obtained from Pointe Scientific Inc. and reconstituted with 15ml of distilled water. Treated cells were harvested and lysed using 1000 ul RIPA (Radioimmunoprecipitation assay) buffer (Cat No: BP-115) and 40 ul Protease inhibitor, by adding 200 ul of RIPA buffer per well. Samples were transferred into 1.5 ml micro-centrifuge tube and sonicated at 44 % amplitude on ice to ensure cells are totally lysed. Tubes containing cells were incubated at 4 ^{o}C for 30 minutes, cells were spun at 3000 g, 4 ^{o}C for 5 minutes and the supernatant collected. A hundred microliter (100 ul) of already reconstituted triglyceride reagent was added to a 96 well micro-titer plate and incubated at 37 ^{o}C for 3 minutes. Ten microliters (10 ul) of samples were added to each well containing the reagent and incubated at 37 ^{o}C for 5 minutes. Caco-2 cell's triglyceride content was determined by spectrophotometry. Absorbance was measured at an optical density of 540 nm.

Determination of Protein Concentration (BCA)

Solution of protein standard with a concentration of 1 mg/ml was prepared. A hundred microliter of distilled water was used as blank. Protein samples were prepared by diluting 10 µl of protein in surfactant in 90 µl of distilled water making a total volume 100 µl. BCA reagent was prepared by mixing reagent A (a solution containing bicinchoninic acid, sodium carbonate, sodium tartrate and sodium bicarbonate in 0.1 N NaOH, pH 11.25) and B (containing 4 % (w/v) CuSO4.5H2O) (Smith *et al*., 1985) in the

ratio 50:1. Two microliter (2 ul) of the BCA working reagent was added to each sample and standard, samples were gently vortexed and incubated at 37^0C for 30 minutes. The blank, protein standard and the unknown samples were transferred to a 96 well spectrophotometric plate and absorbance of all samples was measured at an optical density of 540 nm wavelength using a spectrophotometer. Calibration curve of standard protein sample concentration was plotted against the optical density. The concentration of unknown samples was determined using the standard calibration curve.

Western Blot Analysis

Overnight Caco2 cells treated according to Protocol 1 (overnight cells were overlaid with 100 ul of lipid mixture in 400 ul of growth media), Protocol 2 (overnight cells were washed and treated with 100 ul of lipid mixture in growth media and fresh treatments) and Protocol 3 (overnight cells were washed and treated with 100 ul of lipid mixture growth media only). Caco2 cells treated with 100 ul of lipid in 400 ul complete growth media was used to as control. Plates were incubated for 2 hrs. Cell culture dish was placed on ice. Cells were washed with ice-cold PBS. PBS was aspirated and attached cells were lysed using ice-cold lysis buffer (RIPA buffer (1 ml/10^7 cells/100 mm dish/150 cm^2 flask). Adherent cells were scrapped off the dish using cold plastic cell scrapper. Different cell treatments were gently transferred into pre-cooled micro-centrifuge tubes and centrifuged at 14,000 g for 30 minutes at 4 ^{0}C. The supernatant was separated from pellets and was transferred into new micro-centrifuge tubes and kept on ice. Equal volume of 2x Laemmli Sample Buffer and protein samples were prepared. Proteins were denatured by boiling samples in buffer at 90 ^{0}C for 10 minutes.

Twenty microliters of each protein sample were loaded into each well alongside with molecular weight marker protein standard. Samples were run through 10 % SDS-PAGE gel for 60 minutes at 200 V.

Nitrocellulose membrane was activated in methanol for 1 minute and rinsed in transfer buffer. Resolved protein was transferred to nitrocellulose membrane. The membrane was blocked for 1 hr at room temperature using 3 % bovine serum albumin (BSA) with agitation in Tris-Buffered saline with Tween-20 (TBST). The membrane was incubated with primary antibodies reconstituted with the blocking buffer against *MTP*, FABP, LPAAT (GPAT3) and NPC1L1 overnight in a cold room with agitation. The membrane was washed in TBST and incubated at room temperature for 60 minutes with the recommended dilution of the labeled secondary antibody reconstituted in blocking solution with agitation. The membrane was prepared for chemiluminescence exposure by placing it in 1:1 ratio volume of Super-Signal West Femto Maximum Sensitivity Substrate and allowed to sit for 5 minutes without agitation. The membrane was placed in a plastic wrap (sheet protector) to prevent drying. Chemiluminescence image or normal image exposure for protein detection was carried out.

Statistical Analysis

Experimental data were analyzed using one way analysis of variance (ANOVA). Comparison of individual and group data were performed and multiple range test was carried out to separate means (Bonferonni, Sidak and Scheffe methods). Differences in mean were analyzed at 5 % significant level.

CHAPTER 3

RESULTS

Effect of Oleic Acid on Cell Viability

Approximately 91 % of cells treated with 100 ul of lipid mixture remained viable after the treatment (Table 1). There was a slight reduction in viability to about 80 % of cells treated with 200 ul lipid mixture. Treatment with 300 ul of lipid led to drastic reduction in viability of cells to 44.27 %.

Table 1: Effect of lipid mixture on Caco-2 cell viability (Trypan blue staining) *

Cell/Trypan blue (v/v)	Total number of cells	Number of viable cells	Number of non-viable cells	% viable cells	% non-viable cell
100 ul	1.5×10^6	1.2×10^6	3.15×10^5	90.5 %	9.5 %
200 ul	1.61×10^6	1.13×10^6	4.8×10^5	80.18 %	19.82 %
300 ul	2.06×10^6	5.0×10^5	1.56×10^6	44.27 %	55.73 %

*Values represent mean percentage of viable and non-viable cells (n=3).

Optimum Lipid Accumulation Time

Total Lipid Content Accumulation

Ideal time for lipid accumulation in Caco2 cells was determined by treating differentiated cells for different time frames (60, 120, 180 and 240 minutes) Lipid accumulation increased with time (Figure 3). The total lipid content level of cell was 0.097 at 1hr of incubation and was found to be at its maximum at 2 hrs of incubation with lipid level of 0.184. After 2 hrs the intracellular lipid content level began to decrease. The lipid content level was 0.159 and 0.105 at 3hrs and 4hrs respectively.

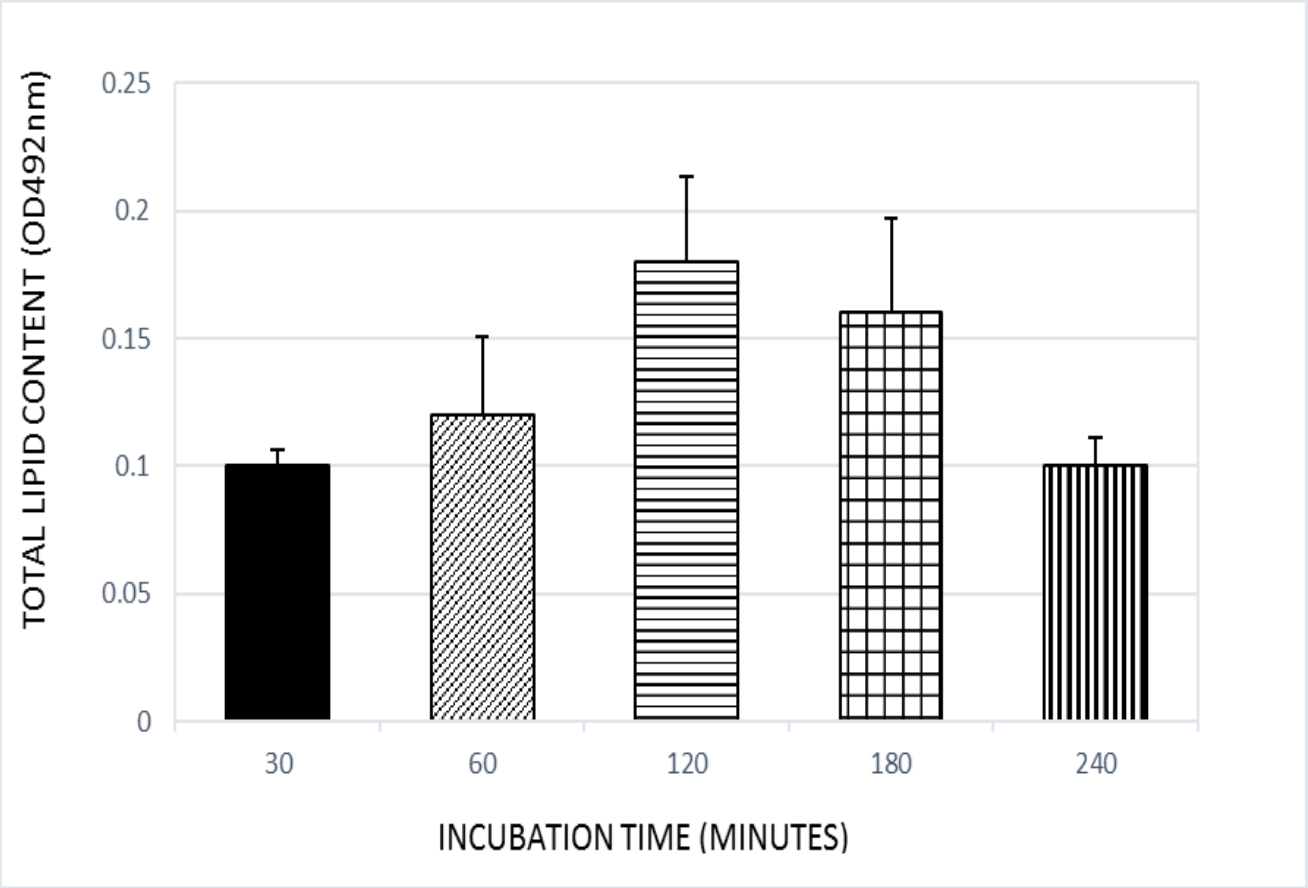

Figure 3. Optimum incubation time for total lipid accumulation in Caco2 cells determined by Oil Red O staining assay. Total lipid content was determined by spectrophotometry and absorbance was measured at optical density (OD) at 492 nm. Each bar represents mean ± SD (n=3).

Intracellular Triglyceride Accumulation

To establish the optimum time for lipids to accumulate in the cell, the triglyceride (TG) content of Caco2 cells was determined using TG reagent (Figure 4). Intracellular accumulated TG for 60, 120, 180 and 240mins were 0.248, 0.283, 0.214 and 0.223 respectively. The intracellular TG level increased until 2 hrs of incubation and was maximum at 2 hrs compared with the control. The TG level began to decrease after 2 hrs of incubation.

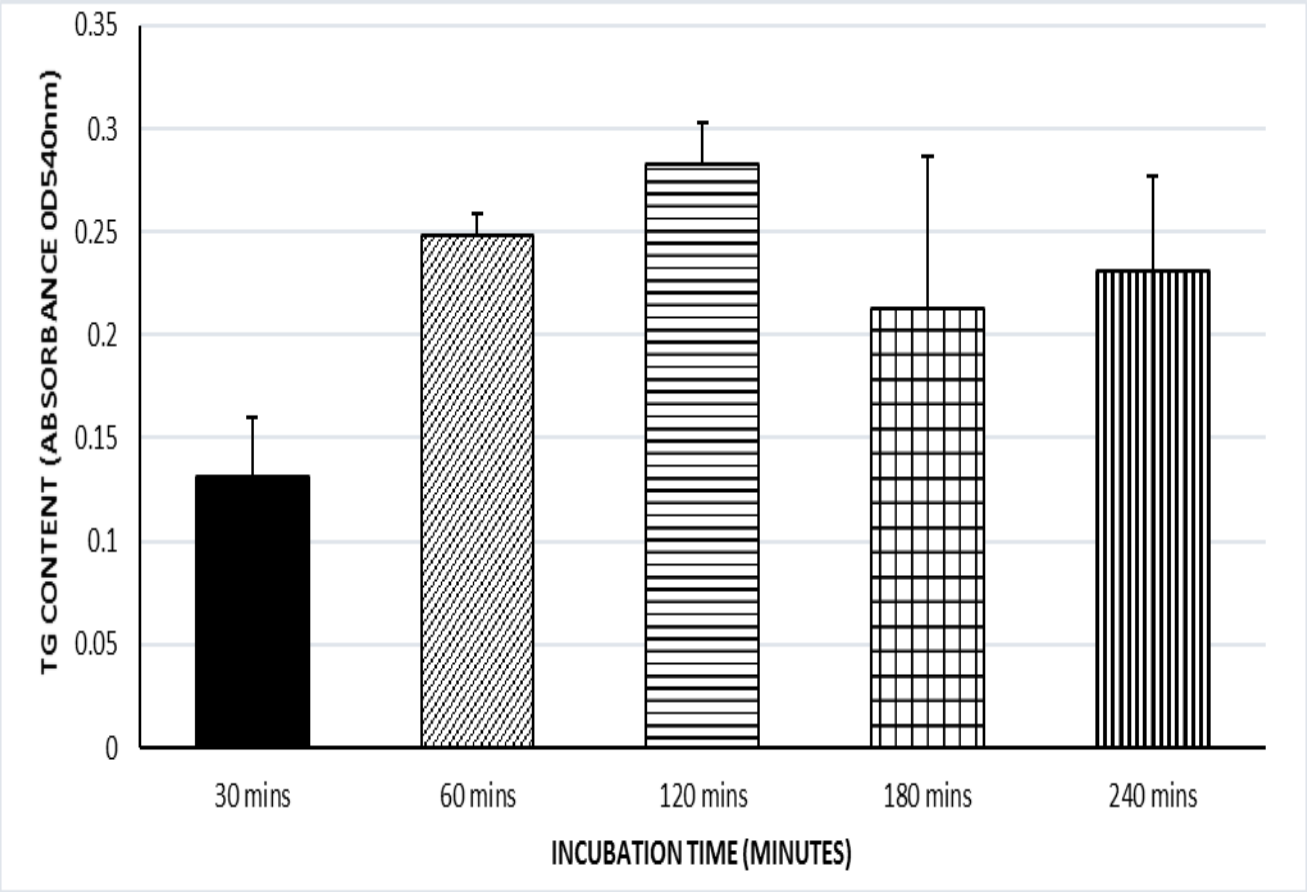

Figure 4. Triglyceride accumulation (TG) in Caco2 cells determined by spectrophotometry at Optical density of 540nm. Each bar represents mean ± SD value of calculated TG level (n=3).

Total Lipid Content Assay

Total Lipid Content of Caco2 Cells Treated According to Protocol 1

In Protocol 1, overnight co-cultured Caco2 cells were overlaid with the lipid mixture without washing off the treatments (Table 2). The TL level of cells overlaid with CF, HIB and LB compared to the control were 0.13, 0.25 and 0.91 respectively (Figure 5). The total lipid level (TL) increased by 0.13 compared to the control in cells treated with FSB, although there was slight decrease of 0.13 and 0.25 in

cells treated with CF and HIB respectively. LB decreased the TL level in Caco2 cells by 0.91 when compared with the control and other treatments.

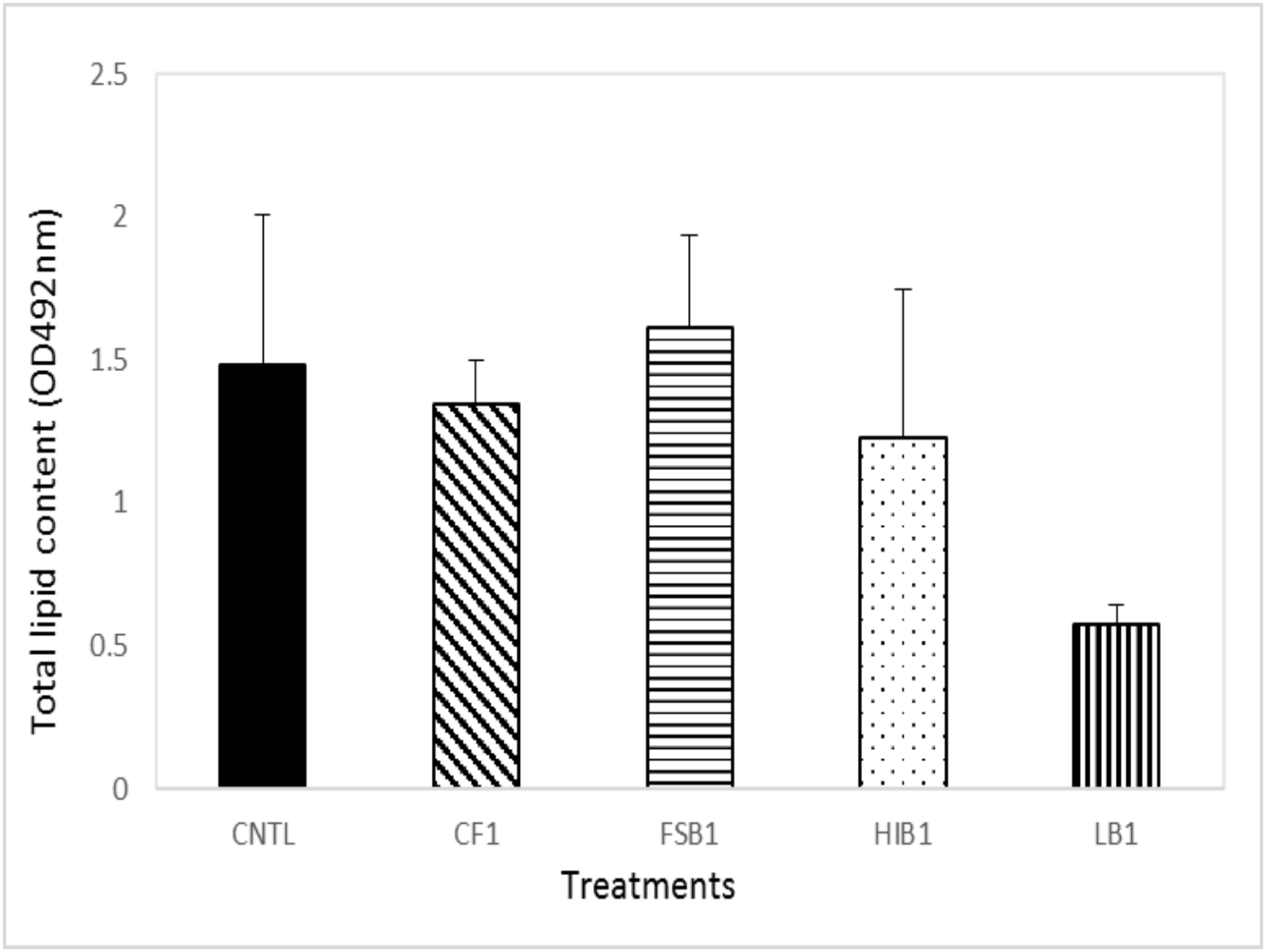

Figure 5. Effect of *Lactobacillus rhamnosus* MRS6AN or its extracts on total cellular lipid level (determined by spectrophotometry) in Caco2 cells treated according to Protocol 1 (after overnight bacterial treatments, cells were overlaid with lipid mixture without washing and incubated for 2 hrs). Optical density was measured at 492 nm. Each bar represents mean ± SD of total cellular lipid content (n=3). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control. Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Table 2: Effect of *L. rhamnosus MRS6AN* or its extracts on total cellular lipid content of Caco2 cells treated according to Protocol 1 (after overnight bacterial treatments, cells were overlaid with lipid mixture without washing and incubated for 2 hrs). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control.

Treatment (200 ul)	**Total Lipid Content (OD492nm)**
Control	1.48
Cytoplasmic fraction	1.35
Filtered Spent broth	1.61
Heat inactivated bacteria	1.23
Live bacteria	0.57

Total Lipid Content of Caco2 Cells Treated According to Protocol 2

In Protocol 2, co-cultured cells were washed and treated with lipid and live bacteria or its extracts. A greater reduction of total lipid content of Caco2 cells was observed in LB and CF treated cells treated according to Protocol 2 compared with the control (Table 3). HIB and FSB treatments did not reduce TL content in cells. The decrease in TL level in LB and CF treated cells was by 0.51 and 0.27 when compared to the control (Figure 6). On the contrary, HIB treated cells showed slight increase in cellular TL content with 0.17.

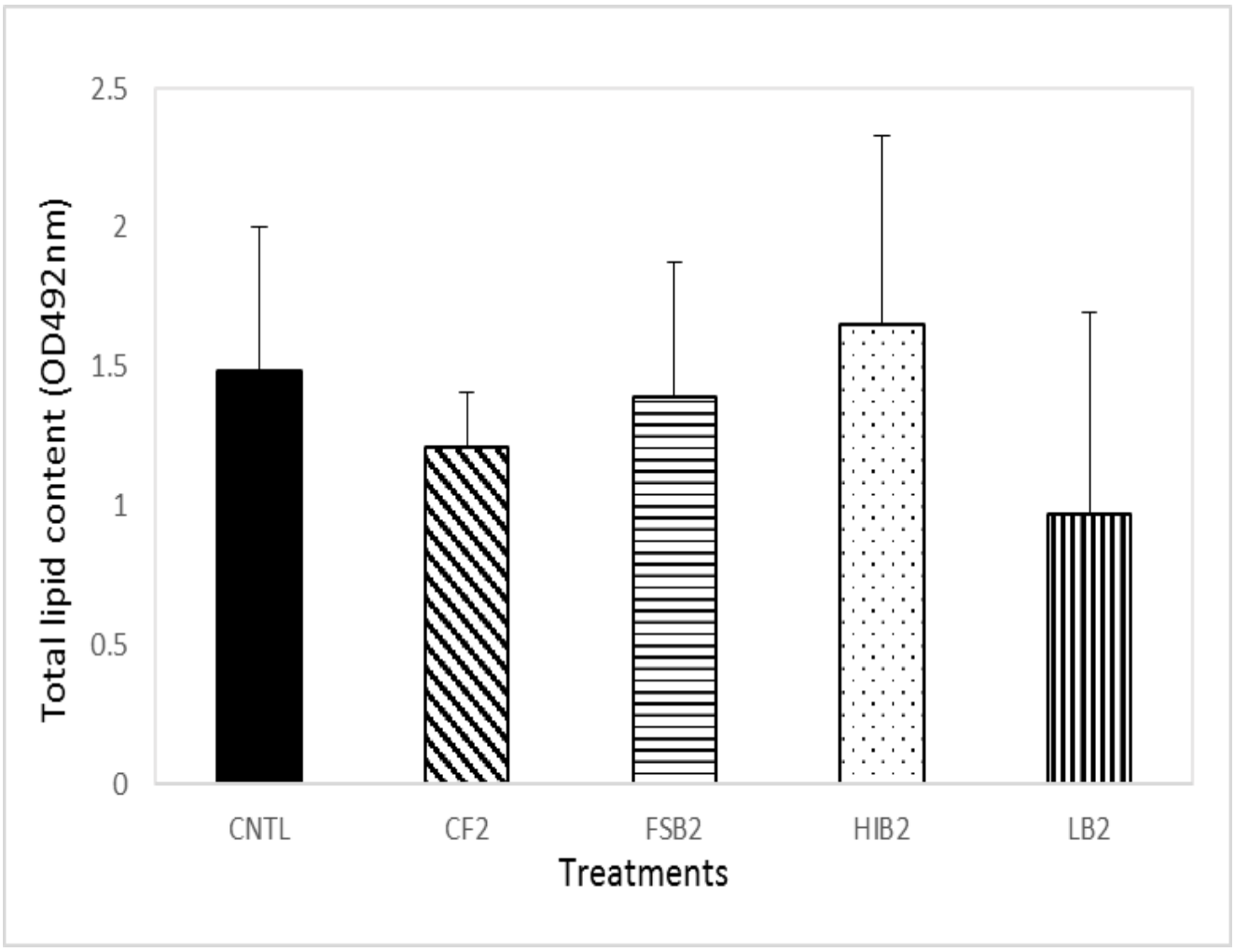

Figure 6. Effect of *Lactobacillus rhamnosus* MRS6AN or its extracts on total cellular lipid content (determined by spectrophotometry) in Caco2 cells treated according to Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipid mixture and fresh bacteria treatment and incubated for 2 hrs). Optical density was measured at 492 nm. Each bar represents mean ± SD value of total cellular lipid content (n=3). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control.

Table 3. Effect of *L. rhamnosus MRS6AN* or its extracts on total cellular lipid content of Caco2 cells treated according to Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipid mixture and fresh bacteria treatment and incubated for 2 hrs). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control.

Treatment (200 ul)	**Total Lipid Content (OD492 nm)**
Control	1.48
Cytoplasmic fraction	1.21
Filtered Spent broth	1.39
Heat inactivated bacteria	1.65
Live bacteria	0.97

Total Lipid Content of Caco2 Cells Treated According to Protocol 3

In Protocol 3, overnight co-cultured cells were washed and treated with 100 ul lipid mixture only. The LB and CF induced reduction in TL content of 0.91 and 0.55 respectively, but was not significantly different when compared to the control (Figure 7). There was little or no change in TL level in the presence of FSB or HIB when compared to the control treatment.

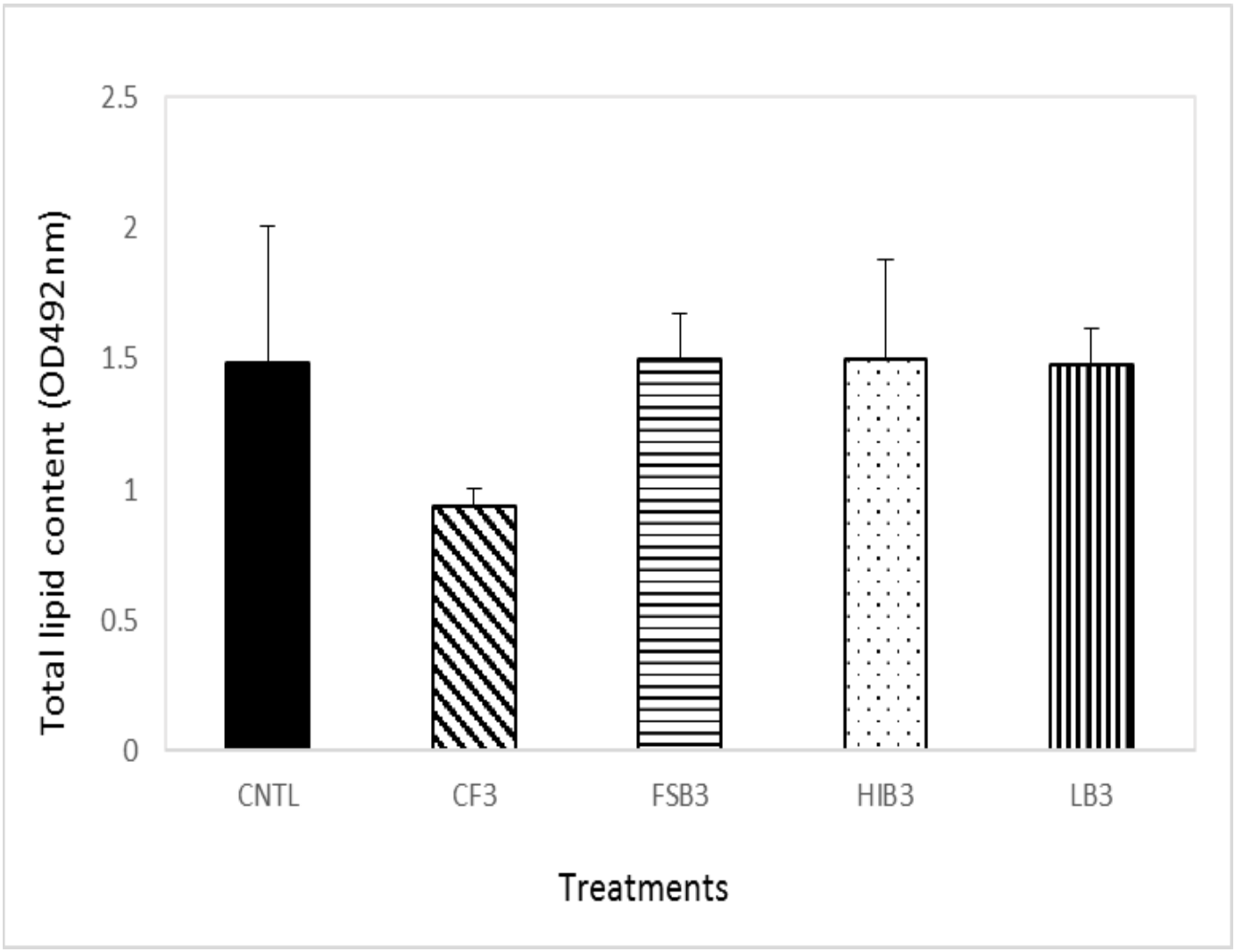

Figure 7. Effect of *Lactobacillus rhamnosus* MRS6AN or its extracts on total cellular lipid content (determined by spectrophotometry) in Caco2 cells treated according to Protocol 3 (After overnight bacterial treatment, cells were washed and treated with only the lipid mixture and incubated for 2 hrs). Optical density was measured at 492 nm. Each bar represents mean ± SD value of calculated total cellular lipid content (n=3). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control. Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Table 4. Effect of *Lactobacillus rhamnosus* MRS6AN or its extracts on total cellular lipid content (determined by spectrophotometry) in Caco2 cells treated according to Protocol 3 (After overnight bacterial treatment, cells were washed and treated with lipid mixture only and incubated for 2 hrs.). Optical density was measured at 492 nm. Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control.

Treatment (200 ul)	**Total Lipid Content (OD492 nm)**
Control	1.48
Cytoplasmic fraction	0.94
Filtered Spent broth	1.50
Heat inactivated bacteria	1.50
Live bacteria	1.48

Comparison Between Effect of Probiotic Treatments on Total Lipid Content between Treatment Protocols.

Cytoplasmic Fraction (CF) Treatment

The total lipid content of Caco2 cells treated with CF (cytoplasmic fraction) were compared across treatment Protocols to determine whether there were differences in their TL content. Although there was no significant difference between CF treated cells, but there was a greater reduction in TL content of cells treated according to Protocol 3 compared to other Protocols and the control treatment. The level of TL in cells treated according to Protocols 1 and 2 were slightly lower when compared to the control treatment.

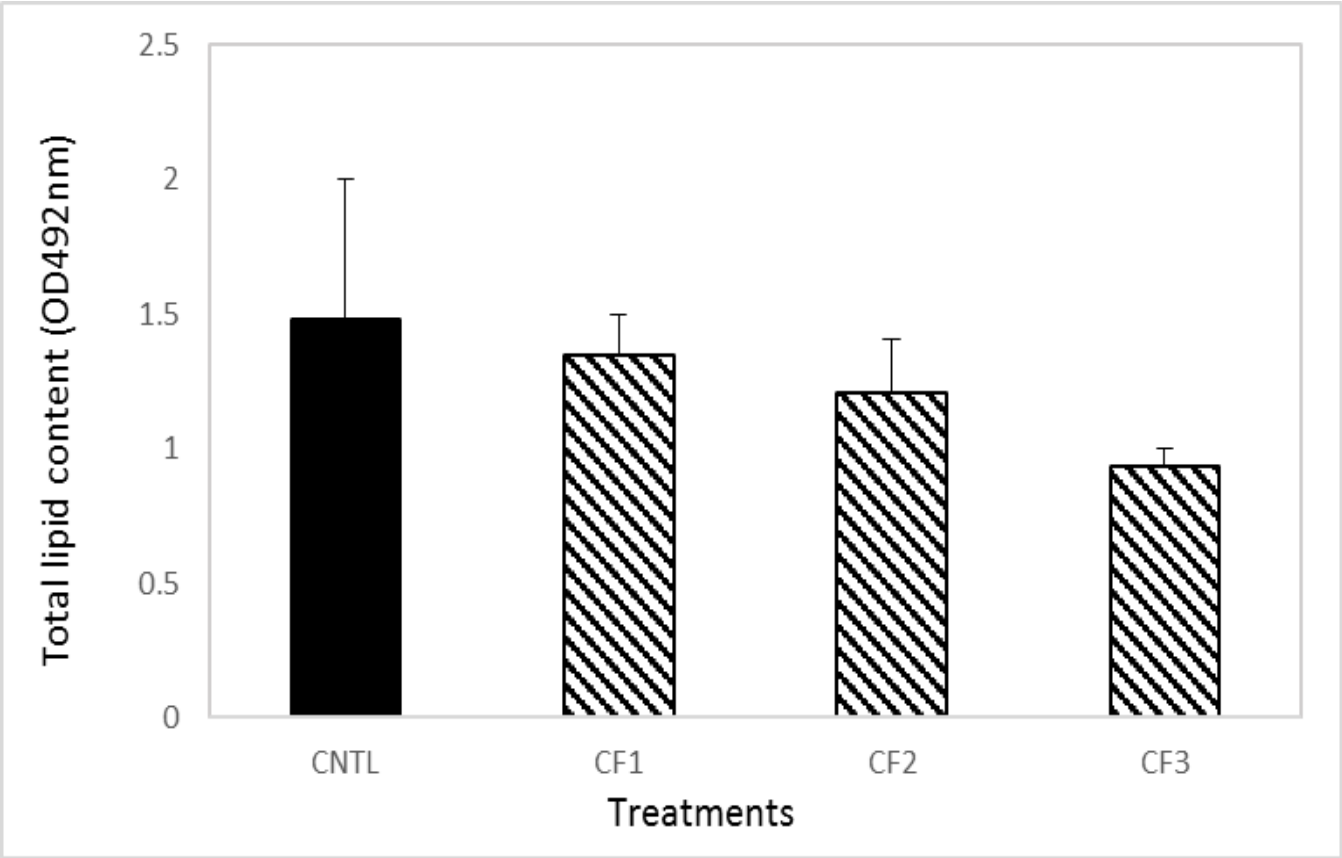

Figure 8. Lipid content of Caco2 cells treated with bacterial cytoplasmic fraction (CF) according to 3 treatment Protocols: Protocol 1 (after overnight bacterial treatment, cells were overlaid with lipids without washing), Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipids and fresh treatments) and Protocol 3 (after overnight bacterial treatment, cells were washed and treated with lipids only). Optical density measurements were determined via spectrophotometry at 492 nm. Each bar represents mean ± SD value of calculated total cellular lipid content (n=3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Filtered Spent Broth (FSB) Treatment

The total lipid content of Caco2 cells treated with FSB (filtered spent broth) were compared in all treatment Protocols to determine whether there were differences in their TL content. There was no significant difference in TL content of Caco2 cells cells between all the Protocols and relative to the control treatment.

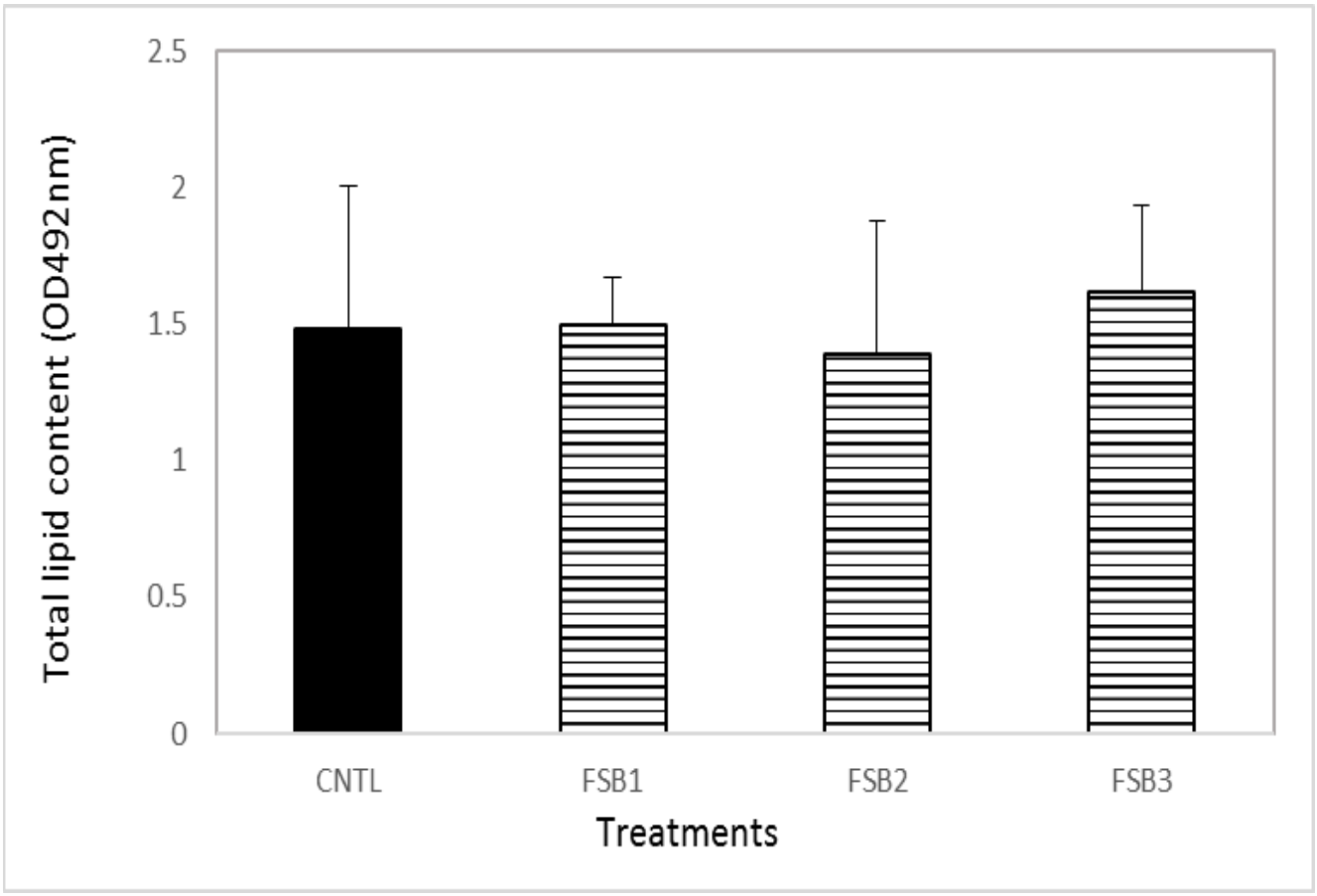

Figure 9. Lipid content for Caco2 cells treated with filtered spent broth (FSB) according to 3 treatment Protocols: Protocol 1 (after overnight bacterial treatment, cells were overlaid with lipids without washing), Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipids and fresh treatments) and Protocol 3 (after overnight bacterial treatment, cells were washed and treated with lipids only). Optical density measurements were determined via spectrophotometry at 492 nm. Each bar represents mean ± SD value of calculated total cellular lipid content (n=3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Heat Inactivated Bacteria (HIB) Treatment

The total lipid content of Caco2 cells treated with HIB (heat inactivated bacteria) were compared across treatment Protocols to determine whether there were differences in their TL content (Figure 10). There was reduction in TL content of cells treated according to Protocol 1 and 3 but was not significantly different compared to other Protocols and relative to the control treatment. There was no reduction in TL content of cells treated according to Protocol 2.

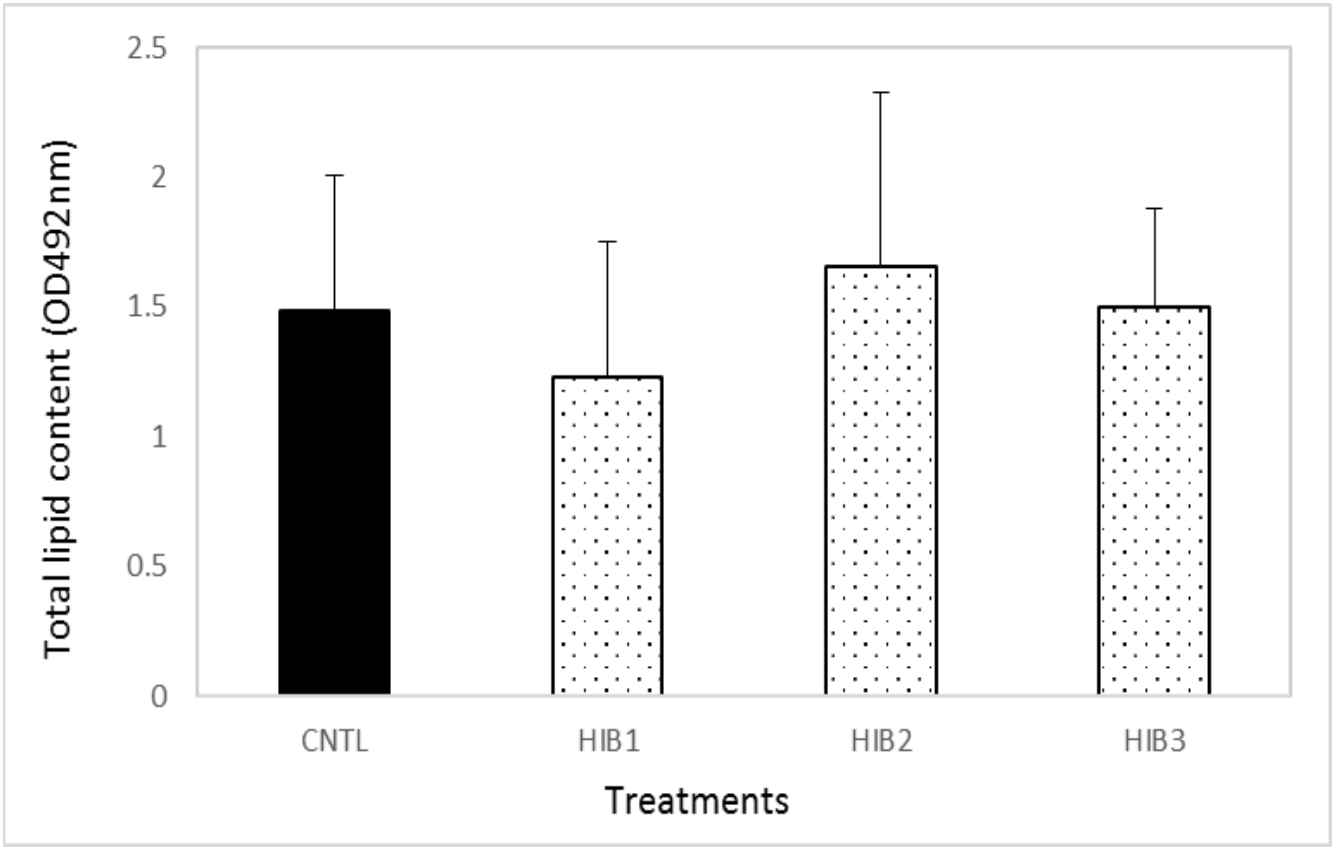

Figure 10. Lipid content of Caco2 cells treated with bacterial heat inactivated bacteria (HIB) according to 3 treatment Protocols: Protocol 1 (after overnight bacterial treatment, cells were overlaid with lipids without washing), Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipids and fresh treatments) and Protocol 3 (after overnight bacterial treatment, cells were washed and treated with lipids only). Optical density measurements were determined via spectrophotometry at 492 nm. Each bar represents mean ± SD value of calculated total cellular lipid content (n=3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Live Bacteria (LB) Treatment

The total lipid content of Caco2 cells treated with LB (live bacteria) were compared across treatment Protocols to determine whether there were differences in their TL content. There was reduction in TL content of cells treated according to Protocol 1 and 2, but TL level was not significantly difference compared to other Protocols and relative to the control treatment. There was however no change in TL content of cells treated according to Protocol 3.

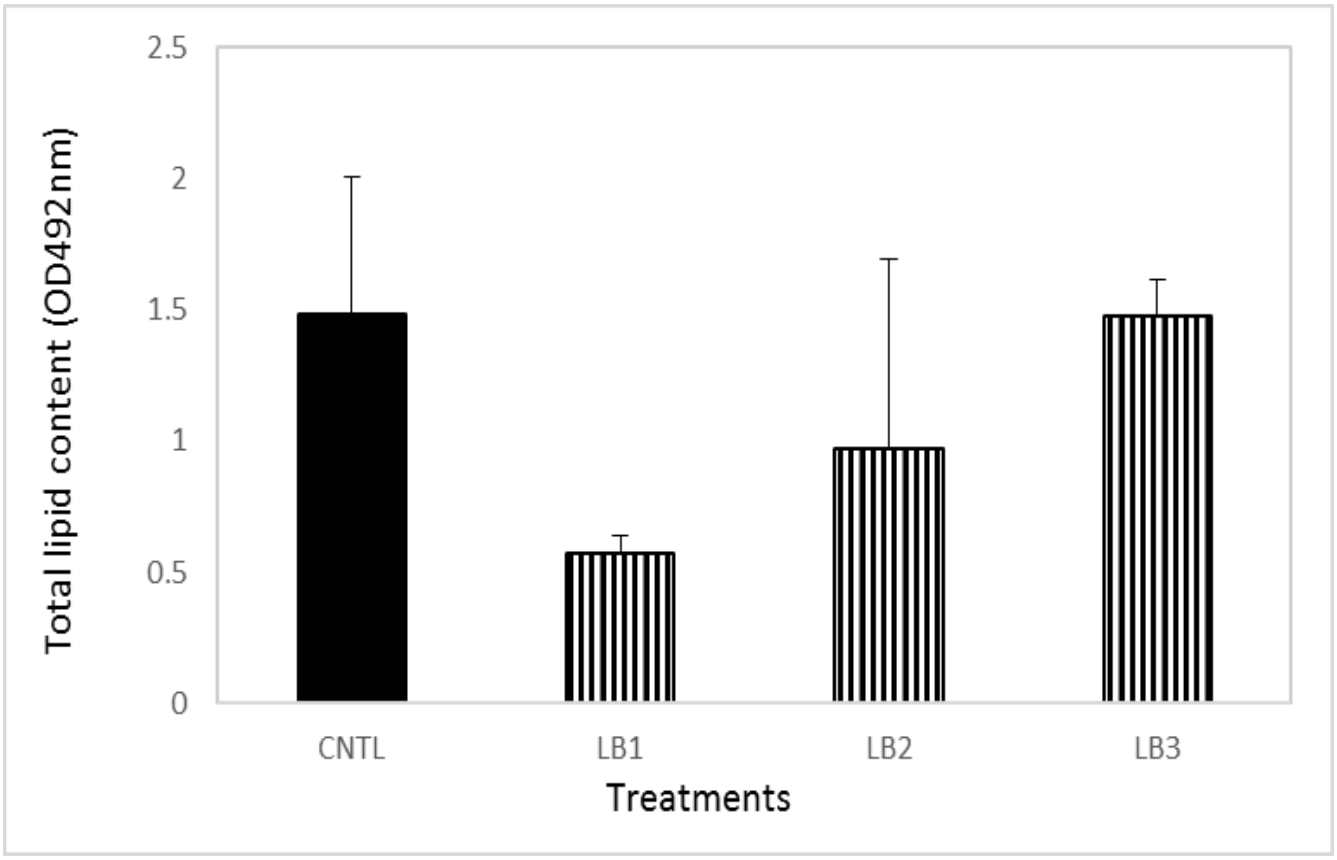

Figure 11. Lipid content of Caco2 cells treated with live *Lactobacillus rhamnosus* MRS6AN(LB) according to 3 treatment Protocols: Protocol 1 (after overnight bacterial treatment, cells were overlaid with lipids without washing), Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipids and fresh treatments) and Protocol 3 (after overnight bacterial treatment, cells were washed and treated with lipids only). Optical density measurements were determined via spectrophotometry at 492 nm. Each bar represents mean ± SD value of calculated total cellular lipid content (n=3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Intracellular Triglyceride Assay

Intracellular Triglyceride Content of Co-Cultured Caco2 Cells Treated According to Protocol 1

In Protocol 1, co-cultured Caco2 cells were overlaid with lipid without washing. A greater and significant reduction of TG accumulation was observed in cells treated with LB compared to the control. Other treatments did not induce significant reduction in intracellular TG content. LB significantly lowered level of TG in cell by 0.1 *(P<0.05)* relative to the control. Cells treated with CF or HIB decreased TG level in cells by 0.03 and 0.06 relative to the control treatment. There was no significant change in TG level of cells treated with FSB relative to the control.

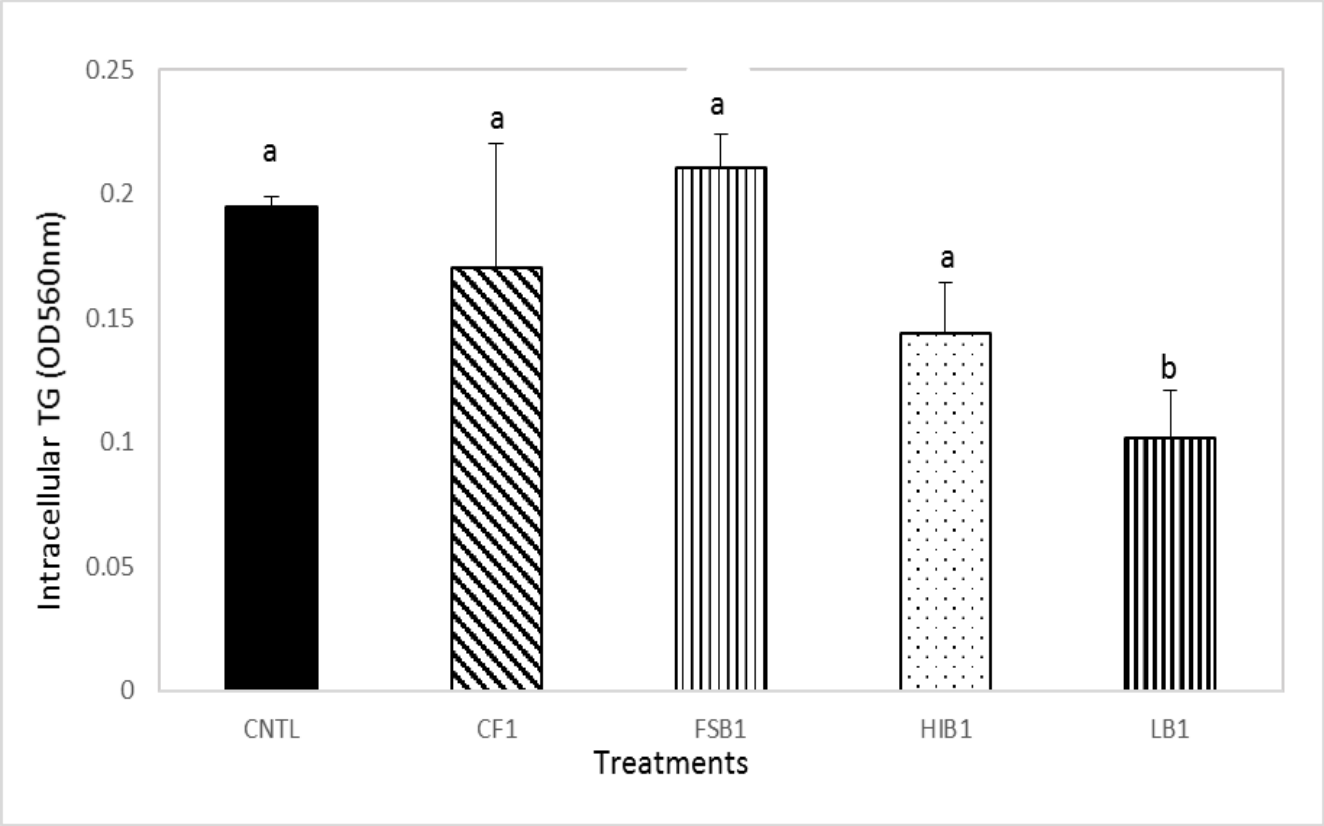

Figure 12. Total intracellular triglyceride level of Caco2 cells treated according to Protocol 1 (after overnight bacterial treatment, cells were overlaid with lipid mixture without washing and incubated for 2 hrs). Quantification of triglyceride content was determined by spectrophotometry at 540nm. Each bar represents mean ± SD value of calculated intracellular triglyceride content (n=3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Table 5. Effect of *L. rhamnosus MRS6AN* or its extracts on total intracellular triglyceride content of Caco2 cells treated according to Protocol 1 (After overnight bacterial treatment, cells were overlaid with lipid mixture without washing and incubated for 2 hrs.). Optical density was measured at 540 nm. Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control.

Treatment (200ul)	**Triglyceride level (OD540 nm)**
Control	0.20
Cytoplasmic fraction	0.17
Filtered Spent broth	0.21
Heat inactivated bacteria	0.14
Live bacteria	0.10

Intracellular Triglyceride Content of Co-Cultured Caco2 Cells Treated According to Protocol 2

In Protocol 2, co-cultured cells were washed and treated with lipid mixture and live bacteria or its extracts. A significant reduction in TG accumulation was observed in LB treated cells compared with the control (Figure 13). LB induced significant reduction in TG level of cell by 0.076 compared to the control treatment ($P < 0.05$). Other treatments did not induce significant change in TG level of Caco2 cells.

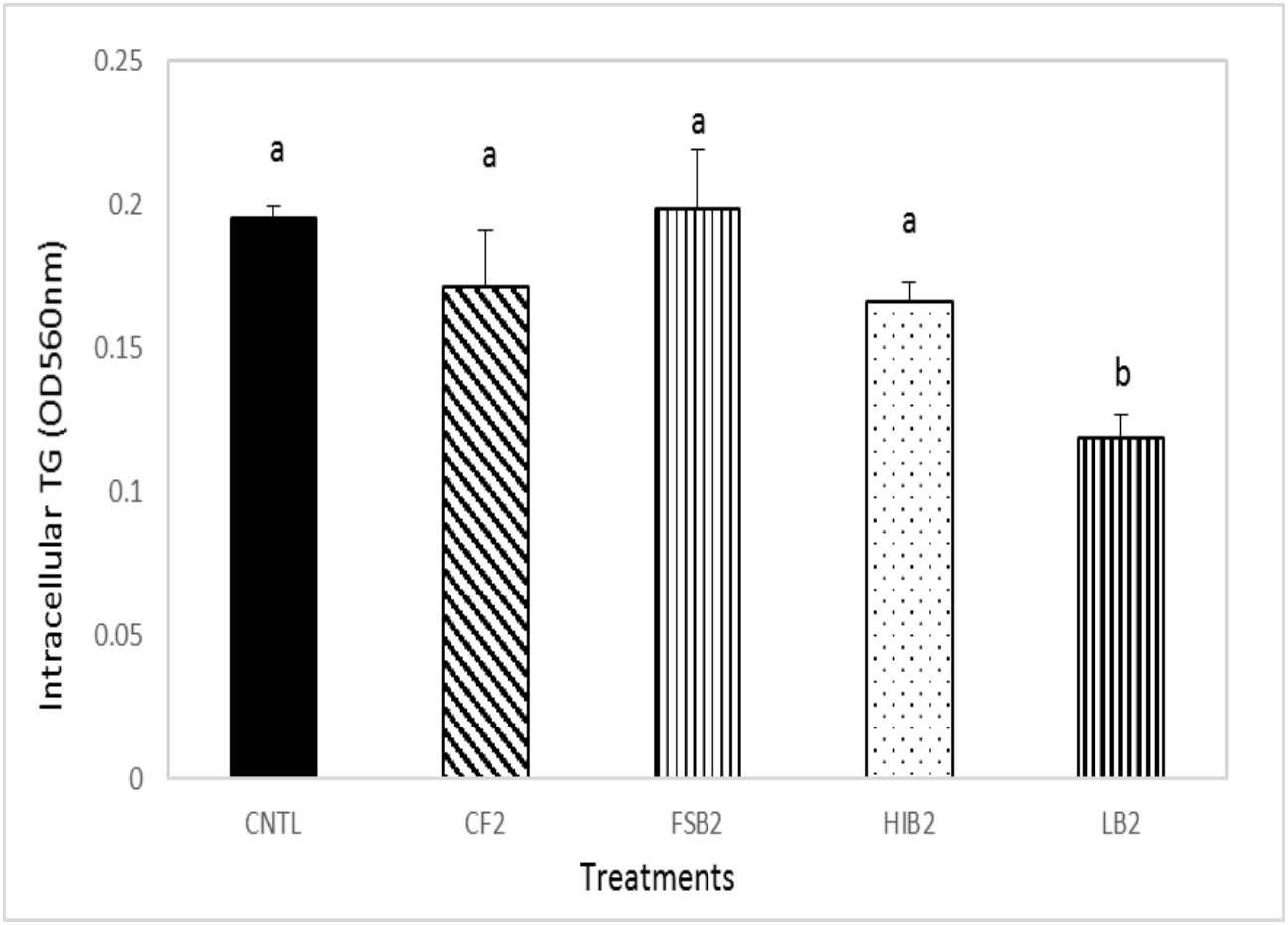

Figure 13. Total intracellular triglyceride level of Caco2 cells treated according to Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipid mixture and fresh bacteria treatments and incubated for 2 hrs.). Quantification of triglyceride content was determined by spectrophotometry at 540nm. Each bar represents mean ± SD value of calculated intracellular triglyceride content (n=3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Table 6. Effect of *L. rhamnosus MRS6AN* or its extracts on total cellular lipid content of Caco2 cells treated according to Protocol 2 (After overnight bacterial treatment, cells were washed and treated with lipid mixture and fresh bacteria treatments and incubated for 2 hrs.). Optical density was measured at 540 nm. Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control.

Treatment (200ul)	**Triglyceride level (OD540 nm)**
Control	0.20
Cytoplasmic fraction	0.17
Filtered Spent broth	0.20
Heat inactivated bacteria	0.17
Live bacteria	0.12

Intracellular triglyceride content of co-cultured Caco2 cells treated according to Protocol 3

In Protocol 3, overnight co-cultured Caco2 cells were washed and treated with fresh lipid mixture. There was no inhibitory effect on TG level of cells treated with CF, FSB and HIB respectively when compared to the control (Figure 14). However, treatment with LB induced slight reduction of 0.05 in the intracellular TG level when compared to the control.

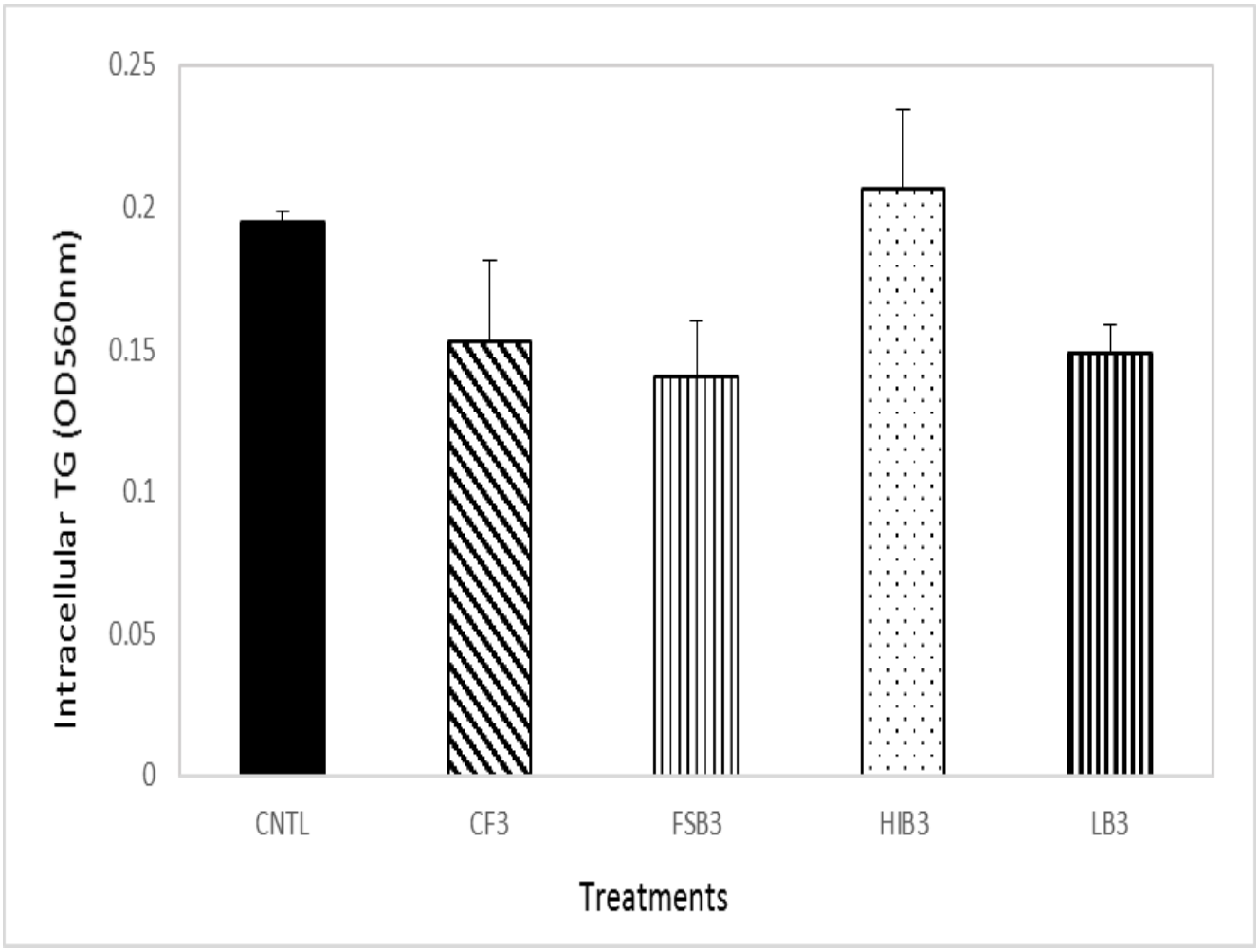

Figure 14. Total intracellular triglyceride level of Caco2 cells treated according to Protocol 3 (after overnight bacterial treatment, cells were washed and treated with lipid mixture only and incubated for 2 hrs). Quantification of triglyceride content was determined by spectrophotometry at 540nm. Each bar represents mean ± SD value of calculated intracellular triglyceride content (n=3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Table 7. Effect of *L. rhamnosus MRS6AN* or its extracts on total cellular lipid content of Caco2 cells treated according to Protocol 3 (after overnight treatment, cells were washed and treated with lipid mixture only and incubated for 2 hrs). Optical density was measured at 540 nm. Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control.

Treatment (200 ul)	**Triglyceride level (OD540 nm)**
Control	0.20
Cytoplasmic fraction	0.16
Filtered Spent broth	0.14
Heat inactivated bacteria	0.21
Live bacteria	0.15

Comparison Between Effects of Probiotic Treatments on Intracellular TG Content between Treatment Protocols.

Effect of Bacterial Cell Extract on Intracellular TG Level in Caco-2 Cell-Lysate

The cytoplasmic fraction (CF) treated cells (treated according to Protocol 3) demonstrated the lowest TG level of 0.05 but with no significance difference compared to other Protocols and the control treatment (Figure 15). However, CF treated cells in Caco2 cells in Protocol 1 (overlaid with lipids without washing) or Protocol 2 (washed and treated with lipids and bacterial treatments) no significant effect on intracellular TG content relative to the control.

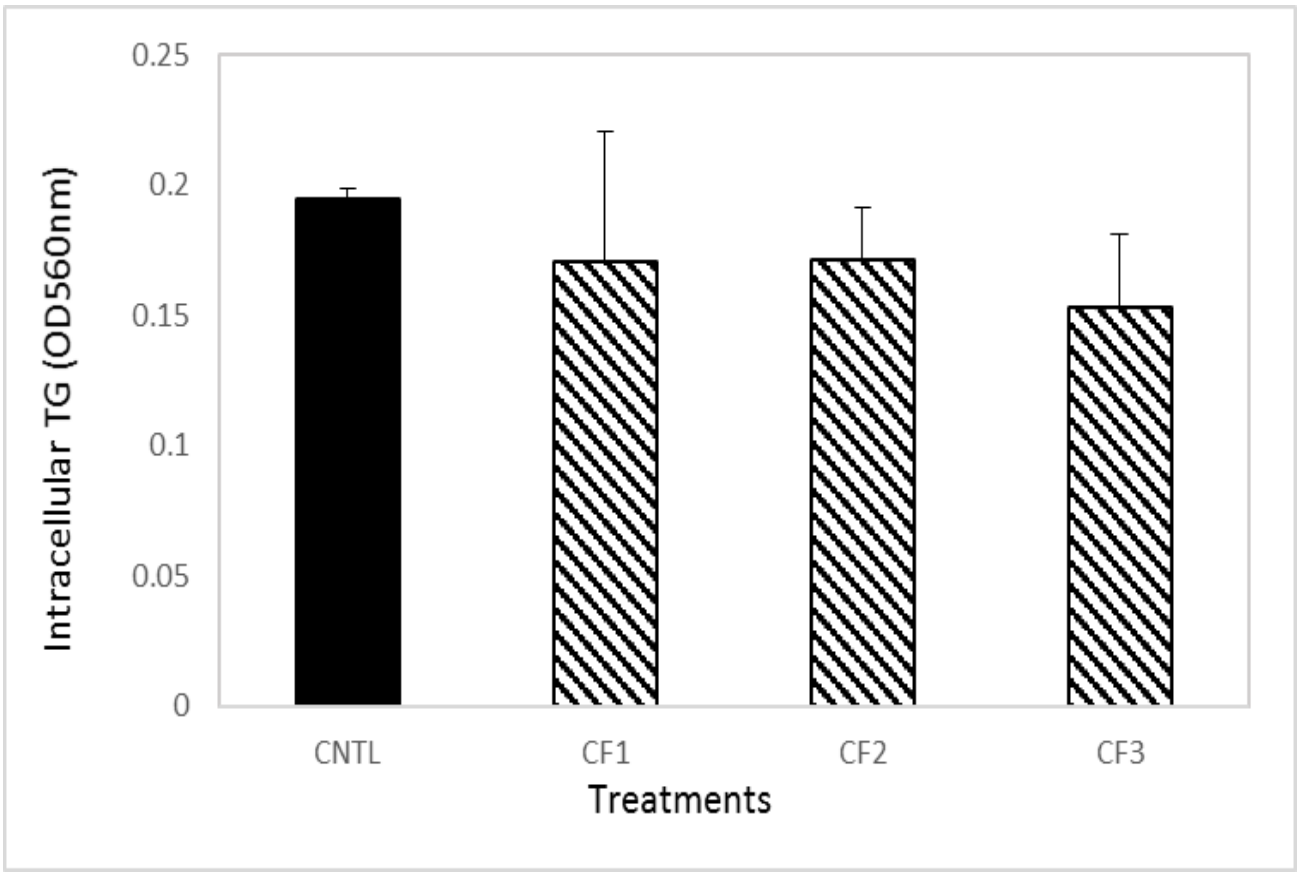

Figure 15. Intracellular triglyceride content of Caco2 cells treated with bacterial cytoplasmic fraction (CF) according to 3 treatment Protocols: Protocol 1 (after overnight bacterial treatment, cells were overlaid with lipids without washing), Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipids and fresh treatments) and Protocol 3 (after overnight bacterial treatment, cells were washed and treated with lipids only). Absorbance was determined via spectrophotometry at optical density of 540 nm. Each bar represents mean ± SD value of calculated intracellular triglyceride content (n=3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Bacterial Filtered Spent Broth on Intracellular TG Level in Caco2 Cells

Bacterial filtered spent broth (FSB) treated cells in Protocol 1 (overlaid with lipids without washing) demonstrated negligible reduction in TG level of 0.06 compared to the control (Figure 16). Although FSB treated Protocols 1 and 2 showed no considerable change in TG level of Caco2 cells, there was however a significant increase in intracellular TG level of cells treated according to protocol 3 compared with cells treated according to protocol 1.

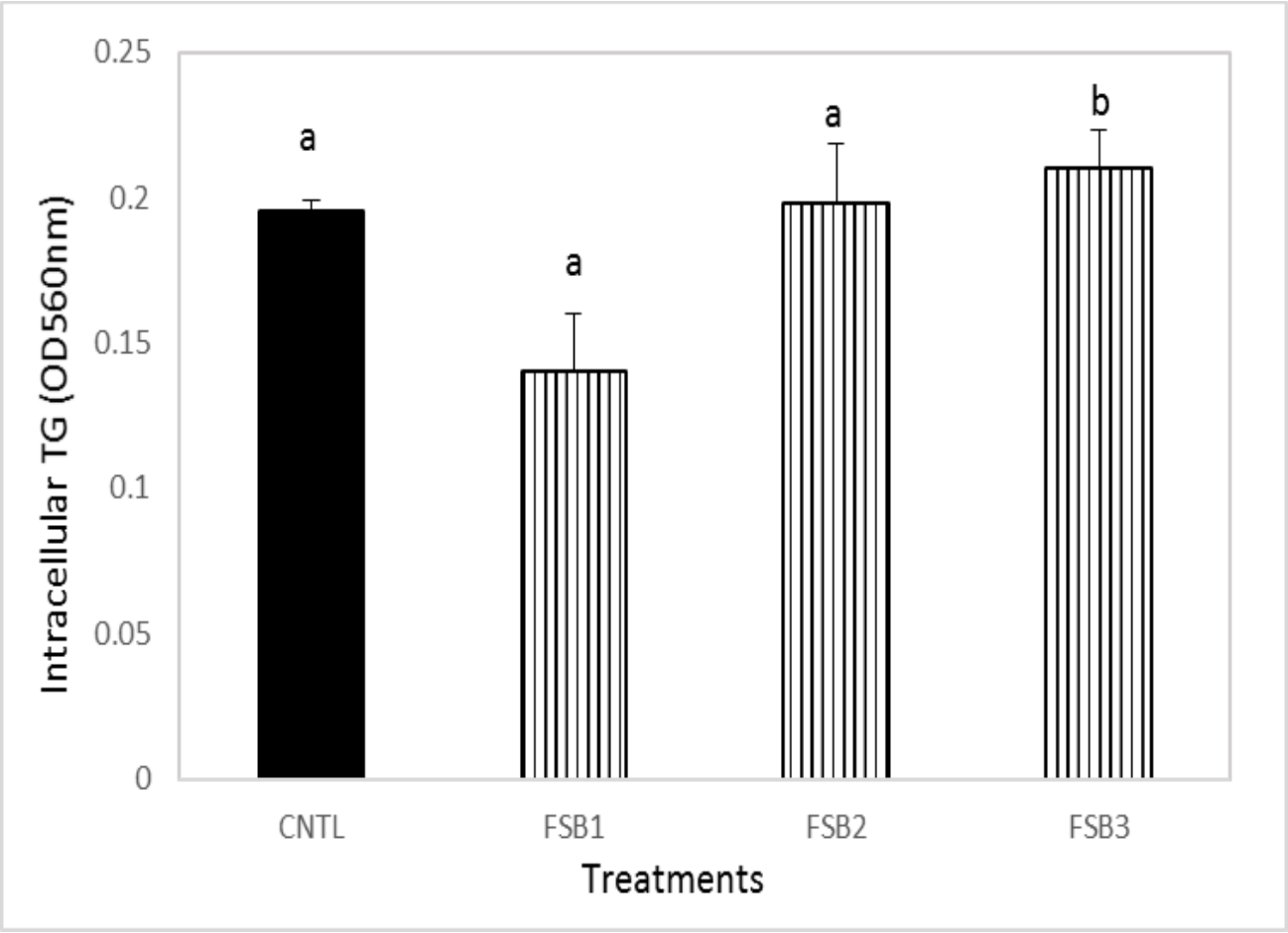

Figure 16. Intracellular triglyceride content of Caco2 cells treated with bacterial filtered spent broth according to 3 treatment Protocols: Protocol 1 (after overnight bacterial treatment, cells were overlaid with lipids without washing), Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipids and fresh treatments) and Protocol 3 (after overnight bacterial treatment, cells were washed and treated with lipids only). Each bar represents mean ± SD value of calculated intracellular triglyceride content (n=3). Absorbance was determined via spectrophotometry at optical density of 540 nm. Each bar represents mean ± SD value of calculated intracellular triglyceride content (n=3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Heat Inactivated Bacteria (HIB) on Intracellular Triglyceride Level

Heat Inactivated bacteria (HIB) treated cells as per Protocol 3 (washed and treated with lipids only) exhibited a significant increase in Caco-2 cell TG level by 0.07 compared to the treatment according to protocol 1 (Figure 17). Cells treated according to all treatment protocols demonstrated no significant change in intracellular TG content relative to the control.

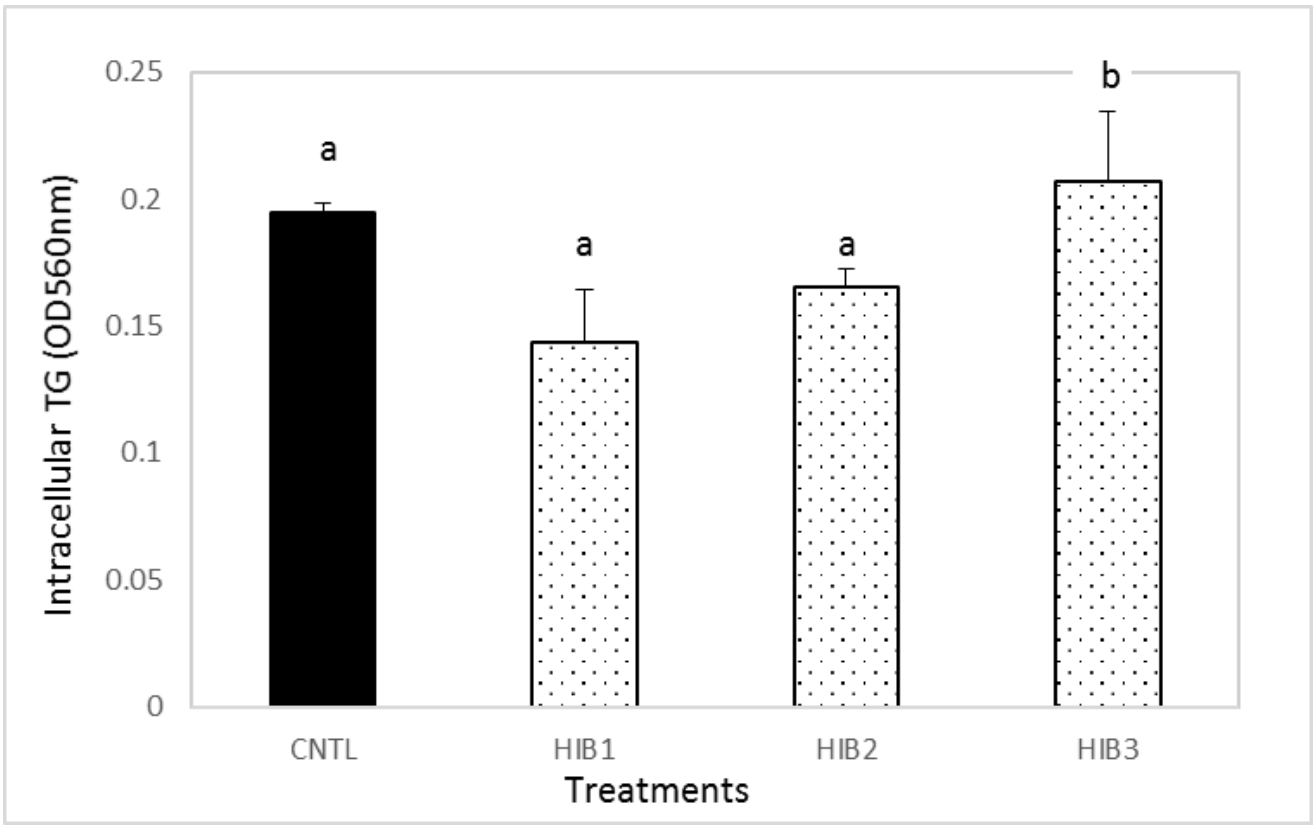

Figure 17. Intracellular triglyceride content of Caco2 cells treated with heat inactivated bacteria (HIB) according to 3 treatment Protocols: Protocol 1 (after overnight bacterial treatment, cells were overlaid with lipids without washing), Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipids and fresh treatments) and Protocol 3 (after overnight bacterial treatment, cells were washed and treated with lipids only). Absorbance was determined via spectrophotometry at optical density of 540 nm. Each bar represents mean ± SD value of calculated intracellular triglyceride content (n=3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Live Bacteria (LB) on Intracellular TG Level in Caco-2 Cell

Live bacteria (LB) applied as per Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipids and fresh treatments) and Protocol 3 (after overnight bacterial treatment, cells were washed and treated with lipids only) significantly reduced TG level by 0.1 and 0.08 relative to the control (Figure 18). However, LB applied as per Protocol 1 (overlaid with lipids without washing) induced the lowest TG level of 0.1 when compared to the control.

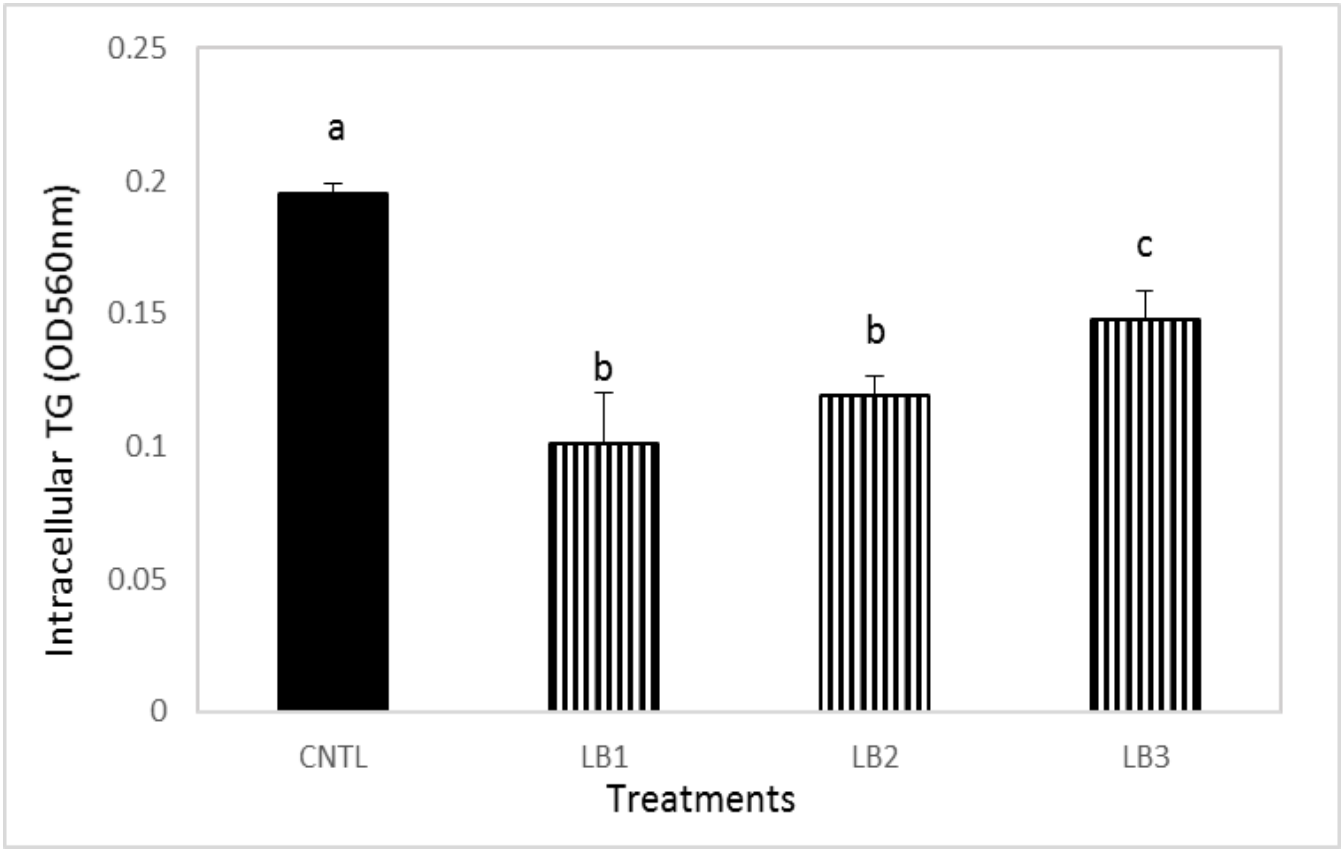

Figure 18. Intracellular triglyceride content of Caco2 cells treated with live *Lactobacillus rhamnosus* MRS6AN (LB) according to 3 treatment Protocols: Protocol 1 (after overnight bacterial treatment, cells were overlaid with lipids without washing), Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipids and fresh treatments) and Protocol 3 (after overnight bacterial treatment, cells were washed and treated with lipids only). Absorbance was determined via spectrophotometry at optical density of 540 nm. Each bar represents mean ± SD value of calculated intracellular triglyceride content (n=3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Probiotic Treatments on MTP Expression

The *MTP* protein expression analysis revealed that LB treatments in all Protocol 1 (after overnight bacterial treatment, cells were overlaid with lipids without washing), Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipids and fresh treatments) and Protocol 3 (after overnight bacterial treatment, cells were washed and treated with lipids only) induced observable reduction in *MTP* expression at 5.50, 4.16 and 5.59-fold respectively compared to the control treatment, with the highest protein reduction being in Protocol 3 (Figure 19 and Table 8). There was little or no change in expression in all cells treated with FSB in Protocols 2 and 3. Heat inactivated bacteria treatment caused slight protein reduction in Protocols 1 and 2 with 1.59 and 1.43-fold reduction respectively.

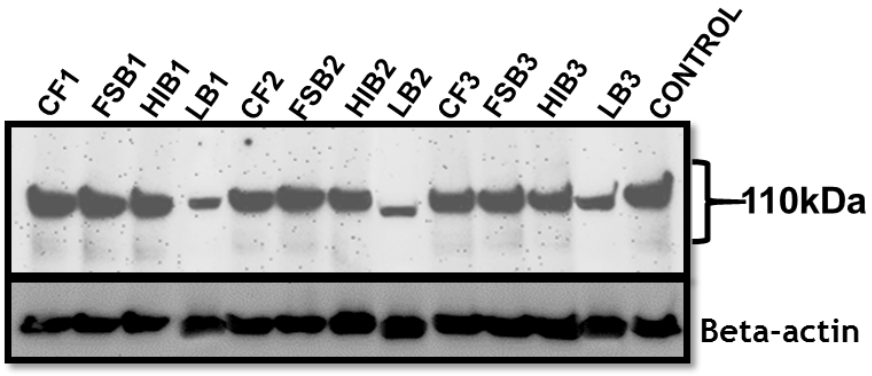

Figure 19. Western blot assay image of *MTP* expression in Caco2 cells
(CF- Cytoplasmic fraction, FSB- Filtered spent broth, HIB- Heat inactivated bacteria, LB- Live bacteria).

MTP Expression of Co-Cultured Caco2 Cells Treated According to Protocol 1

Co-cultured Caco2 cells were overlaid with lipid without washing, after an overnight incubation period (Figure 20). A significant reduction in *MTP* expression was observed in cells treated with LB compared to other treatments and the control. Other treatments did not induce significant change in *MTP* expression of cells. LB significantly lowered *MTP* expression in cell by 6.35 *(P<0.05)* relative to the control. Cells treated with CF, FSB or HIB did not show significant change in *MTP* expression relative to the control.

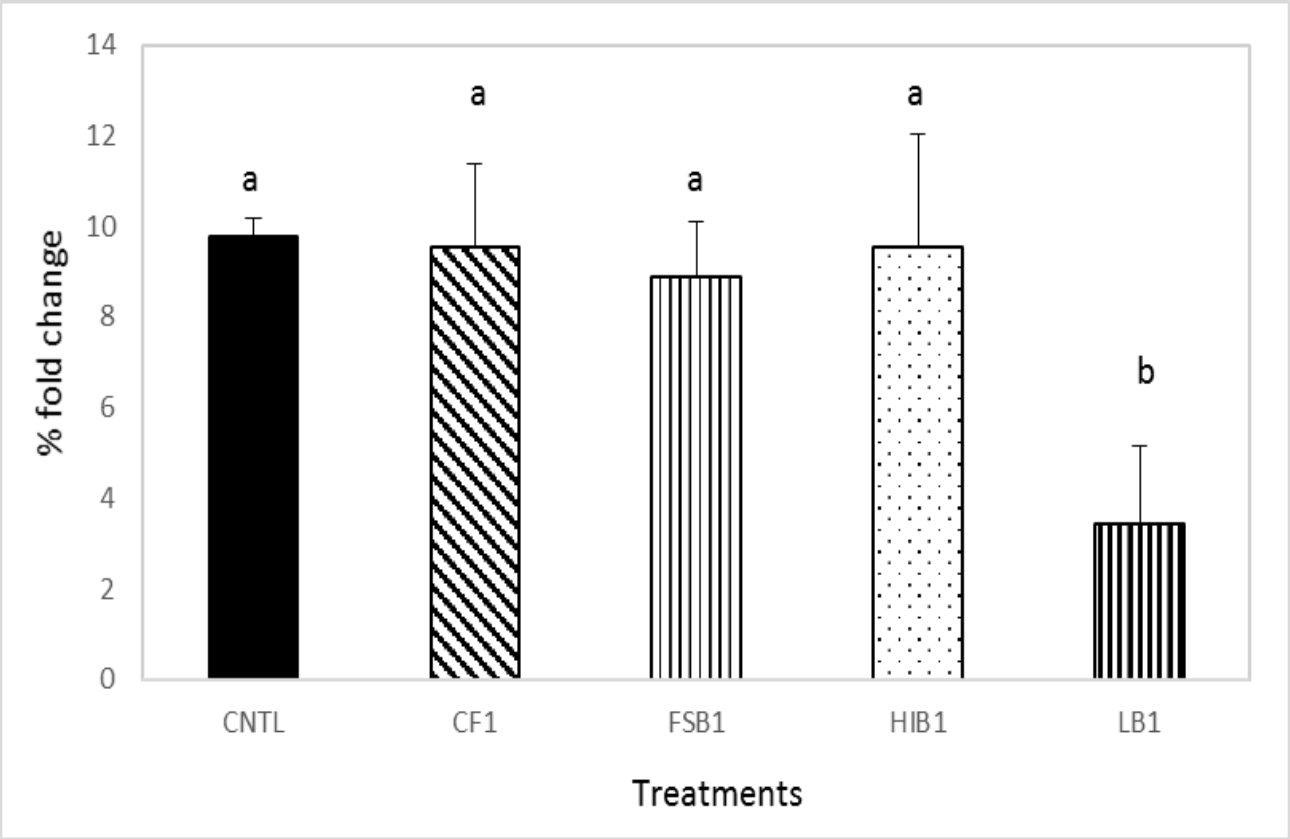

Figure 20. Effect of *Lactobacillus rhamnosus* MRS6AN or its extracts on *MTP* expression in Caco2 cells treated according to Protocol 1 (after overnight bacterial treatment, cells were overlaid with lipid mixture without washing and incubated for 2 hrs). Each bar represents mean ± SD value of percent fold change (n=3). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control. Bars labelled with different letters are statistically different from each other *(P≤0.05).*

MTP Expression of Co-Cultured Caco2 Cells Treated According to Protocol 2

Overnight treated cells were washed 3 times in PBS, and treated with fresh bacteria treatments. Changes in *MTP* expression was not significantly different among all treatments and the control (Figure 21). Nevertheless, LB treatment slightly lowered *MTP* expression in cell by 4.4 relative to the control.

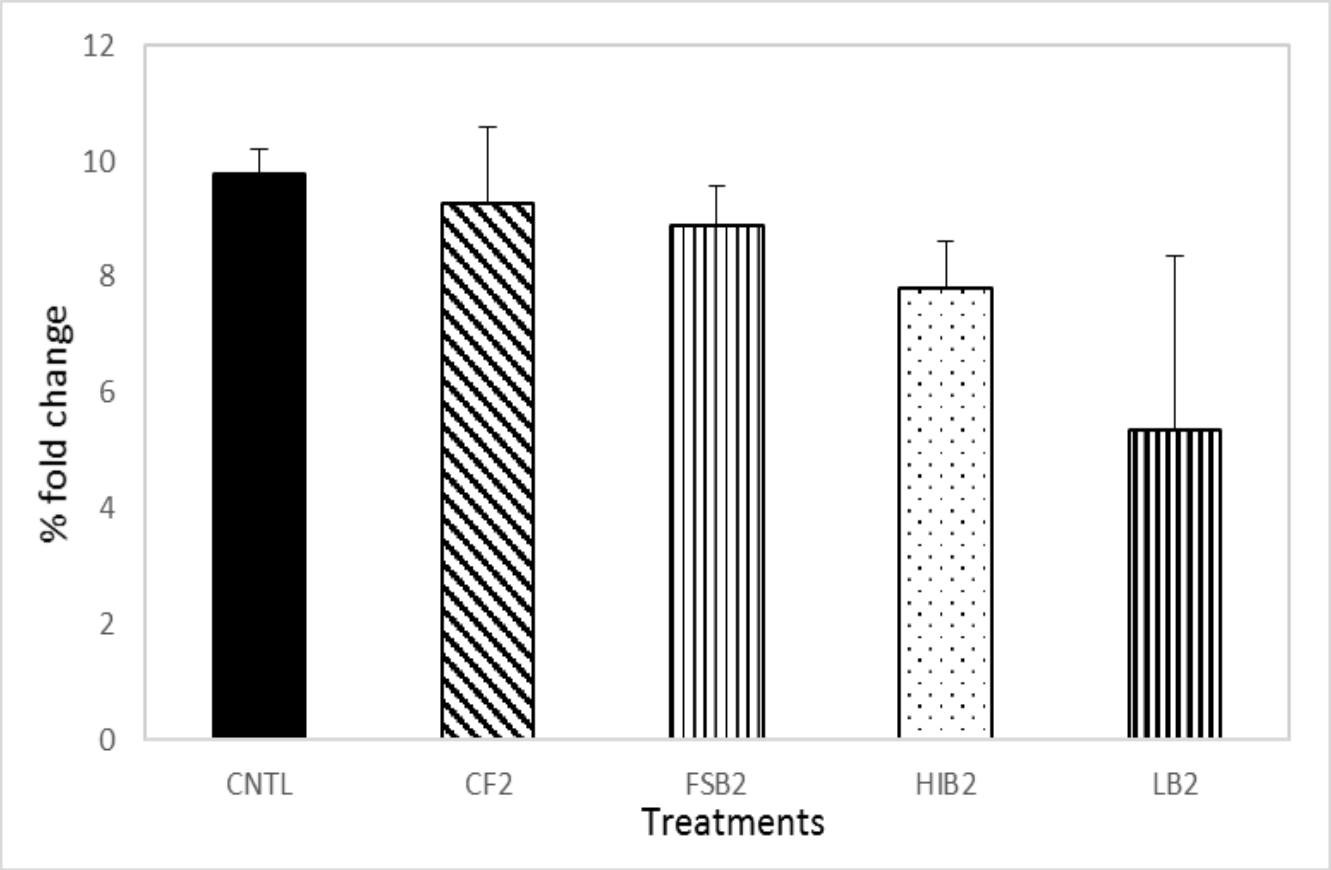

Figure 21. Effect of *Lactobacillus rhamnosus* MRS6AN or its extracts on *MTP* expression in Caco2 cells treated according to Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipid mixture and fresh bacterial treatments and incubated for 2 hrs). Each bar represents mean ± SD value of percent fold change (n=3). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control. Bars labelled with different letters are statistically different from each other *(P≤0.05).*

MTP Expression of Co-Cultured Caco2 Cells Treated According to Protocol 3

Caco2 cells treated according to protocol 3 (overnight co-cultured cells were washed and treated lipids only) showed significant reduction in *MTP* expression in cells treated with LB compared to FSB treated cells and the control by 5.3 and 5.94 respectively. Other treatments did not induce significant change in *MTP* expression of cells (Figure 22).

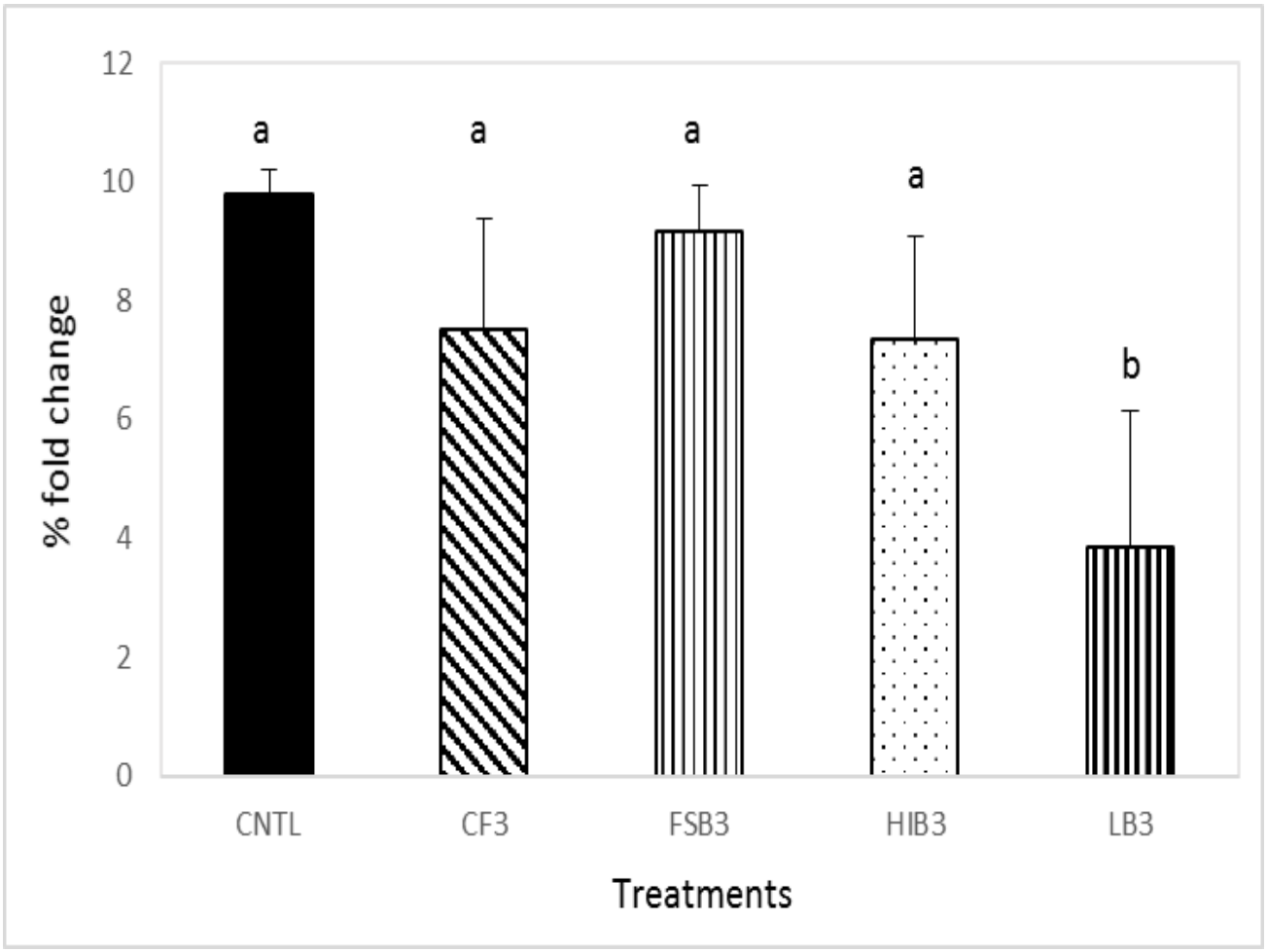

Figure 22. Effect of *Lactobacillus rhamnosus* MRS6AN or its extracts on *MTP* expression in Caco2 cells treated according to Protocol 3 (after overnight bacterial treatment, cells were washed and treated with lipid mixture only and incubated for 2 hrs). Each bar represents mean ± SD value of percent fold change (n=3). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control. Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of bacterial cytoplasmic fraction (CF) on MTP expression

There were no significant differences in *MTP* expression across all Protocols in Caco2 cells treated with bacteria cytoplasmic fraction (CF) and relative to the control treatment (Figure 23).

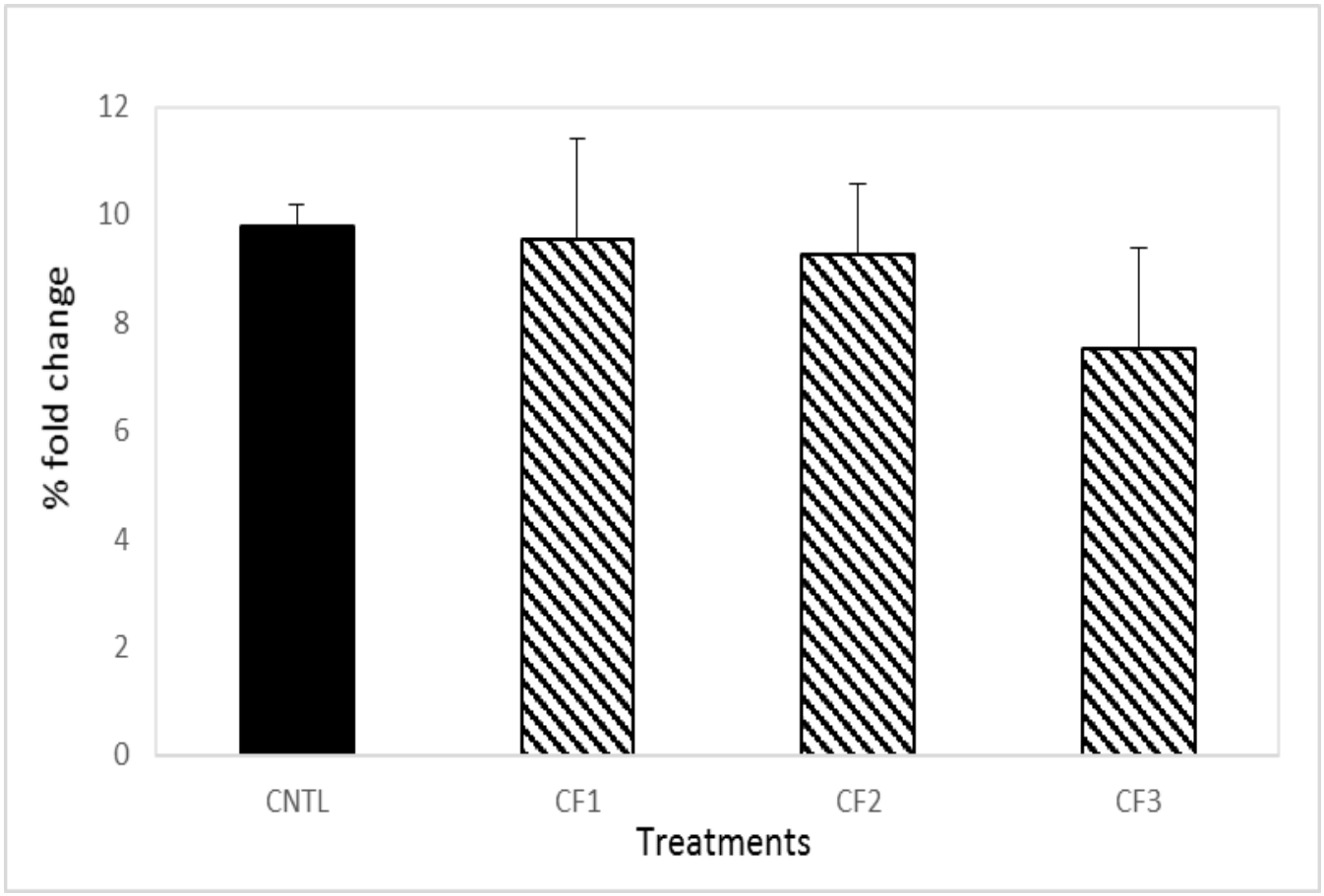

Figure 23. Effects of bacterial cell extracts (CF) on *MTP* expression in Caco2 cells. The CF was applied under three Protocol (Protocol 1: overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs, Protocol 2: overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs, Protocol 3: overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs). Each bar represents mean fold change relative to the control treatment (n=2 to 3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Bacterial Filtered Spent Broth (FSB) on MTP Expression

The presence of filtered spent broth (FSB) did not induce any significant change in *MTP* expression level in Caco2 cells across all protocols (Figure 24).

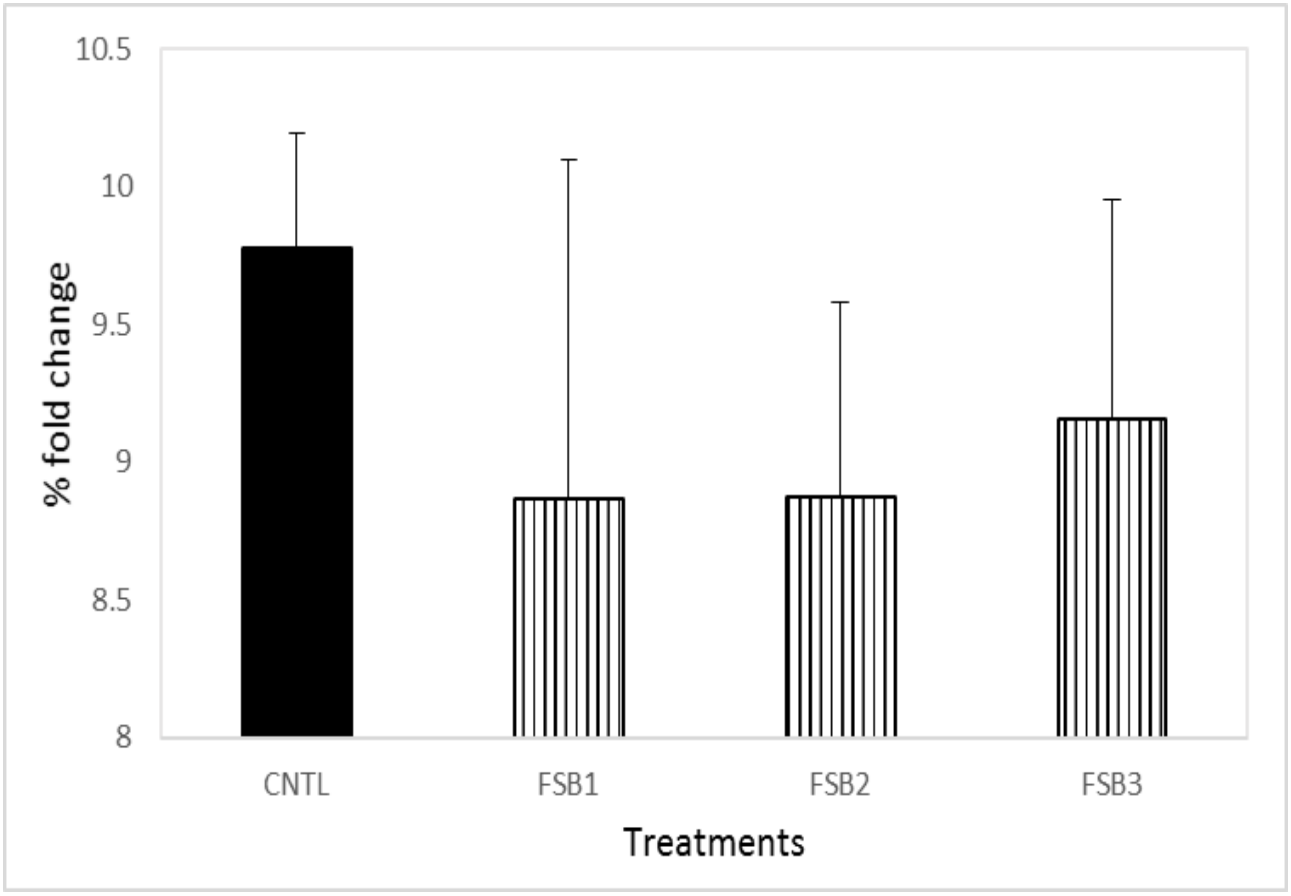

Figure 24. Effects of bacterial filtered spent broth (FSB) on *MTP* expression in Caco2 cells. The FSB was applied under three Protocol (Protocol 1: overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs, Protocol 2: overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs, Protocol 3: overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs). Each bar represents mean fold change relative to the control treatment (n=2 to 3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Heat Inactivated Bacteria (HIB) on MTP Expression

Lactobacillus rhamnosus was heat inactivated for 15 minutes at 90^{o}C to determine the effect of heat killed bacteria on MTP expression in Caco2 cells. The result showed that heat inactivated bacteria induced no significant change in *MTP* expression of Protocol 1 (overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs), Protocol 2 (overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs) and Protocol 3 (overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs) and relative to the control treatment (Figure 25).

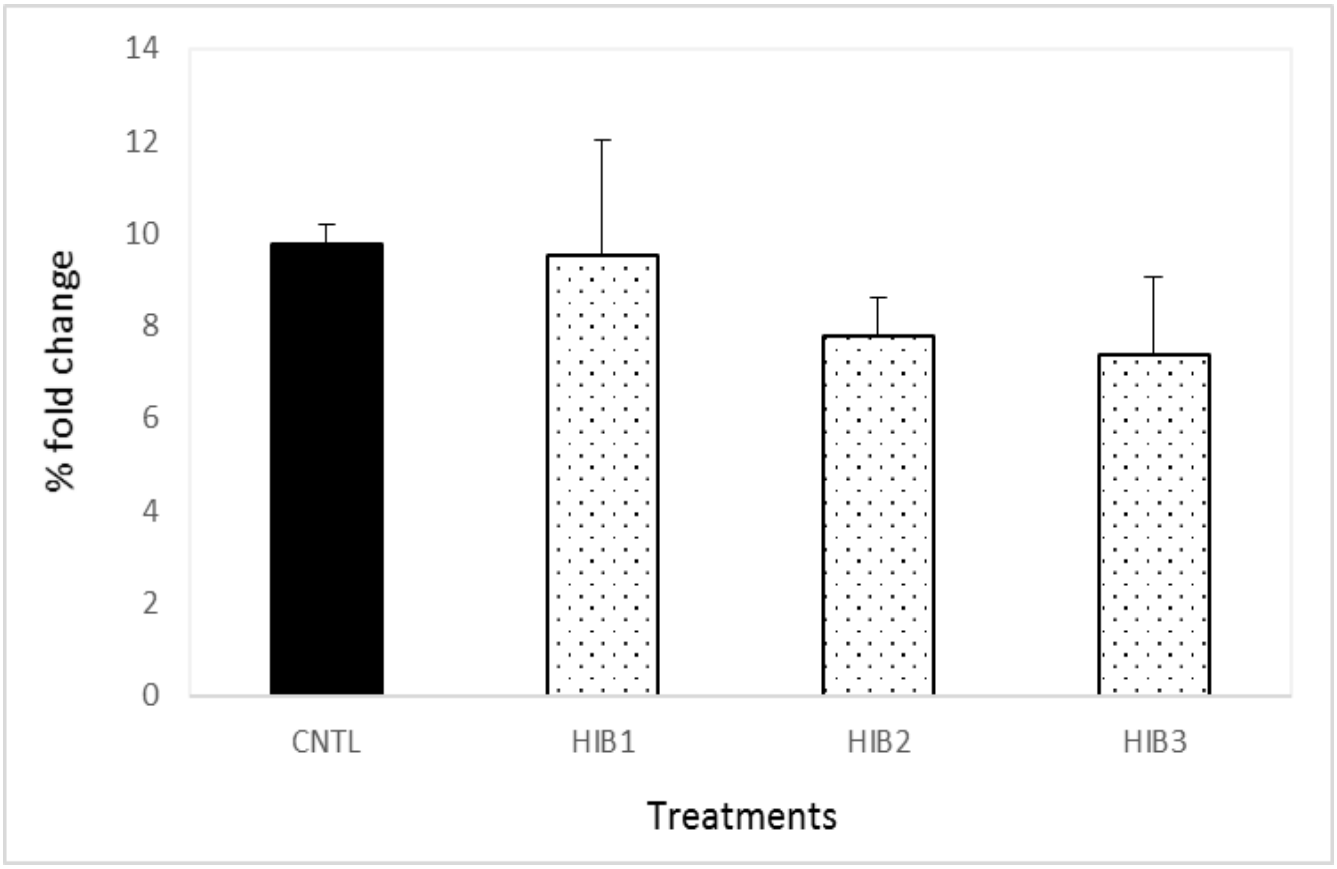

Figure 25. Comparison of effects of heat inactivated bacteria (HIB) on *MTP* expression in Caco2 cells. The HIB was applied under three Protocol (Protocol 1: overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs, Protocol 2: overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs, Protocol 3: overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs). Each bar represents mean fold change relative to the control treatment (n=2 to 3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Bacterial Live Bacteria (LB) on MTP Expression

Lactobacillus rhamnosus MRS6AN significantly reduced *MTP* expression in Protocols 1 (overlaid with lipids without washing) and 3 (washed and treated with fresh bacterial treatments) when compared to the control treatment, nevertheless, Caco2 cells treated as per Protocol 1 induced the lowest *MTP* expression by 6.35 percentage fold-change relative to the control treatment (Figure 23). There was no considerable difference in *MTP* expression among the 3 protocols.

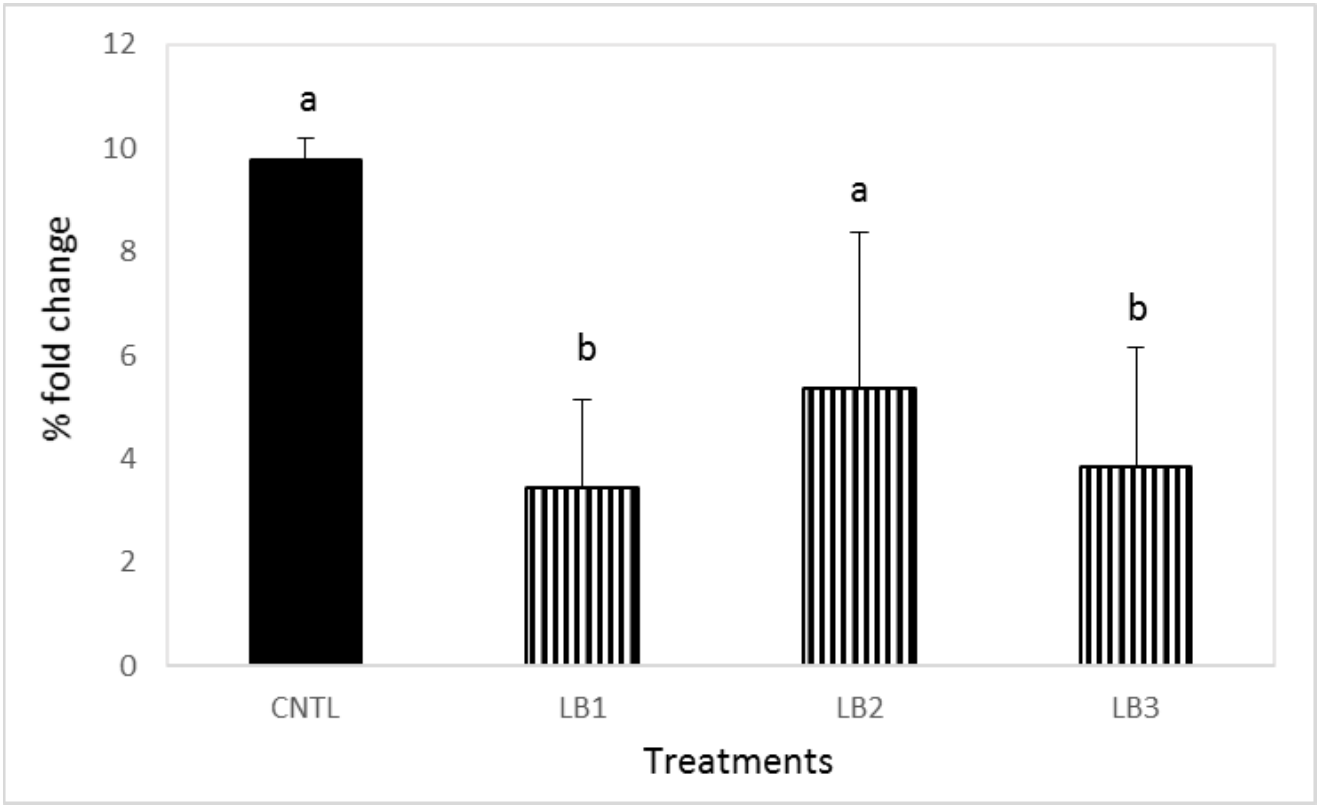

Figure 26. Effects of live bacteria (LB) on *MTP* expression in Caco2 cells. LB was applied under three Protocol (Protocol 1: overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs, Protocol 2: overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs, Protocol 3: overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs). Each bar represents mean fold change relative to the control treatment (n=2 to 3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Table 8. Effect of live *L. rhamnosus* MRS6AN, heat inactivated bacteria, filtered spent broth or its cytoplasmic fraction on *MTP* expression in Caco2 cells.

TREATMENT	CNTRL	CF1	CF2	CF3	FSB1	FSB2	FSB3	HIB1	HIB2	HIB3	LB1	LB2	LB3
MTP REL. EXPRESSION*	9.78	9.56	9.25	7.53	8.87	8.88	9.15	9.53	7.79	7.37	3.43	5.36	3.84

*Expression values are mean fold change, where n=1 to 4. Mean fold change represent protein expression relative to the control.

Effect of Probiotic Treatments on GPAT3 Expression

GPAT3 expression was significantly reduced in LB treated cells according to protocol 1 (overnight co-culture Caco2 cells overlaid with lipids without washing and incubated for 2 hrs), 2 (co-cultured cells washed and treated with fresh bacterial treatments) and 3 (overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs) compared to other treatments and the control (Figure 27 and Table 9) Furthermore, LB in Protocols 1, 2 and 3 lowered expression of GPAT3 by 7.32, 7.40 and 4.92-fold change respectively relative to the control treatment. Other treatments induced no significant effect on *GPAT3* expression in all protocols relative to the control.

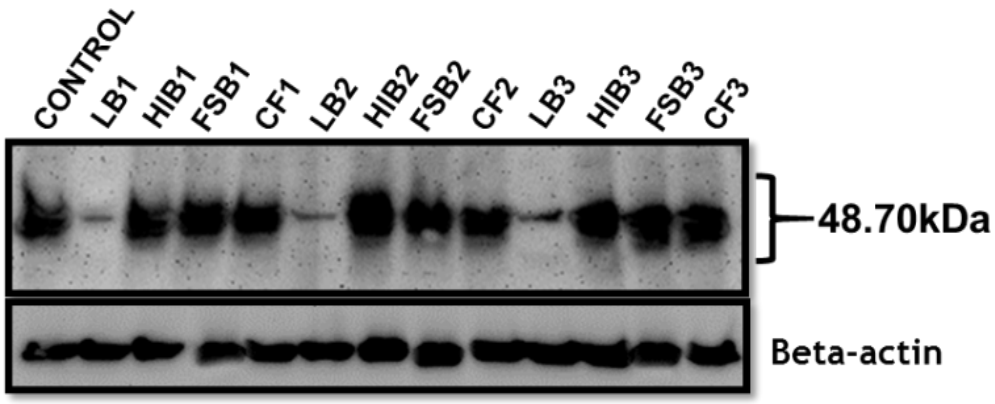

Figure 27. Western blot assay image of GPAT3 expression in Caco2 cells
(CF- Cytoplasmic fraction, FSB- Filtered spent broth, HIB- Heat inactivated bacteria, LB- Live bacteria)

GPAT3 expression of co-cultured Caco2 cells treated according to Protocol 1

Co-cultured Caco2 cells were overlaid with lipid without washing, after an overnight incubation period. Significant reduction in *GPAT3* expression was observed in Caco2 cells treated with LB compared to other treatments and the control (Figure 28). Other treatments did not induce significant change in *GPAT3* expression in cells.

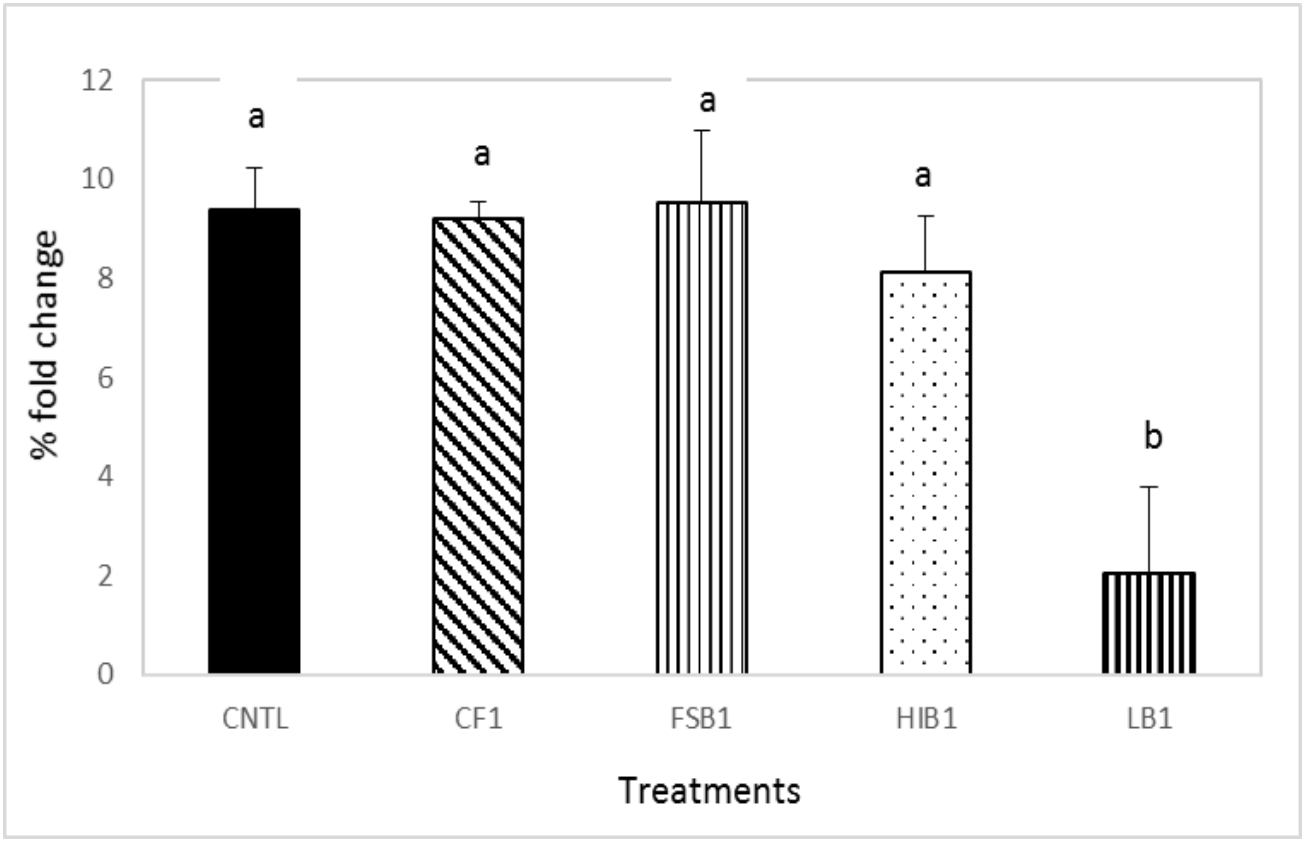

Figure 28. Effect of *Lactobacillus rhamnosus* MRS6AN or its extracts on *MTP* expression in Caco2 cells treated according to Protocol 1 (after overnight bacterial treatment, cells were overlaid with lipid mixture without washing and incubated for 2 hrs). Each bar represents mean ± SD value of percent fold change (n=3). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control. Bars labelled with different letters are statistically different from each other *(P≤0.05).*

GPAT3 Expression of Co-Cultured Caco2 Cells Treated According to Protocol 2

Overnight co-cultured cells treated according to protocol 2 (cells washed and treated with fresh bacteria treatments) showed significant reduction in *GPAT3* expression when treated with LB compared to other treatments and the control (Figure 29). There was however no significant change in *GPAT3* expression in other treatments relative to the control.

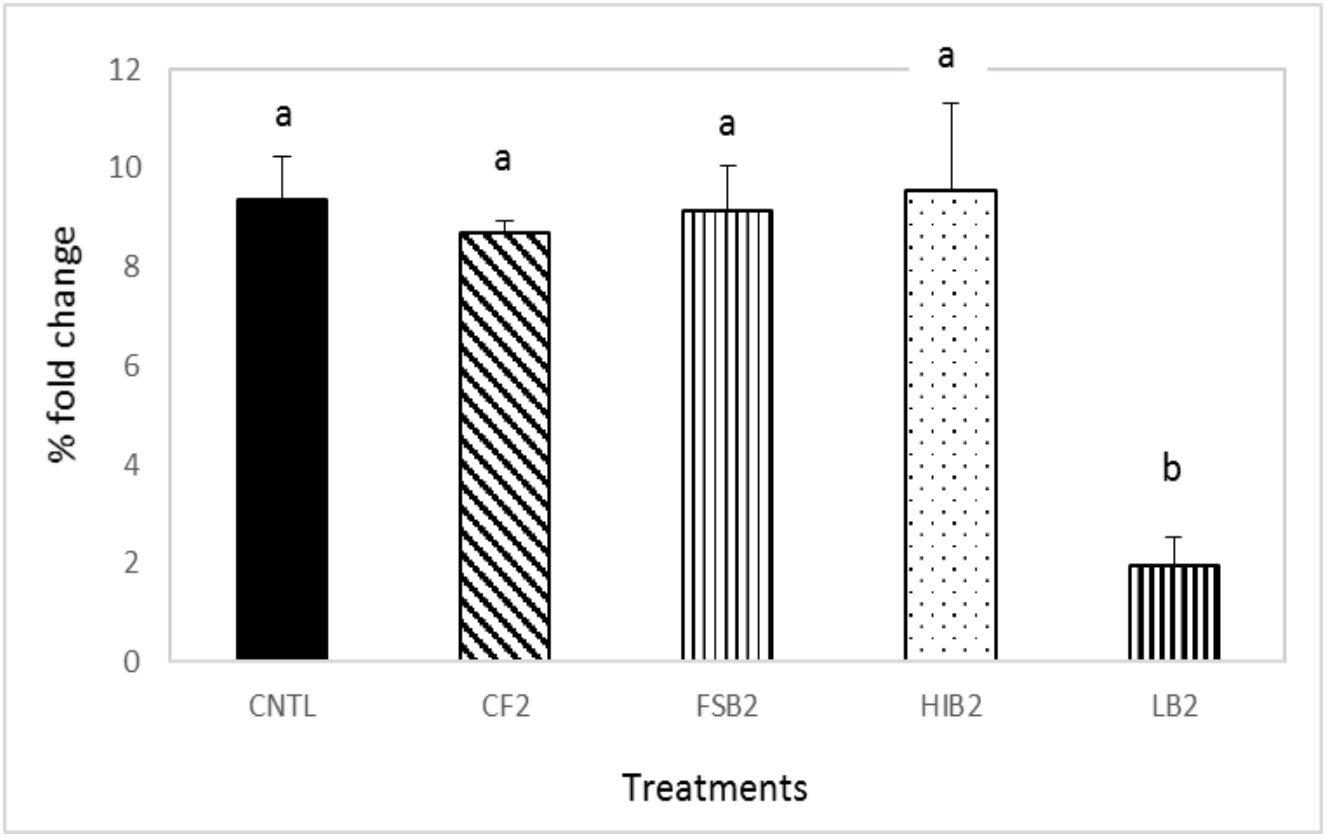

Figure 29. Effect of *Lactobacillus rhamnosus* MRS6AN or its extracts on *MTP* expression in Caco2 cells treated according to Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipid mixture and fresh bacterial treatments and incubated for 2 hrs). Each bar represents mean ± SD value of percent fold change (n=3). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control. Bars labelled with different letters are statistically different from each other *(P≤0.05).*

GPAT3 Expression of Co-Cultured Caco2 Cells Treated According to Protocol 3

LB treated Caco2 cells treated according to protocol 3 (overnight co-cultured cells were washed and treated lipids only) demonstrated significant reduction in *GPAT3* expression compared to other treatments and the control (Figure 30). Other treatments did not induce significant change in *GPAT3* expression of cells.

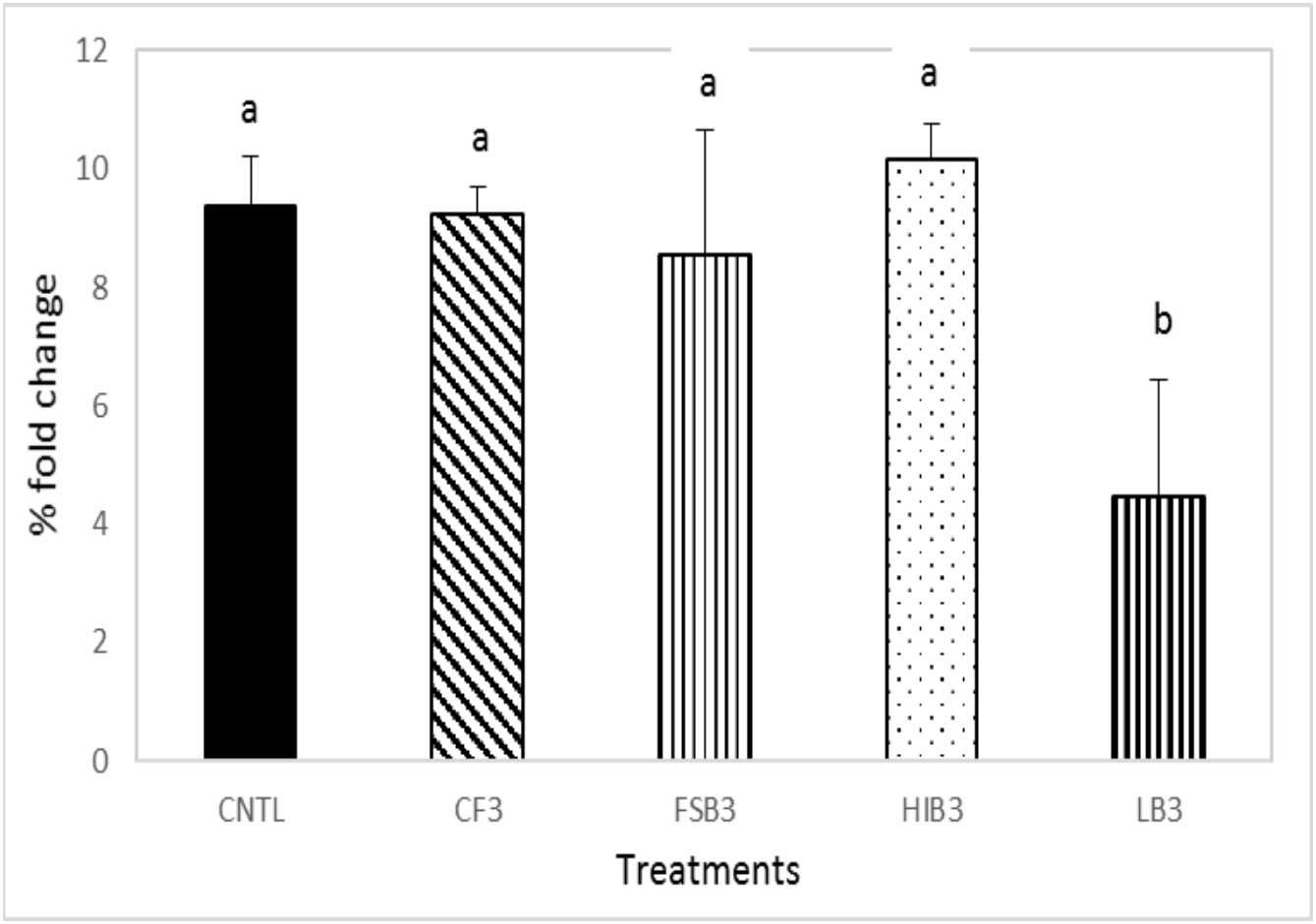

Figure 30. Effect of *Lactobacillus rhamnosus* MRS6AN or its extracts on *MTP* expression in Caco2 cells treated according to Protocol 3 (after overnight bacterial treatment, cells were washed and treated with lipid mixture only and incubated for 2 hrs). Each bar represents mean ± SD value of percent fold change (n=3). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control. Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Bacterial Cytoplasmic Fraction (CF) on GPAT3 Expression

Bacterial cytoplasmic fraction across all Protocols had no significant effect on *GPAT3* expression (Figure 31).

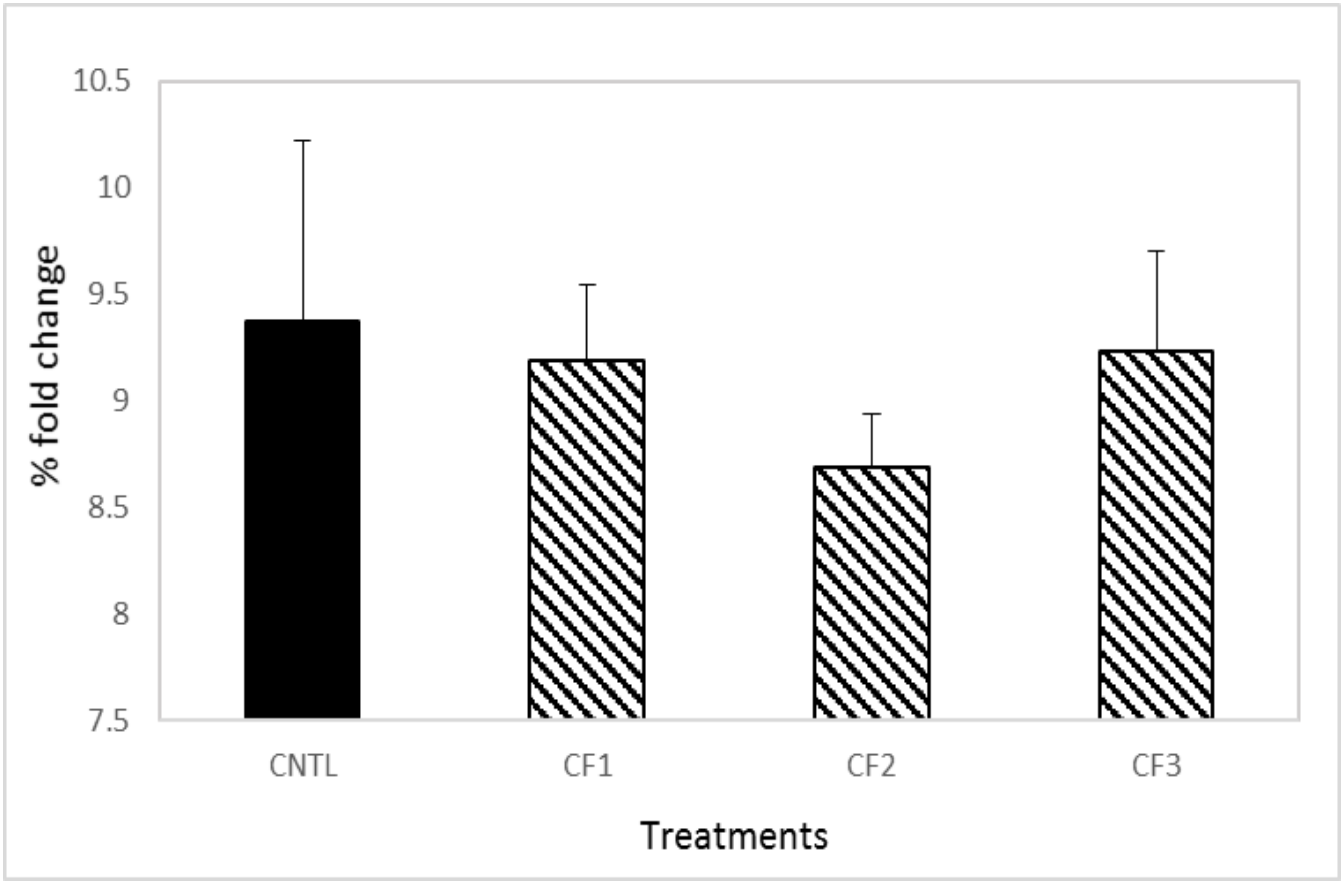

Figure 31. Comparison between the effects of bacteria cell extracts (CF) on GPAT3 expression in Caco2 cells. The CF was applied under three Protocol (Protocol 1: overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs, Protocol 2: overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs, Protocol 3: overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs). Each bar represents mean fold change relative to the control treatment (n=2 to 3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Bacterial Filtered Spent Broth (FSB) on GPAT3 Enzyme

There was no significant change in *GPAT3* expression level in Protocols 1, 2 and 3 compared to the control (Figure 32).

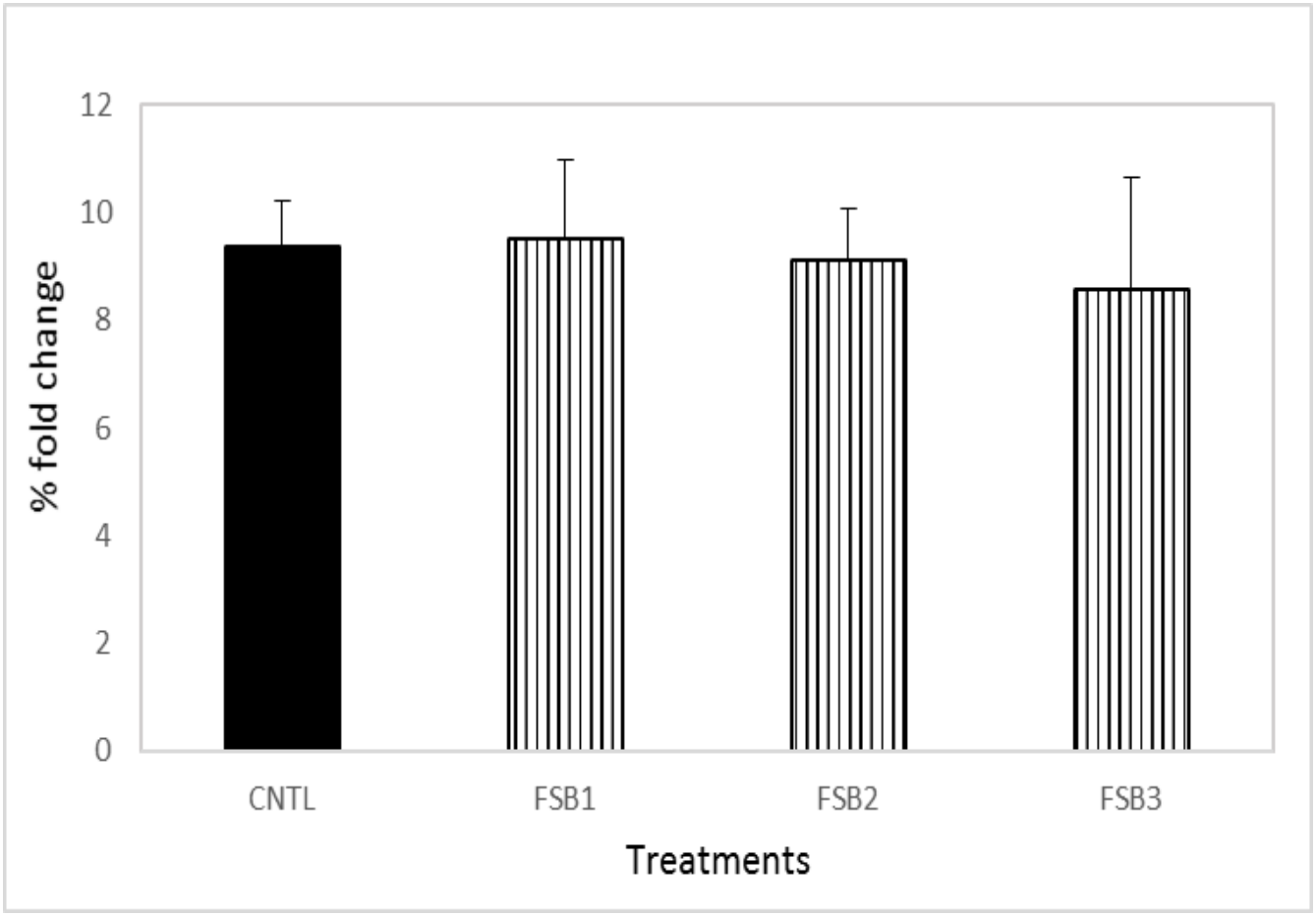

Figure 32. Comparison between the effects of bacterial filtered spent broth (FSB) on GPAT3 expression in Caco2 cells, The FSB was applied under three Protocol (Protocol 1: overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs, Protocol 2: overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs, Protocol 3: overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs). Each bar represents mean fold change relative to the control treatment (n=2 to 3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Heat Inactivated Bacteria (HIB) on GPAT3 Enzyme

In the presence of heat inactivated bacteria, there was no significant effect on the expression level of *GPAT3* across Protocols relative to the control treatment. However, there was a slight reduction in expression in cells as per Protocol 1 (1.80-fold change relative to the control) (Figure 33).

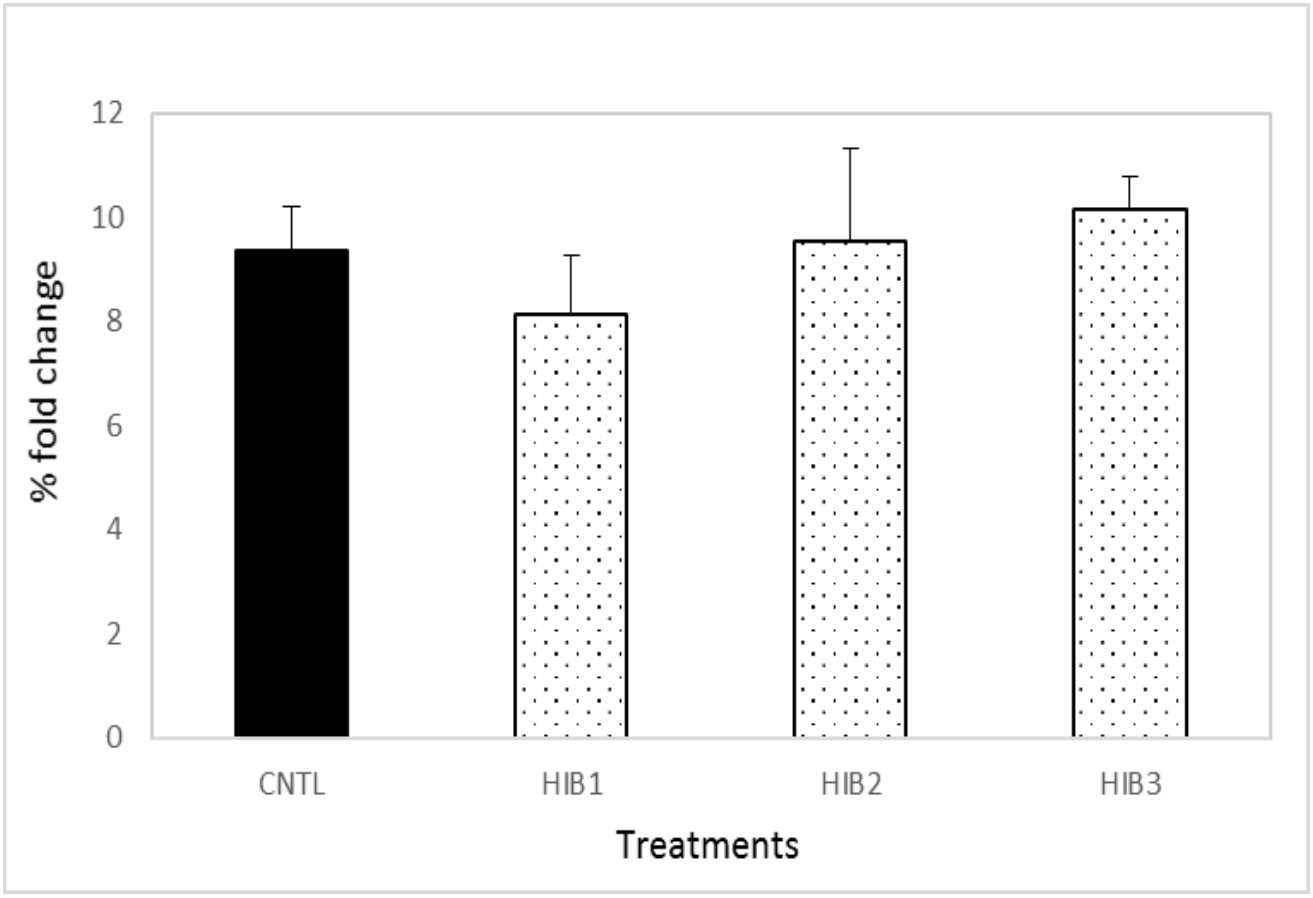

Figure 33. Effects of heat inactivated bacteria (HIB) on GPAT3 expression in Caco2 cells. The HIB was applied under three Protocol (Protocol 1: overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs, Protocol 2: overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs, Protocol 3: overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs). Each bar represents mean fold change relative to the control treatment (n=2 to 3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Live Bacteria (LB) on GPAT3 Enzyme.

There was significant down-regulation of *GPAT3* expression in LB treated cells across all Protocols relative to the control with the lowest expression in cells treated according to protocol 1 (cells overlaid with lipids without washing) (Figure 34). Although, there were slight changes in *GPAT3* expression between LB treated protocols, there was however no significant difference in *GPAT3* expression between LB treated protocols.

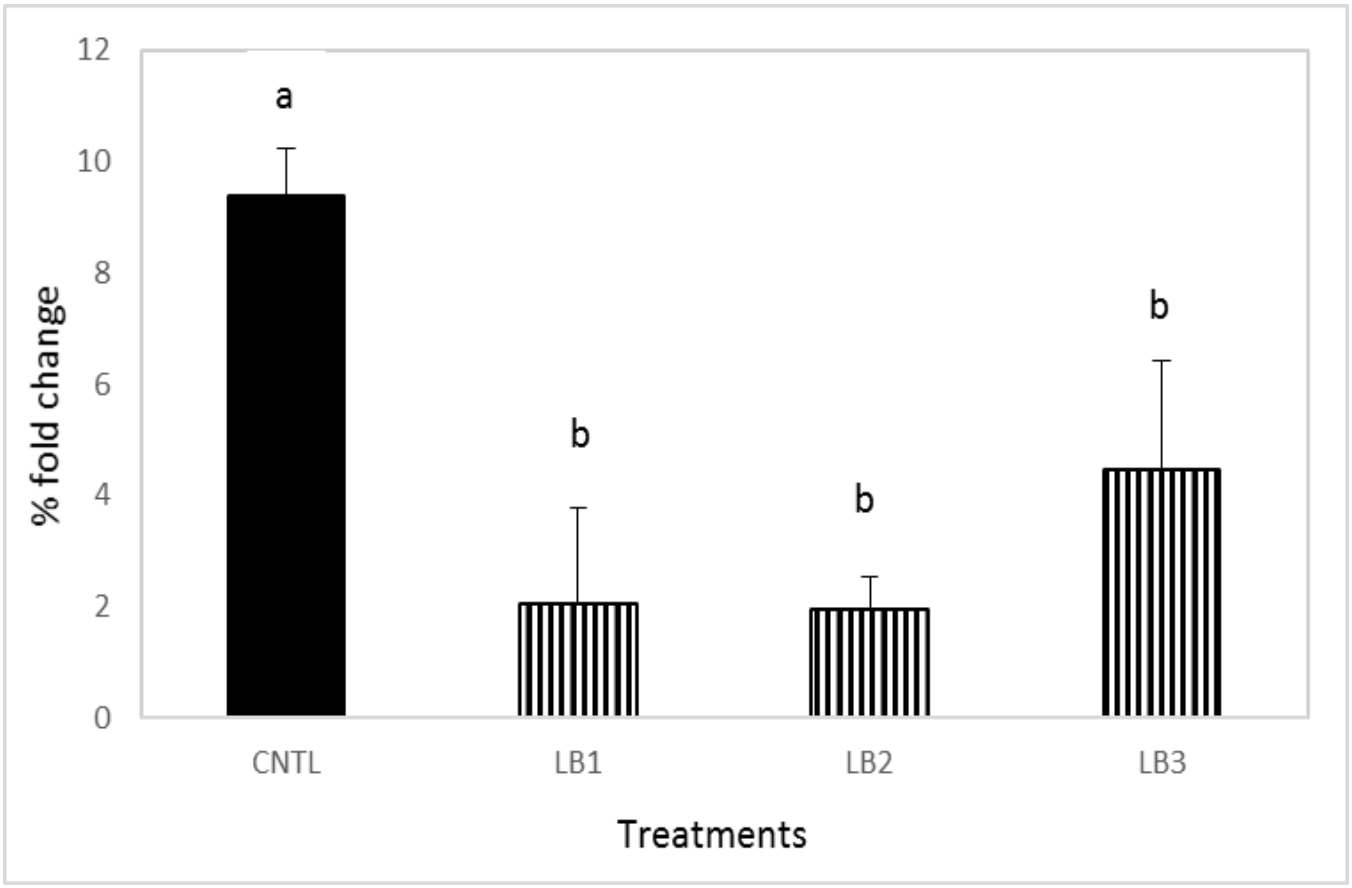

Figure 34. Comparison between the effects of live *L. rhamnosus* MRS6AN (LB) on GPAT3 expression in Caco2 cells. The LB was applied under three Protocol (Protocol 1: overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs, Protocol 2: overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs, Protocol 3: overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs). Each bar represents mean fold change relative to the control treatment (n=2 to 3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Table 9. Effect of *L. rhamnosus* MRS6AN, heat inactivated bacteria, filtered spent broth or its cytoplasmic fraction on GPAT3 expression in Caco-2 cells.

TREATMENT	CNTRL	CF1	CF2	CF3	FSB1	FSB2	FSB3	HIB1	HIB2	HIB3	LB1	LB2	LB3
GPAT3 REL. EXPRESSION*	9.37	9.19	8.69	9.24	9.52	9.12	8.56	8.14	9.55	10.15	2.05	1.97	4.45

*Expression values are mean fold change, where n=2, Mean fold change represent protein expression relative to the control.

Effect of Probiotic Treatment on I-FABP Expression

I-FABP expression in LB treated Caco2 cells in Protocol 2 (overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs) treatment was lowest with 8.9-fold change relative to the control group (Figure 35 and Table 10). Also, there were 3.2 and 5.0 reduction in I-FABP expression in LB treated cells in Protocol 1 (overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs) and 3 (overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs) relative to the control. CF treated Protocol 1 demonstrated considerably low expression of I-FABP, however CF, HIB and FSB treated Protocol 3 lowered I-FABP expression by 1.8, 2.6 and 1.3 compared to the control.

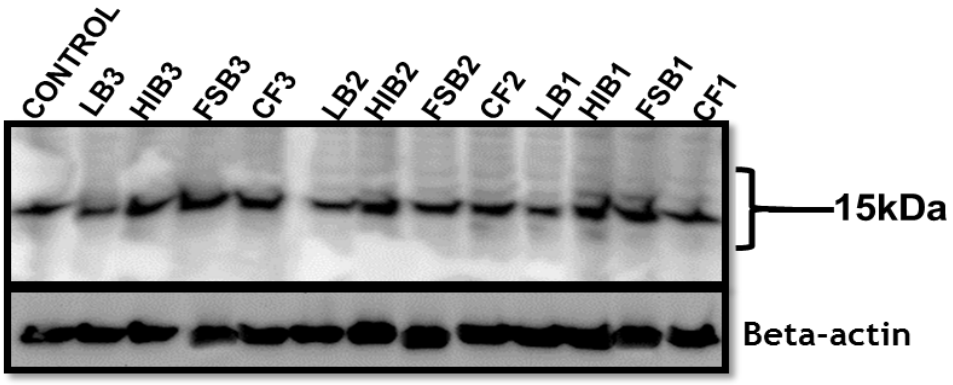

Figure 35. Western blot assay image of I-FABP expression in Caco2 cells
(CF- Cytoplasmic fraction, FSB- Filtered spent broth, HIB- Heat inactivated bacteria, LB- Live bacteria)

I-FABP Expression of Co-Cultured Caco2 Cells Treated According to Protocol 1

Co-cultured Caco2 cells were overlaid with lipid without washing, after an overnight incubation period. There was no significant change in *I-FABP* expression in Caco2 cells treated with CF, FSB and HIB compared to other treatments and the control (Figure 36). However, cells treated with LB induce significant reduction in *I-FABP* expression in cells by 7.92 relative to the control, although there was no significance change in *I-FABP* expression between all treatments.

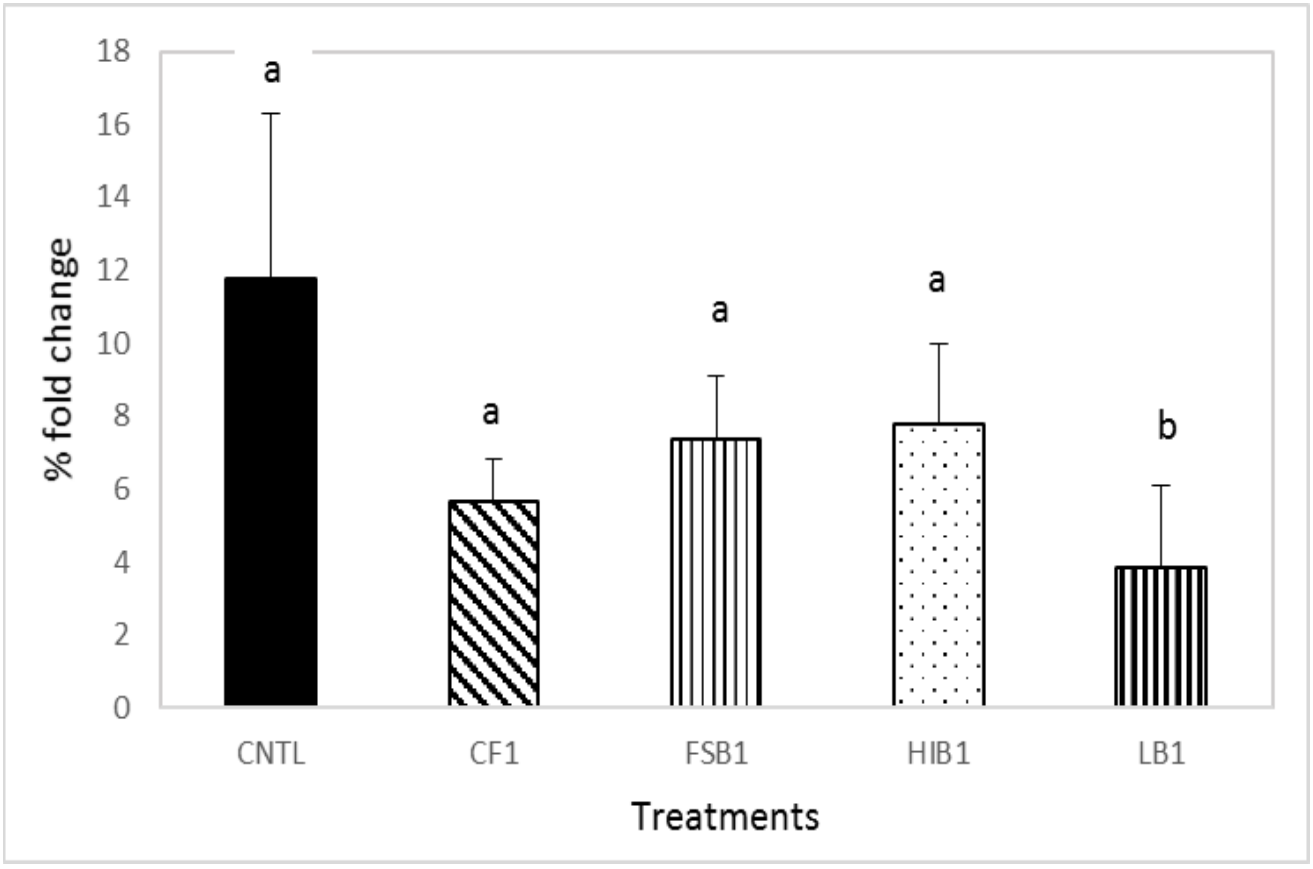

Figure 36. Effect of *Lactobacillus rhamnosus* MRS6AN or its extracts on *MTP* expression in Caco2 cells treated according to Protocol 1 (after overnight bacterial treatment, cells were overlaid with lipid mixture without washing and incubated for 2 hrs). Each bar represents mean ± SD value of percent fold change (n=3). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control. Bars labelled with different letters are statistically different from each other *(P≤0.05).*

I-FABP Expression of Co-Cultured Caco2 Cells Treated According to Protocol 2

Co-cultured Caco2 cells were washed and treated with lipid and fresh bacteria treatments, after an overnight incubation period. Significant reduction in *I-FABP* expression was observed in Caco2 cells treated with LB compared to the control. Other treatments did not induce significant change in *I-FABP* expression in cells relative to the control and there was also no significant variance among all treatments within the protocol (Figure 37).

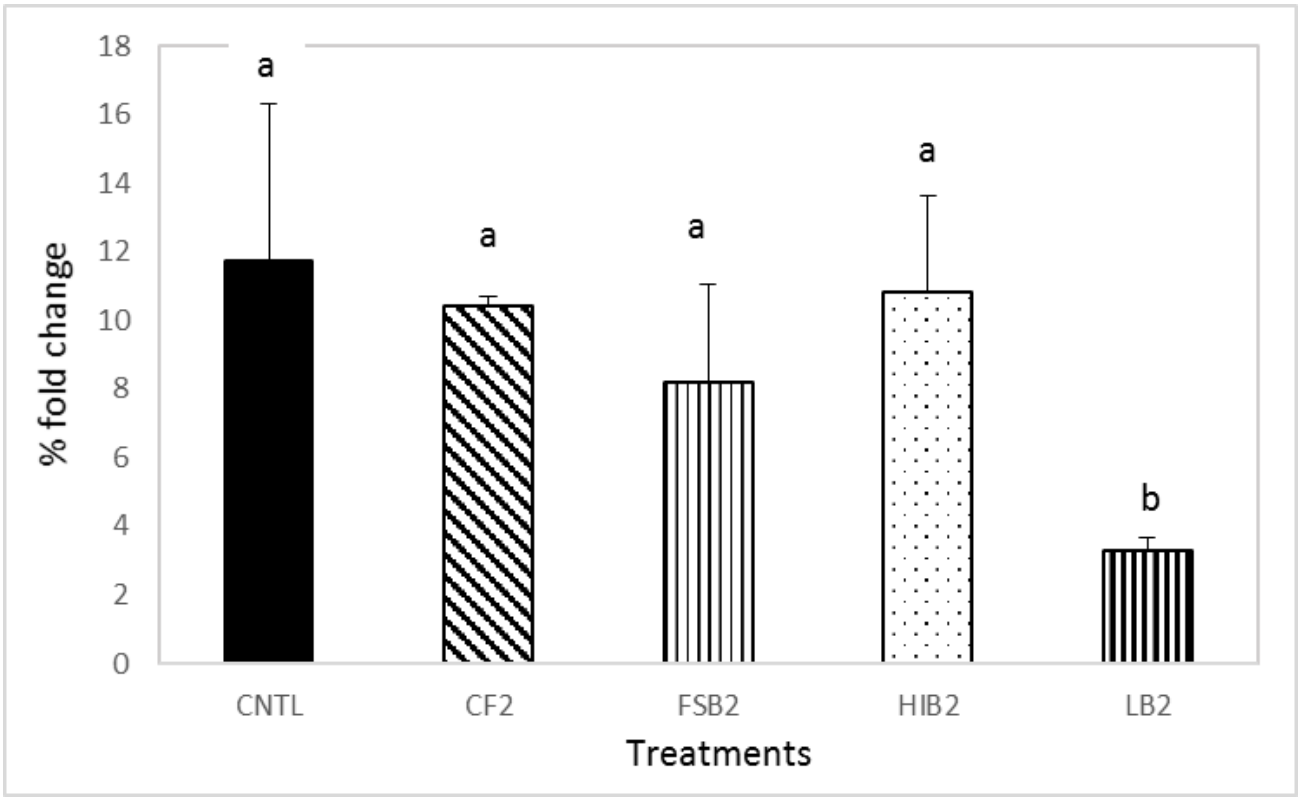

Figure 37. Effect of *Lactobacillus rhamnosus* MRS6AN or its extracts on *MTP* expression in Caco2 cells treated according to Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipid mixture and fresh bacterial treatments and incubated for 2 hrs). Each bar represents mean ± SD value of percent fold change (n=3). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control. Bars labelled with different letters are statistically different from each other *(P≤0.05).*

I-FABP Expression of Co-Cultured Caco2 Cells Treated According to Protocol 3

There was a significant reduction in *I-FABP* expression in LB treated Caco2 cells according to protocol 3 (overnight co-cultured cells were washed and treated lipids only) compared to the control (Figure 38). Other bacterial treatments however did not cause any significant change in *I-FABP* expression of cells relative to the control.

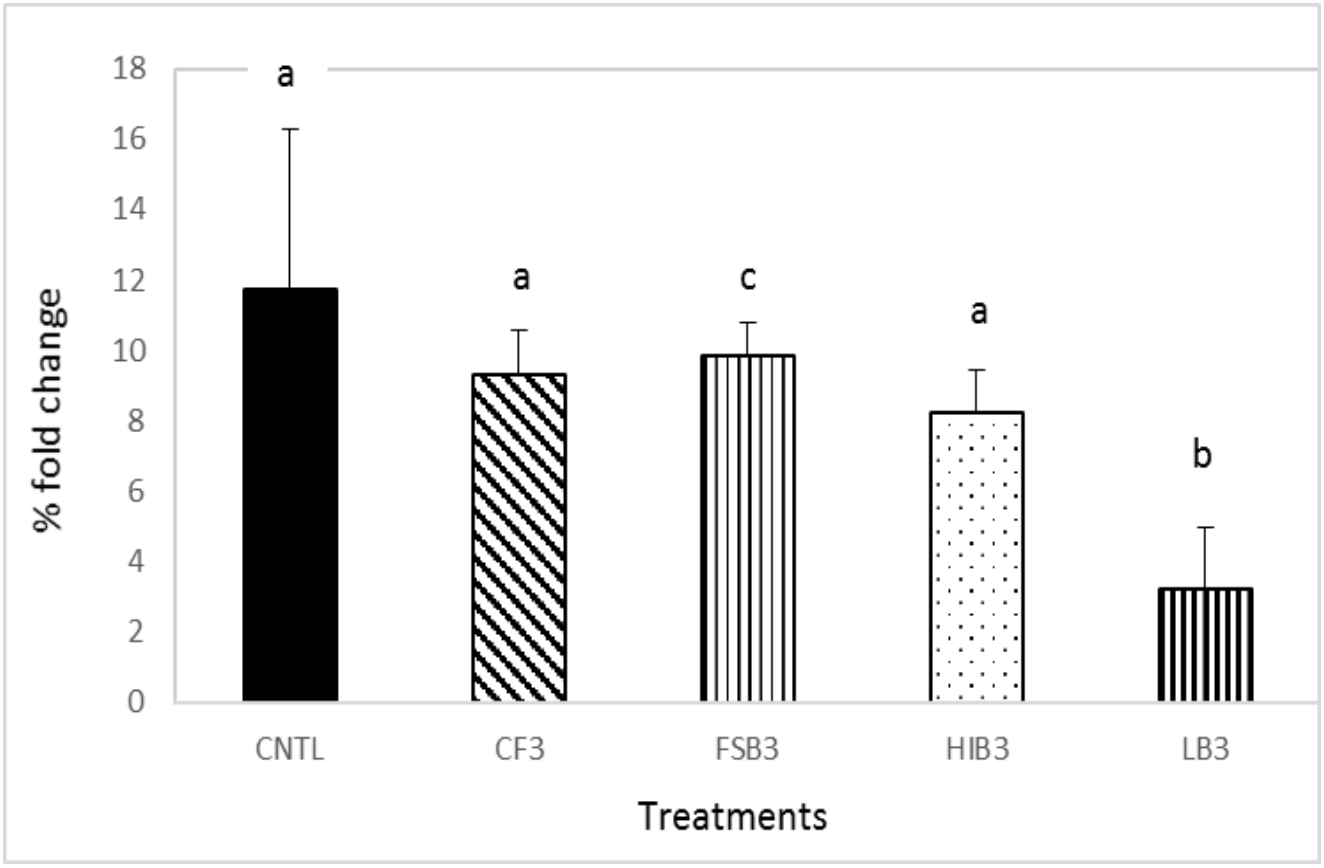

Figure 38. Effect of *Lactobacillus rhamnosus* MRS6AN or its extracts on *MTP* expression in Caco2 cells treated according to Protocol 3 (after overnight bacterial treatment, cells were washed and treated with lipid mixture only and incubated for 2 hrs). Each bar represents mean ± SD value of percent fold change (n=3). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control. Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Bacterial Cytoplasmic Fraction (CF) on I-FABP Expression

CF treatments across all protocols did not cause significant change in *I-FABP* expression in Caco2 cells relative to the control, Furthermore, there was no significant change observed in I-FABP CF treated cells across all protocols (Figure 39).

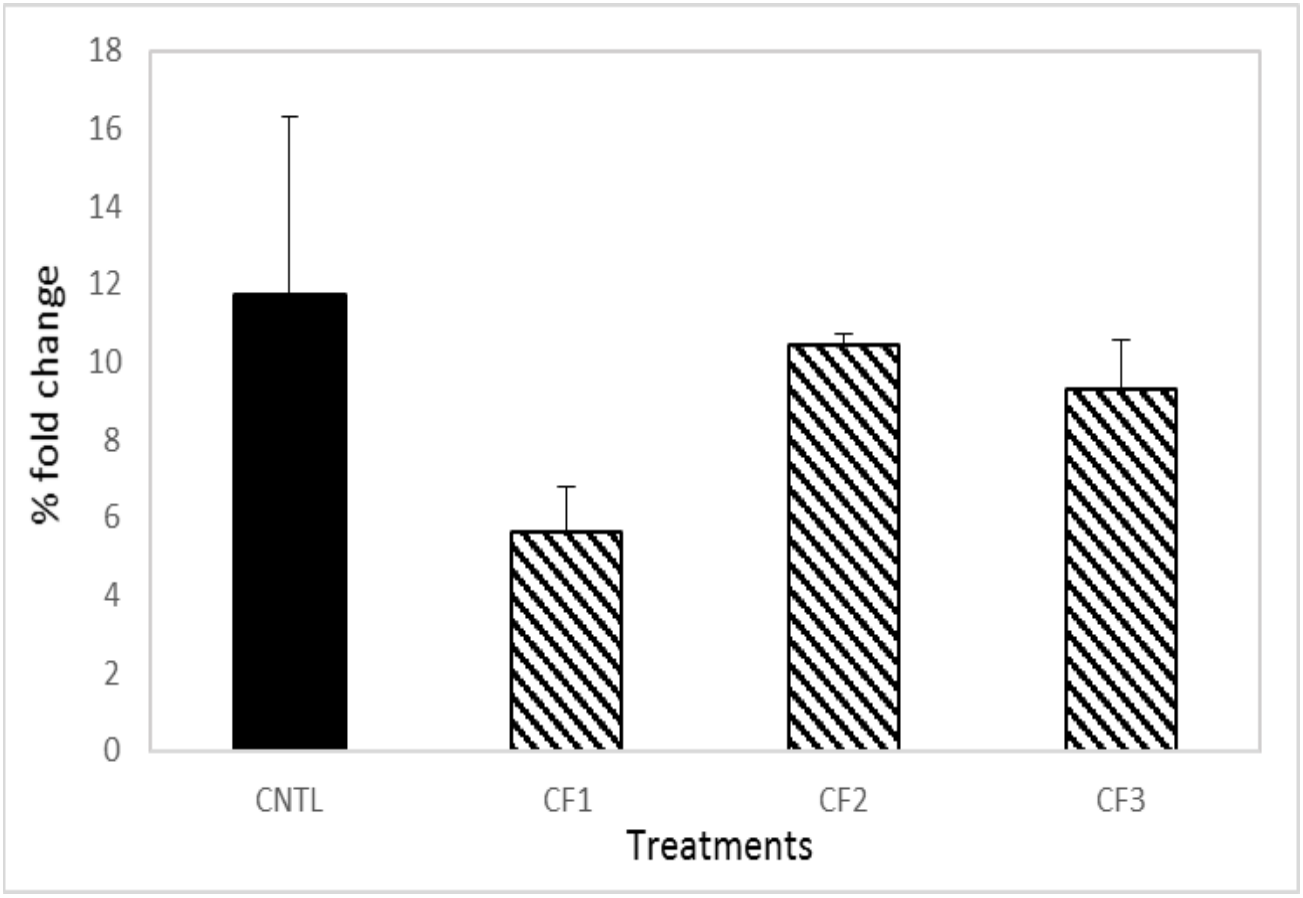

Figure 39: Evaluation of Intestinal-fatty acid binding protein (I-FABP) expression when overnight co-cultured Caco2 cells were treated with bacteria cytoplasmic fraction (CF). The CF was applied under three Protocol (Protocol 1: overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs, Protocol 2: overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs, Protocol 3: overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs). Each bar represents mean fold change relative to the control treatment (n=2 to 3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Bacterial Filtered Spent Broth (FSB) on I-FABP Expression

Effect of bacterial filtered spent broth on I-FABP expression was evaluated across all Protocols, and they all demonstrated no significant effect on I-FABP expression level relative to the control. There was no significant variance in *I-FABP* expression across all protocols treated with bacterial filtered spent broth (FSB) (Figure 40).

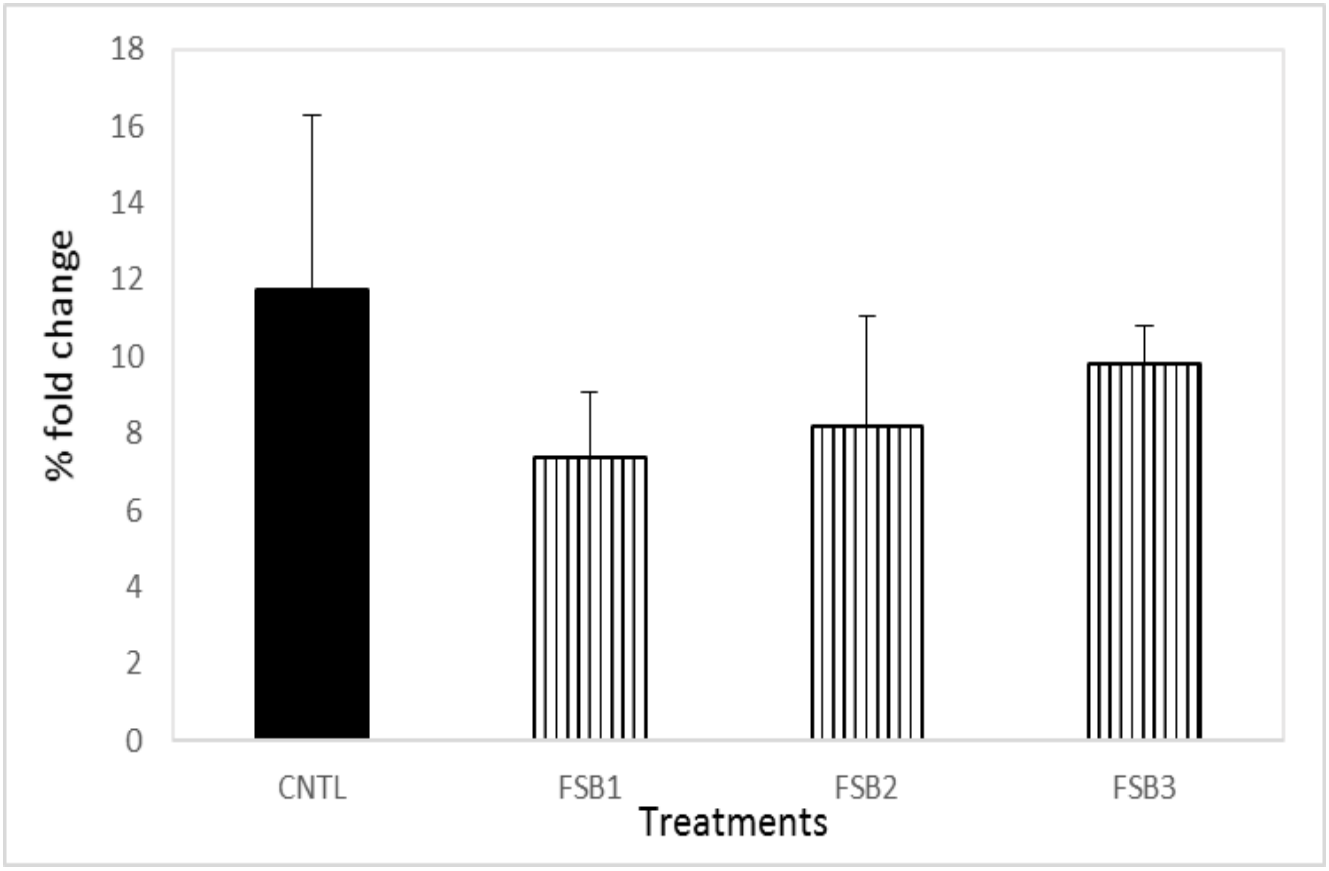

Figure 40: Evaluation of Intestinal-fatty acid binding protein (I-FABP) expression when overnight co-cultured Caco2 cells were treated with bacteria filtered spent broth (FSB). The FSB was applied under three Protocol (Protocol 1: overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs, Protocol 2: overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs, Protocol 3: overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs). Each bar represents mean fold change relative to the control treatment (n=2 to 3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Heat Inactivated Bacteria (HIB) on I-FABP Expression

Expression of *I-FABP* was not significantly affected by the presence of heat inactivated bacteria across all protocols, likewise there was no significant difference in *I-FABP* expression in all FSB treated protocols relative to the control (Figure 41).

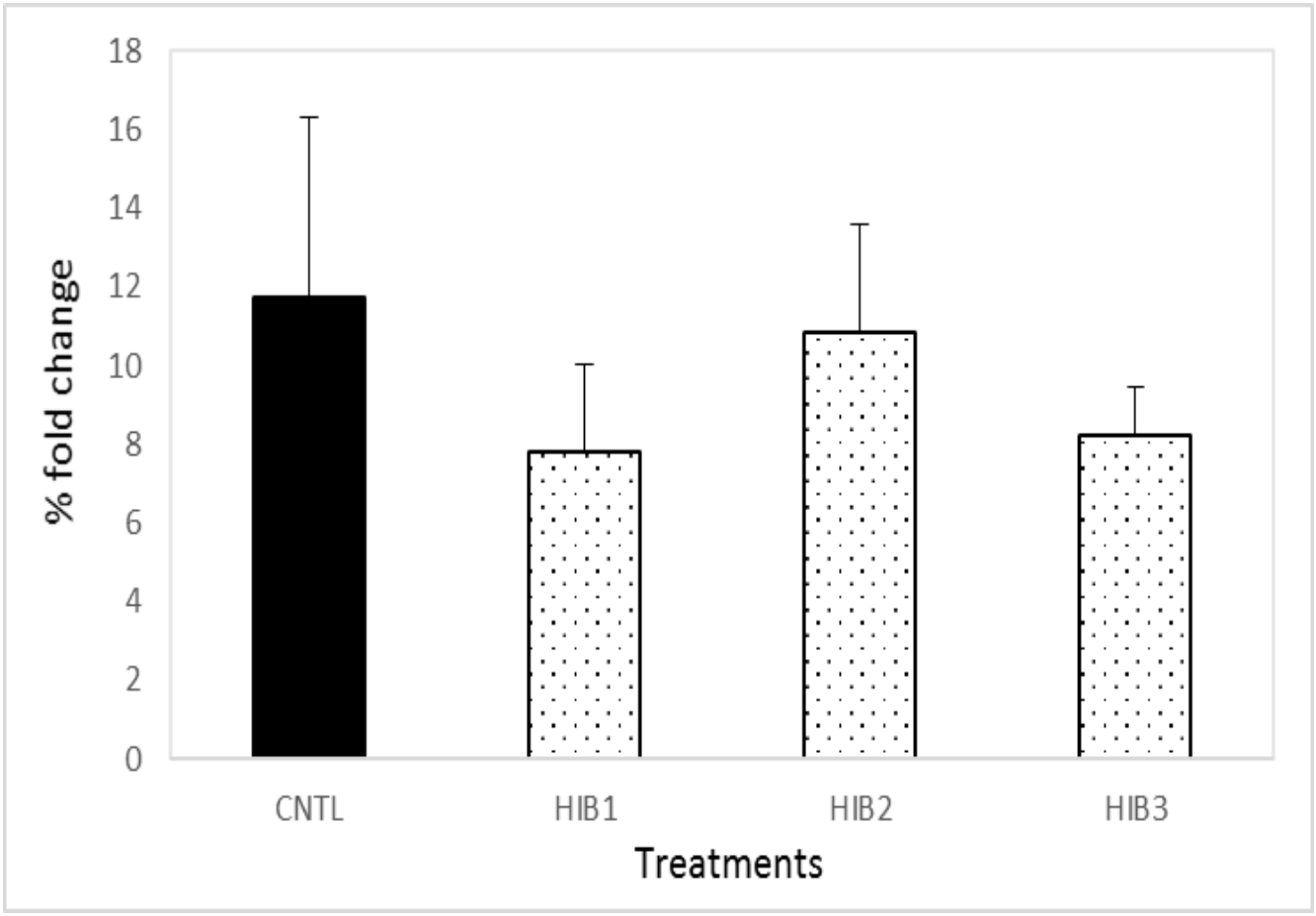

Figure 41: Evaluation of Intestinal-fatty acid binding protein (I-FABP) expression when overnight co-cultured Caco2 cells were treated with heat inactivated bacteria (HIB). The HIB was applied under three Protocol (Protocol 1: overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs, Protocol 2: overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs, Protocol 3: overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs). Each bar represents mean fold change relative to the control treatment (n=2 to 3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Live L. Rhamnosus (LB) on I-FABP Expression

Consistent with the significant effect of live MRS6AN on lipid transport associated protein expression, *I-FABP* level was significantly reduced in all protocols treated cells with live bacteria relative to the control (Figure 42). with the highest reduction of 7.3 percent fold decrease in Protocol 2 (cells washed and treated with lipids and fresh bacterial treatments). There was no significant difference between all LB protocol treatments.

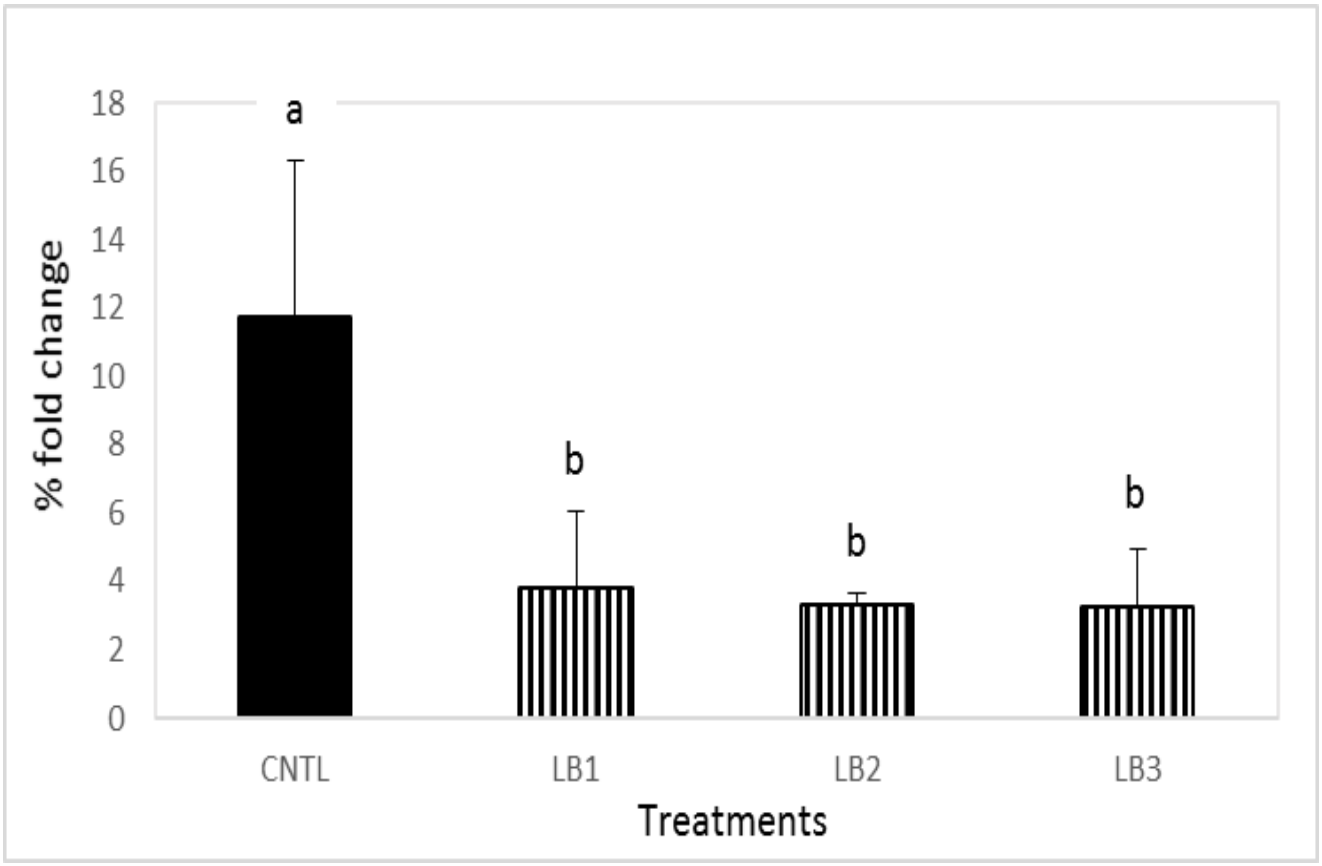

Figure 42. Evaluation of Intestinal-fatty acid binding protein (I-FABP) expression when overnight co-cultured Caco2 cells were treated with live bacteria (LB). The LB was applied under three Protocol (Protocol 1: overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs, Protocol 2: overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs, Protocol 3: overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs). Each bar represents mean fold change relative to the control treatment (n= 3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Table 10: Effect of *L. rhamnosus* MRS6AN, heat inactivated bacteria, filtered spent broth or its cytoplasmic fraction on I-FABP expression in Caco-2 cell.

TREATMENT	CNTRL	CF1	CF2	CF3	FSB1	FSB2	FSB3	HIB1	HIB2	HIB3	LB1	LB2	LB3
I-FABP REL. EXPRESSION*	11.75	5.63	10.43	9.28	7.35	8.18	9.83	7.79	10.82	8.23	3.83	3.31	3.23

*Expression values are mean fold change, where n=3, Mean fold change represent protein expression relative to the positive control.

Effect of Probiotic Treatment on NPC1L1 Expression

This transport protein belongs to the sterol transporter family. The expression of NPC1L1 was significantly reduced by LB in Protocol 1 (cells overlaid with lipid mixture) by 5.7 percent fold decrease. Reduction in expression was also induced in LB treated Protocol 2 and 3 by 4.9 and 3.2 relative to the control. Bacterial filtered spent broth (FSB) in Protocol 3 had no significant effect on NPC1L1 expression (Figure 43 and Table 11). There was reduction in Protocols 1 and 2 with 3.7 and 2.4-fold decrease in expression relative to the control treatment. Heat inactivated bacteria also slightly down-regulated NPC1L1 expression in Protocols 1 (cells overlaid with lipids without washing) and Protocol 2 (cells washed and treated with lipids and fresh bacteria treatments) by 2.8 and 2.0, with less reduction in Protocol 3 (cells wash and treated with lipids only) by 1.5-fold decrease.

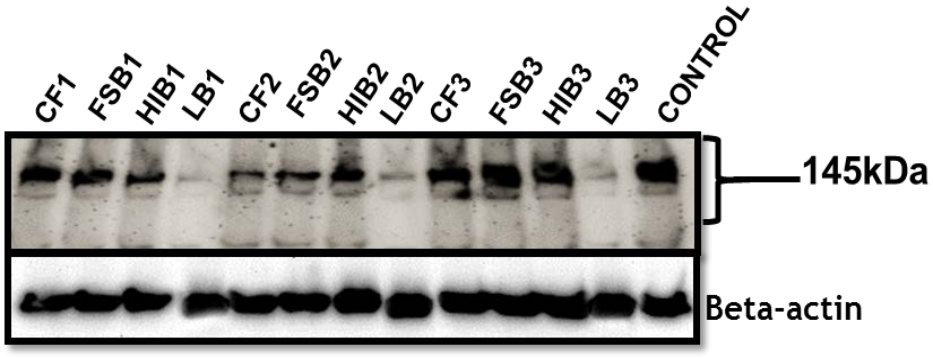

Figure 43. Western blot assay image of NPC1L1 expression in Caco2 cells
(CF- Cytoplasmic fraction, FSB- Filtered spent broth, HIB- Heat inactivated bacteria, LB- Live bacteria)

NPC1L1 Expression of Co-Cultured Caco2 Cells Treated According to Protocol 1

NPC1L1 level of overnight co-cultured Caco2 cells overlaid with lipids without washing was determined. There was no significant change in *NPC1L1* expression in Caco2 cells treated with CF, FSB and HIB compared to other treatments and the control (Figure 44). But LB treatment induce significant reduction in *NPC1L1* expression in Caco2 cells by 6.36 relative to the control, even though there was no significance change in *NPC1L1 protein level* between all treatments in the protocol group.

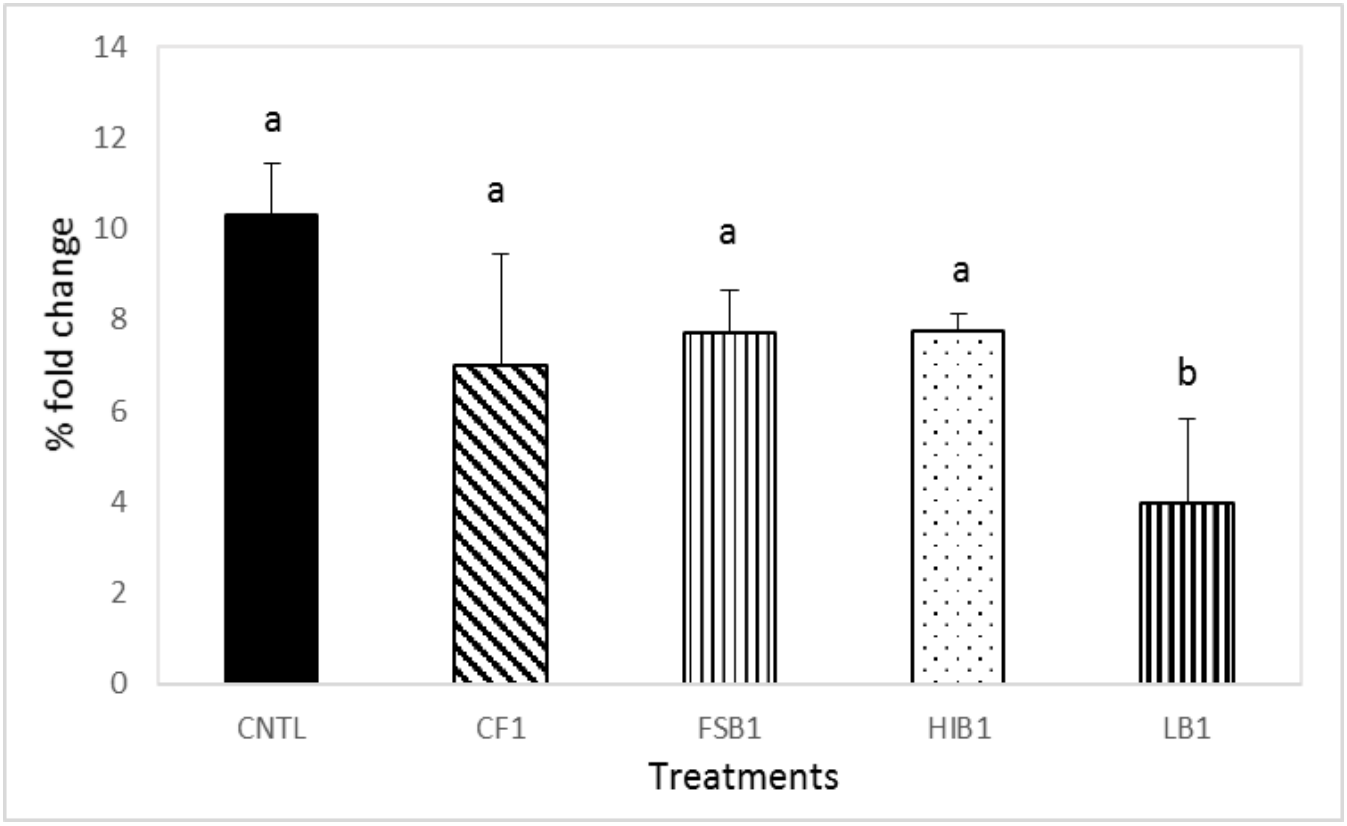

Figure 44. Effect of *Lactobacillus rhamnosus* MRS6AN or its extracts on *MTP* expression in Caco2 cells treated according to Protocol 1 (after overnight bacterial treatment, cells were overlaid with lipid mixture without washing and incubated for 2 hrs). Each bar represents mean ± SD value of percent fold change (n=3). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control. Bars labelled with different letters are statistically different from each other *(P≤0.05).*

NPC1L1 Expression of Co-Cultured Caco2 Cells Treated According to Protocol 2

Overnight co-cultured Caco2 cells treated with LB showed significant reduction in *NPC1L1* expression compared with CF, FSB and HIB treated cells (Figure 45). The expression level of NPC1L1 LB treated cells was also significantly reduced by 5.88 relative to the control, although there was no significance change in *NPC1L1* expression between treatments with CF, FSB and HIB.

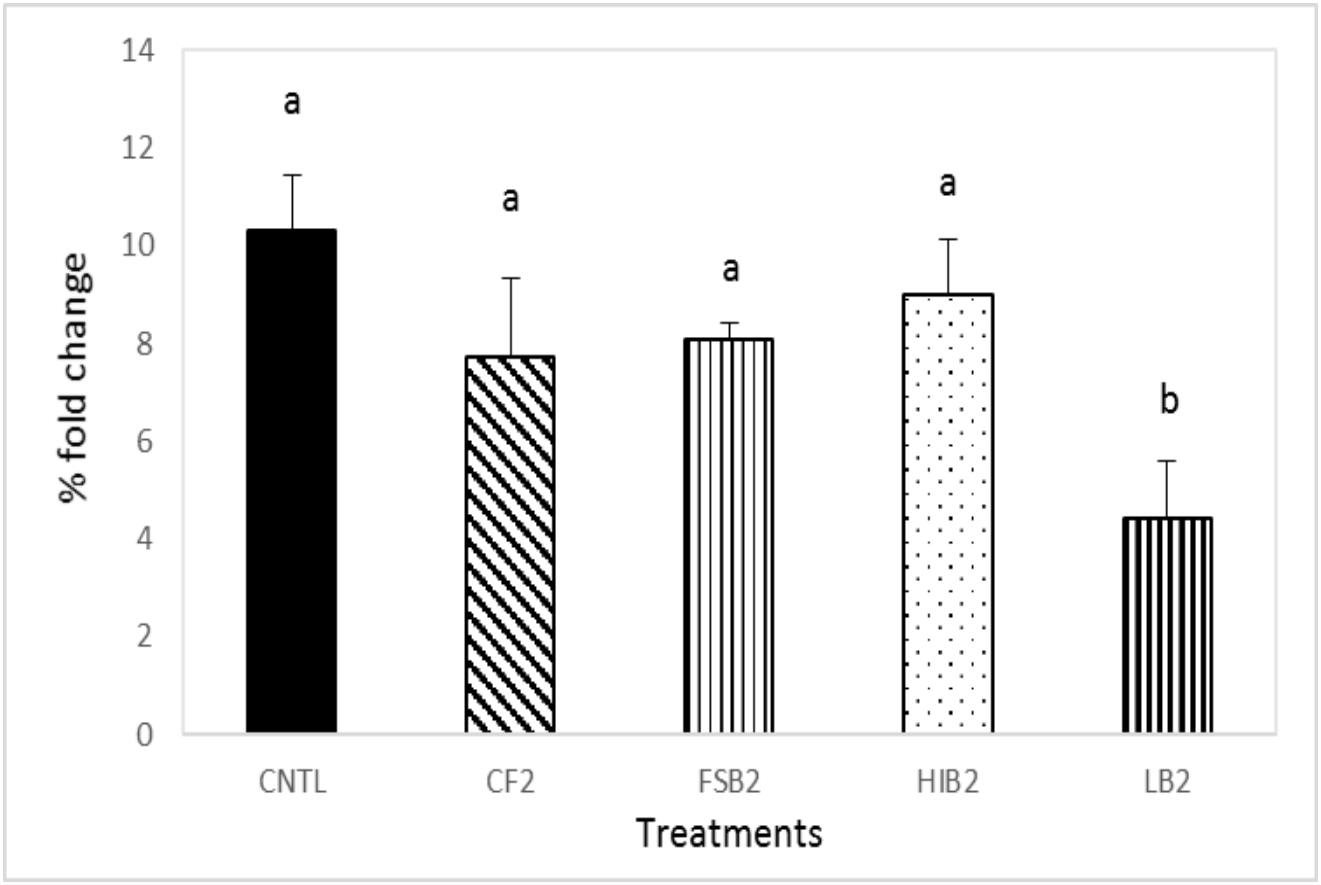

Figure 45. Effect of *Lactobacillus rhamnosus* MRS6AN or its extracts on *MTP* expression in Caco2 cells treated according to Protocol 2 (after overnight bacterial treatment, cells were washed and treated with lipid mixture and fresh bacterial treatments and incubated for 2 hrs). Each bar represents mean ± SD value of percent fold change (n=3). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control. Bars labelled with different letters are statistically different from each other *(P≤0.05).*

NPC1L1 Expression of Co-Cultured Caco2 Cells Treated According to Protocol 3

Caco2 cells treated according to protocol 3 (overnight co-cultured cells were washed and treated lipids only) showed significant reduction in *NPC1L1* protein level in Caco2 cells treated with LB compared to CF, FSB and HIB treated cells by 5.4, 5.7 and 5.1 respectively, similarly significant decrease in NPC1L1 protein level was observed in LB treated cells by 5.88 compared with the control (Figure 46). There was no significant difference in *NPC1L1* expression between other bacteria treatments.

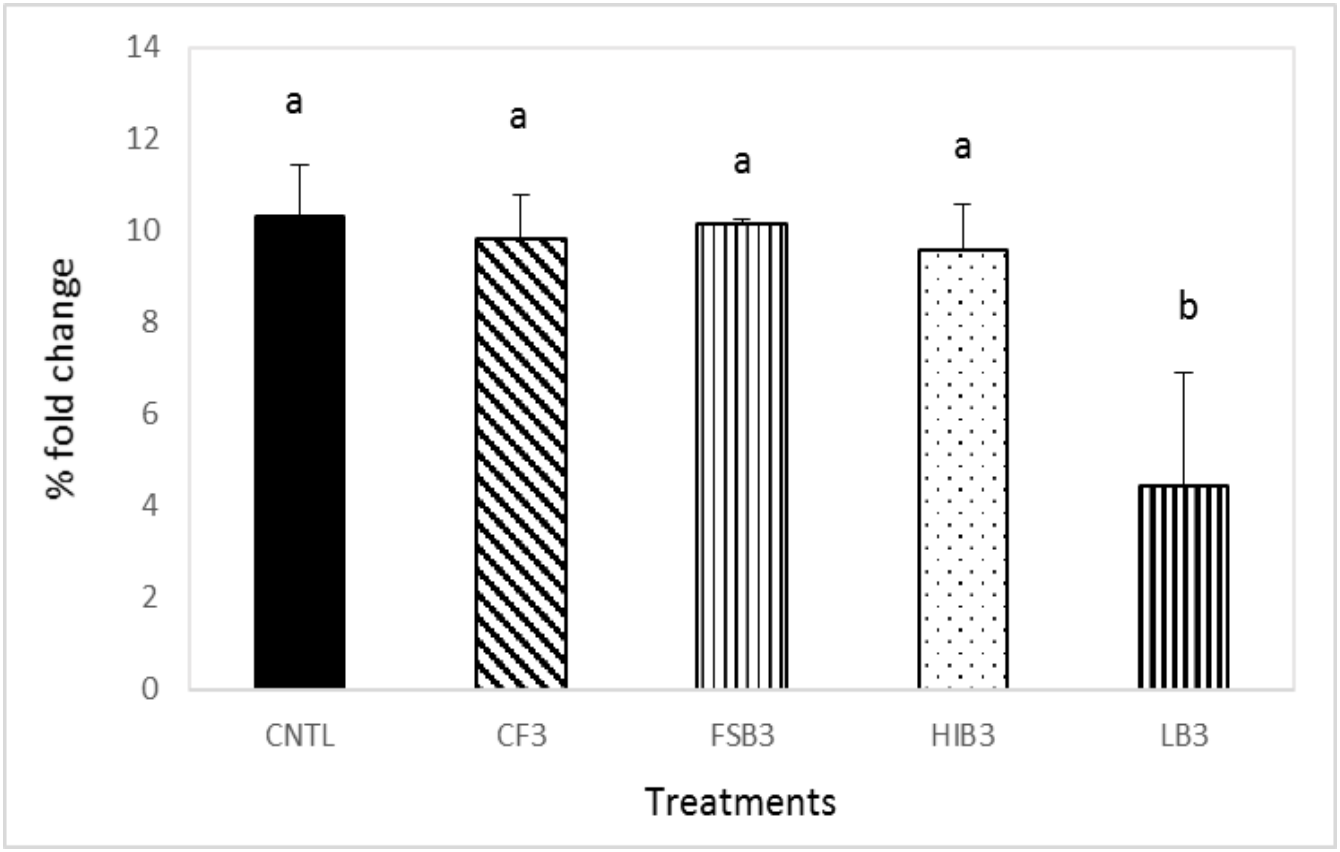

Figure 46. Effect of *Lactobacillus rhamnosus* MRS6AN or its extracts on *MTP* expression in Caco2 cells treated according to Protocol 3 (after overnight bacterial treatment, cells were washed and treated with lipid mixture only and incubated for 2 hrs). Each bar represents mean ± SD value of percent fold change (n=3). Treatments include CF (cytoplasmic fraction), FSB (filtered spent broth), HIB (heat inactivated bacteria), LB (live bacteria) and the control. Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Bacterial Cytoplasmic Fraction (CF) on NPC1L1 Expression

Expression of sterol transporter NPC1L1 was not significantly affected in Caco2 cells between Protocols 1 (cells overlaid with lipids without washing) and Protocol 2 (cells washed and treated with lipids and fresh bacteria treatments) and Protocol 3 (cells washed and treated with lipids only) and relative to the control treatment (Figure 47).

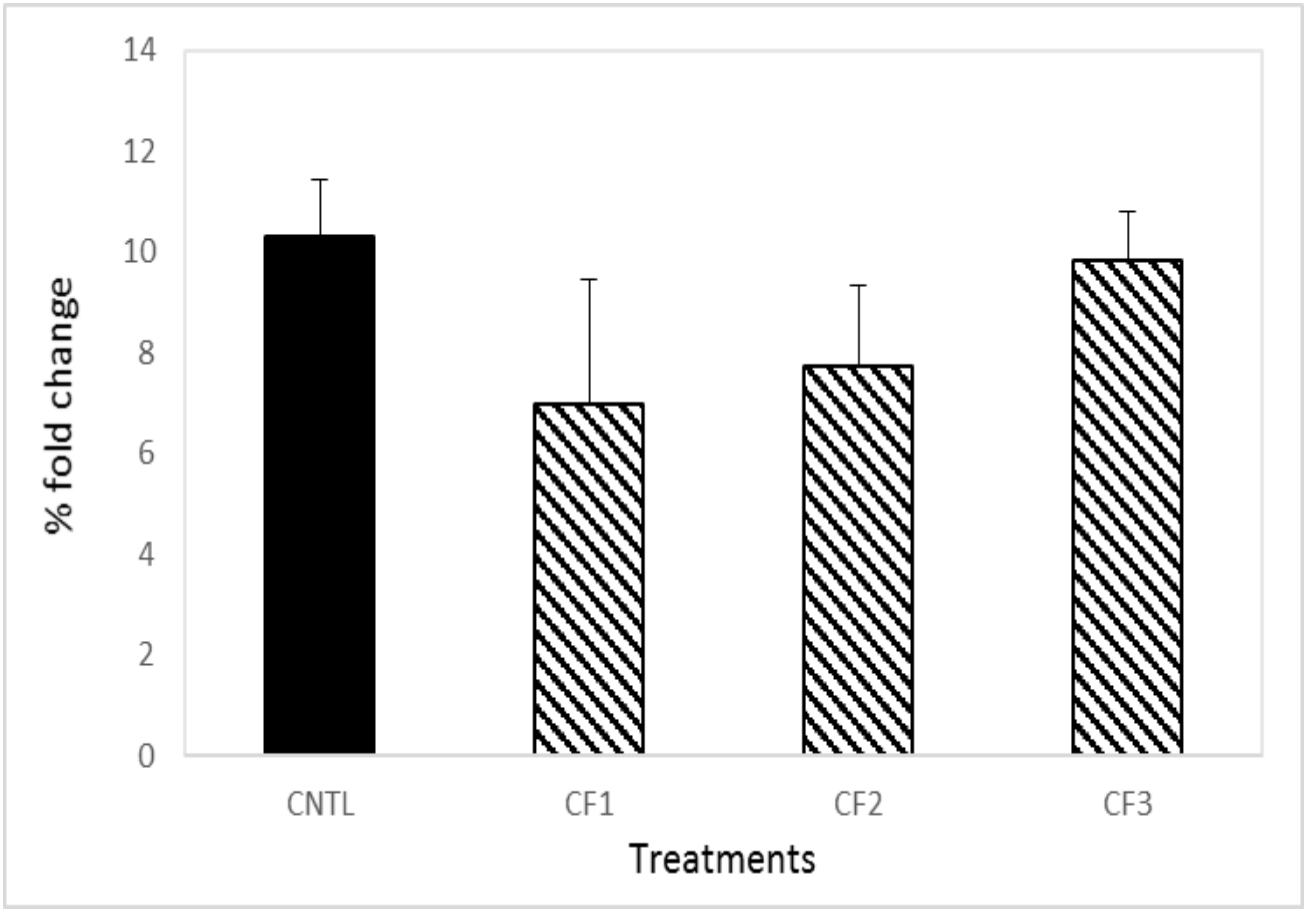

Figure 47. Evaluation of the effect of bacteria cell extract (CF) on NPC1L1 expression in Caco2 cells across Protocol treatments. The CF was applied under three Protocol (Protocol 1: overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs, Protocol 2: overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs, Protocol 3: overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs). Each bar represents mean fold change relative to the control treatment (n=2 to 3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Bacterial Filtered Spent Broth (FSB) on NPC1L1 Expression

Bacterial filtered spent broth induced a significant reduction in NPC1L1 expression in cells from Protocol 1 (cells overlaid with lipids without washing) and Protocol 2 (overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments) relative to the control treatment (Figure 48). There was significant difference between FSB treated cells according to protocol 1 and Protocol 3 (overnight co-culture Caco2 cells washed and treated with lipids only). Caco2 cells treated according to protocol 3 showed no significant difference in NPC1L1 expression compared to protocol 2 and control treatment.

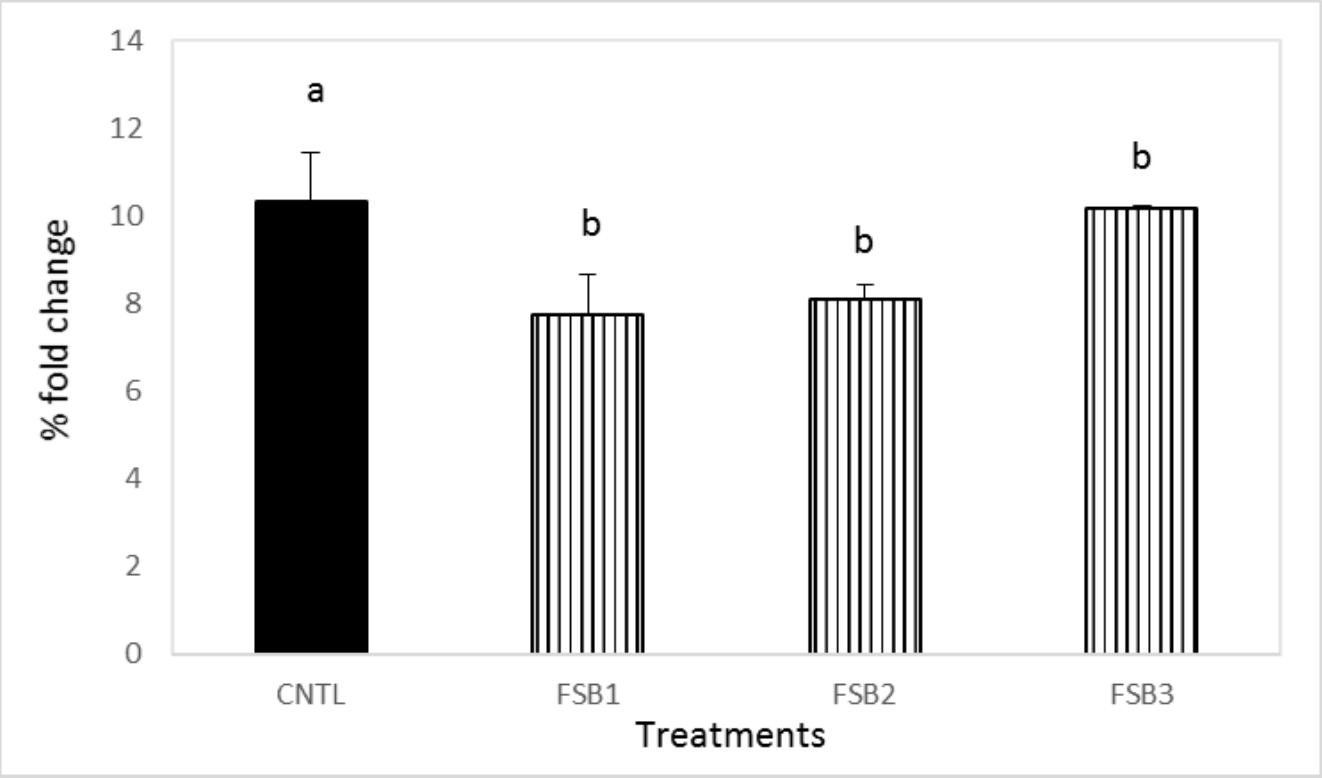

Figure 48. Evaluation of the effect of bacteria filtered spent broth (FSB) on NPC1L1 expression in Caco2 cells across Protocol treatments. The FSB was applied under three Protocol (Protocol 1: overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs, Protocol 2: overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs, Protocol 3: overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs). Each bar represents mean fold change relative to the control treatment (n= 3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Heat Inactivated Bacteria (HIB) on NPC1L1 Expression

NPC1L1 expression level although showed slight reduction in HIB treated Protocol 1 (overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs.), it was not significantly different form other protocols and when compared with the control treatment (Figure 49). Likewise, heat inactivated bacteria (HIB) had no significant effect on the expression of NPC1L1 between all the protocols.

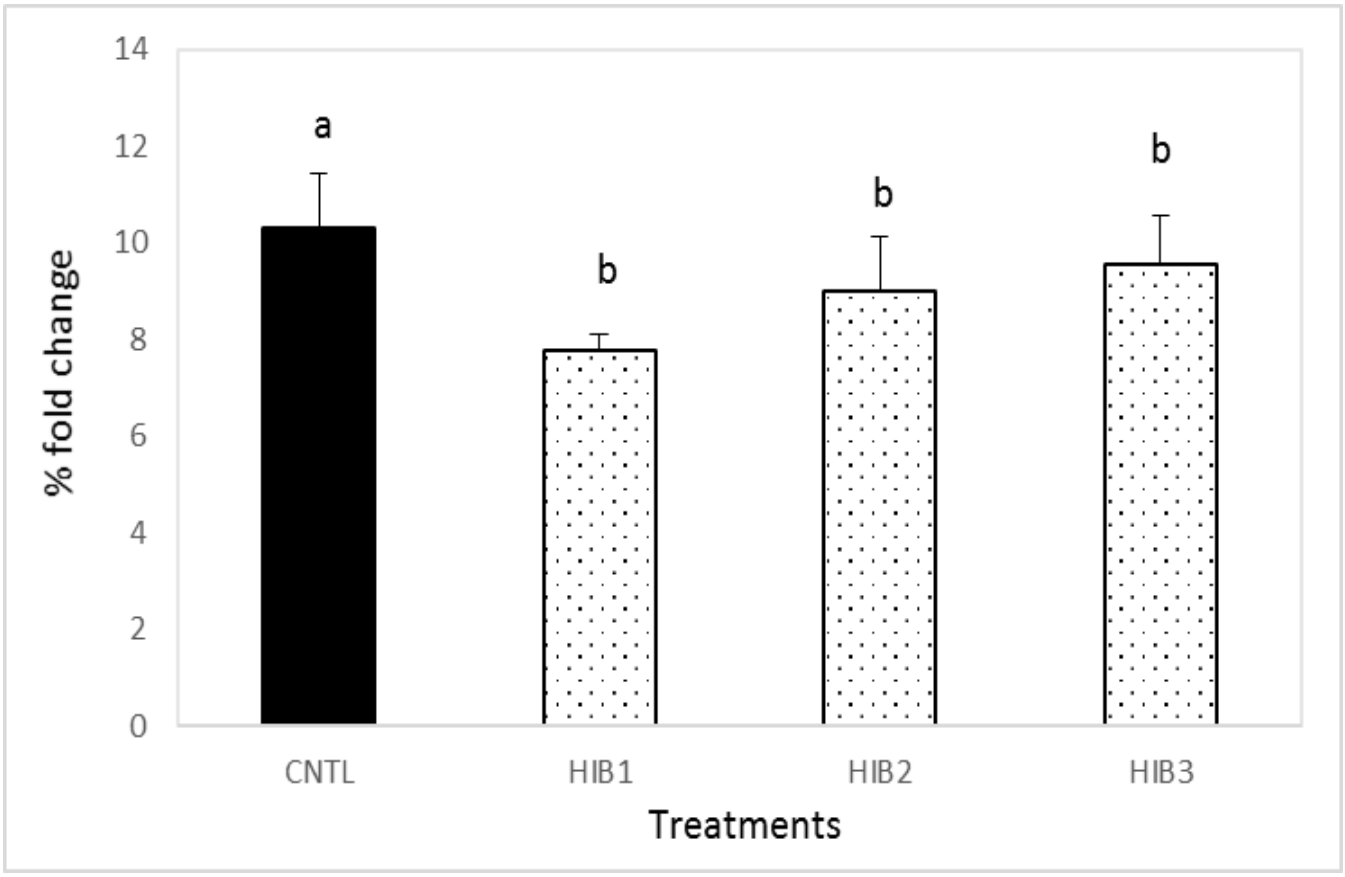

Figure 49. Evaluation of the effect of heat inactivated bacteria (HIB) on NPC1L1 expression in Caco2 cells across Protocol treatments. The HIB was applied under three Protocol (Protocol 1: overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs, Protocol 2: overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs, Protocol 3: overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs) Each bar represents mean fold change relative to the control treatment (n= 3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Effect of Live Bacteria (LB) on NPC1L1 Expression.

Live *Lactobacillus rhamnosus* (LB) significantly reduced the expression of NPC1L1 in all the treatment Protocols compared to the control treatment (Figure 50). There was however no considerable difference in *NPC1L1* protein level between all the protocol treatments.

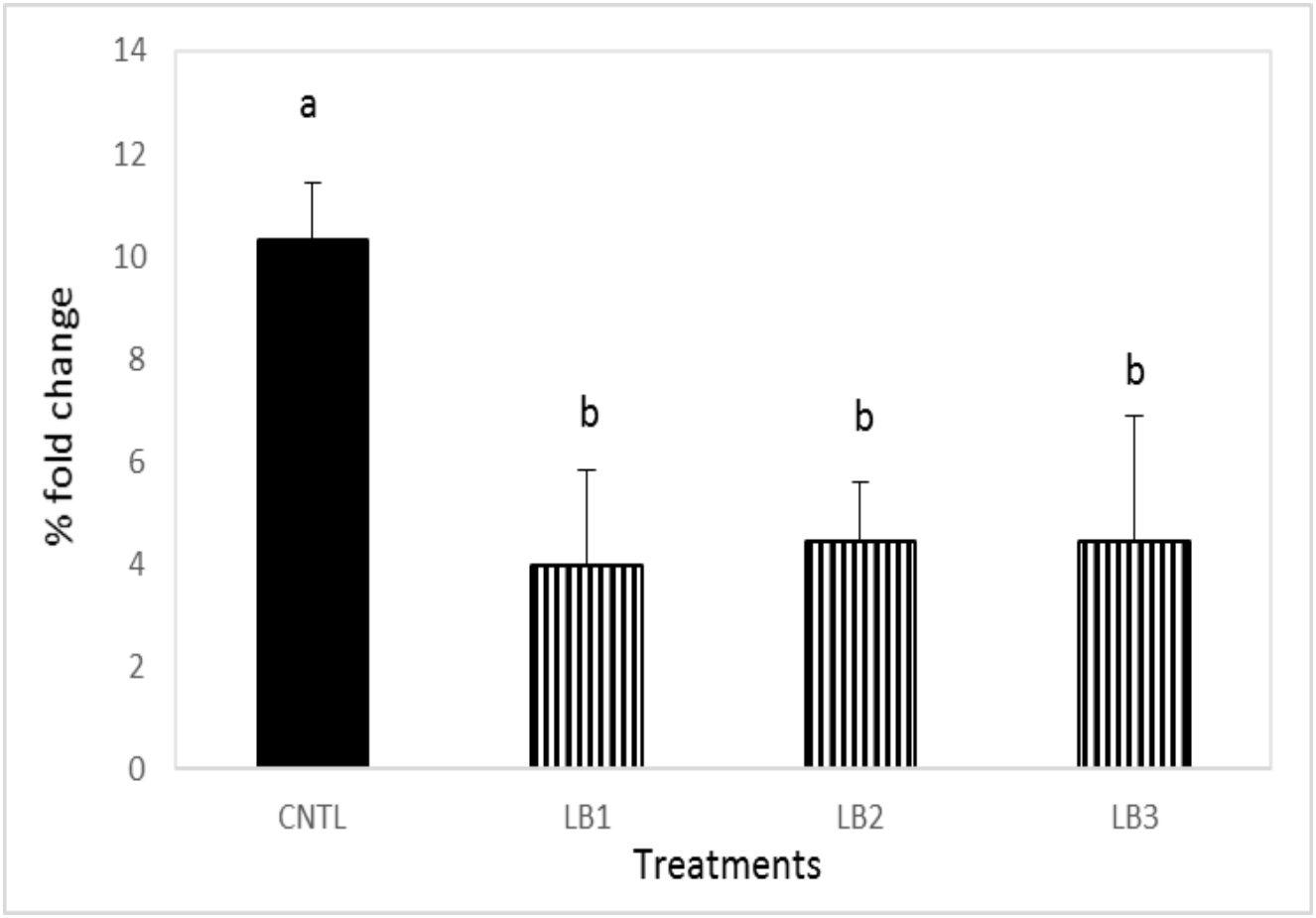

Figure 50. Evaluation of the effect of bacteria cell extract on NPC1L1 expression in Caco2 cells across Protocol treatments. The LB was applied under three Protocol (Protocol 1: overnight co-culture Caco2 cells overlaid and incubated with lipids for 2 hrs., Protocol 2: overnight co-culture Caco2 cells washed, treated with lipids and fresh bacteria treatments and incubated for 2 hrs., Protocol 3: overnight co-culture Caco2 cells washed and incubated with lipids only for 2 hrs.). Each bar represents mean fold change relative to the control treatment (n=2 to 3). Bars labelled with different letters are statistically different from each other *(P≤0.05).*

Table 11. Effect of *L. rhamnosus* MRS6AN, heat inactivated bacteria, filtered spent broth or its cytoplasmic fraction on NPC1L1 expression in Caco-2 cell.

TREATMENT	CNTRL	CF1	CF2	CF3	FSB1	FSB2	FSB3	HIB1	HIB2	HIB3	LB1	LB2	LB3
NPC1L1 REL. EXPRESSION*	10.32	6.99	7.71	9.82	7.73	8.10	10.15	7.76	9.01	9.58	3.96	4.43	4.44

*Expression values are mean fold change, where n=3, Mean fold change represent protein expression relative to the positive control.

CHAPTER 4

DISCUSSION

This study was carried out in response to the prevailing level of diet induced obesity, hepatic associated dysfunctions, coronary heart disease and insulin resistance induced diabetes. The main purpose of this study is to develop a feasible alternative therapy using probiotic *Lactobacillus rhamnosus* to reduce the prevalence of health abnormalities such as obesity, type II diabetes and hyperlipidemia (Kang et al. 2013). The fact that not only Lactobacillus sp. can deconjugate bile acid (for example bile acids frequently are conjugated to sulfate or glucoronate to allow easier excretion) suggest that other members of the intestinal microbiota may also influence intestinal lipid profile (Shimada K et al. 1969). Several research studies have validated the intestinal lipid modulation effect of the genus lactobacilli. Reduction in total cellular lipid content and intracellular triglyceride level of cells was shown in cells treated with lipids alongside live bacteria bacterial, cytoplasmic fraction or its filtered spent broth. The pro-active influence of live Lactobacillus sp. cannot be overemphasized, although significant reduction in total cellular lipid content had been shown to influenced by probiotic isolates (Kang et al. 2013). Disparities may exist based on strains and genera of probiotics involved (Santos et al. 2013). Greany et al. (2004) have shown that daily administration of *L. plantarum* strain dosage was enough to significantly reduce cholesterol in Sprague Dawley rats, but L. acidophilus and *Bifidobacterium longnum* strains could not significantly induce change in Sprague Dawley rat dietary lipid. Zhang and his colleagues in 2012 showed that a strain of *Lactobacillus plantarum* NR74 induced more reduction in cellular cholesterol in Caco2 cells compared to LXR agonist, a nuclear receptor responsible for control of transcription factors of genes liable for uptake, transport and excretion of dietary cholesterol (Zhang et al. 2013).

Reduction in the level of free fatty acids (FFA) in gut had been shown to be induced by a *L. rhamnosus* strain, but this process was facilitated when strain of *L. acidophilus* was mutagenized by N-methyl-N-nitro-N-nitroso guanidine (NTG), a palmitate absorption stimulant that causes the bacteria to readily absorb free fatty acids (FFA) (Chung et al. 2016). This indicates a feasible way to engineer probiotics towards inhibition of diet induced obesity (Chung et al. 2016).

There were no significant changes in the level of total cellular lipid content (TL) of Caco2 cells in all treatment protocols, this could indicate that the TL content of treated cell accounts for total lipids containing within the cell and extracellular lipid contents of the cell membrane.

It has further been reported that oral administration of probiotic bacteria could reduce the level of blood triglycerides by assimilation of dietary lipid by bacteria, inhibition of hepatic cholesterol and triglyceride by SCFA and deconjugation of bile acid by secretion of hydrolase enzyme (Rastislav et al. 2013). Intracellular triglyceride level reduction by *L. rhamnosus* MRS6AN in the present study could be due to such mechanisms. *L. plantarum* has been reported to induce significant reduction in serum triglyceride compared to other probiotic species after an oral probiotic administration (Rastislav et al. 2013). Caco2 cells treated with live bacteria (LB) according to protocol 1 (overnight co-cultured cells were overlaid directly with lipids without washing) and 2 (overnight co-cultured cells were washed and treated with lipids and fresh bacteria treatments) demonstrated significantly low level of intracellular triglyceride, these results indicate that the presence of live bacteria might be needed to elicit any change intracellular TG level. Although these two protocols presented significant difference in intracellular TG content, however, Caco2 cells treated according to protocol 1 demonstrated lower level of intracellular TG compared to protocol 2 treatment. This could indicate that probiotic flora need to colonize the gastrointestinal tract and their presence might serve as a bio-sequestrant thereby reducing the quantity of lipids absorbed by

enterocytes (Chung et al. 2016). This finding suggests that the presence of live bacteria might be needed for lipid uptake to be regulated.

Reduction in triglyceride level by live probiotics might also be because of the instigation of lipase activities in the lumen during triglyceride assembly which decreases lipid absorption and alternatively enhances lipid catabolism and antioxidant activities (Harisa et al. 2009).

Facinelli et al. (2015) observed reduction in triglyceride level in zebrafish model treated orally with *Lactobacillus rhamnosus*. This was suggested to be due to expression of genes involved in TG metabolism and lipolysis (Huang and Zheng 2010). During lipid uptake in the intestine, there are 2 main routes through which triacylglycerol is synthesized: monoacylglycerol and glycerol-3-phosphate. Glycerol-3-phosphate pathway is the primary route which is catalyzed by a GPAT3, a member of the GPAT family (Khatun 2015). In the present study, significant reduction in the expression of GPAT3 was observed in live bacteria treated cell across all protocol treatments. Because triglyceride level was inhibited by live bacteria, reduction of GPAT3 enzymatic activity might be due to the reduced uptake of dietary triglyceride by Caco2 cells.

Enterocytes transiently store dietary triglycerides as cytosolic lipid droplet as they are being absorbed (Zhu 2009). GPAT3 enzymes are overly expressed at the apical region of enterocytes during this process. Triglyceride level was not affected in fasted GPAT3 knock-out mice and the wild type. However, fed wild type mice exhibited reduced triglyceride levels compared to the knock-out (Khatun 2015). Result in the present study demonstrated reduced triglyceride levels with a corresponding decrease in GPAT3 expression. This could indicate that activation and expression of GPAT3 might be associated with influx of triglyceride into enterocytes. A study that was carried out on the possible mechanism behind the reduction in ER stress during lipid uptake after administration of dosage of *L. rhamnosus* GG revealed that GPAT3 enzyme alongside other lipid metabolism associated protein were downregulated in skeletal

muscles of LGG fed diabetic mice resulting in reduced lipotoxicity (Park et al. 2015). Apart from probiotics, dietary polyphenol had also been shown to reduce GPAT3 mRNA level in which increases fatty acid oxidation and reduced FA esterification (Baboota et al. 2013).

In the present study, there was a reduction in *MTP* expression in Caco2 cells treated with live bacteria across all treatment Protocols. It has been reported that ablation or "knock-out" of intestinal *MTP* increases intestinal lipid absorption and reduces plasma lipoproteins (Iqbal et al. 2014). The present study investigates effects of probiotic treatment on TG accumulation*, MTP* expression might not really be affected since it is responsible for assembly and transfer of lipoprotein as chylomicrons for excretion and not triglyceride accumulation (Jamil et al. 1995) and (Iqbal et al. 2014). Iqbal et al. (2014) stressed that deletion of intestinal *MTP* led to a decrease in total plasma triglyceride and cholesterol level. A reduction in *MTP* expression in the present study was probably triggered by the reduction in lipid absorption by the Caco2 cells.

The high expression level of intestinal fatty acid binding protein (I-FABP) in the enterocytic cytosol is responsible for 95 % of lipids that are taken up by enterocytes (Storch et al. 2010). This cytosolic water-soluble protein readily binds fatty acid with high affinity thereby facilitating lipid uptake (Richieri et al. 1994) and (Richieri et al. 2000). The relative expression of *I-FABP* was significantly reduced in the present study, in cells treated with live bacteria (LB) but no considerable change in cells treated with heat inactivated bacteria (HIB), filtered spent broth (FSB) and cytoplasmic fraction (CF). This suggests that inhibitory activity of *L. rhamnosus* MRS6AN might require the presence of the whole bacteria. Strains of *L. rhamnosus* had been reported to inhibit atherosclerosis by reduction in *I-FABP* expression with subsequent reduced intestinal lipid profile of high fat diet fed mice (Chan et al. 2016). They have also been shown to help in the regulation of unbounded fatty acid by binding excess free fatty acid (Atshaves et al. 2010). This

supports the idea that its expression is upregulated during high fat diet uptake (Bass 1990). Evaluation of the prophylactic effect of *L. acidophilus* on necrotizing enterocolitis (NEC) and its role in I-FABP activation in a study carried out by Francis and his team in 2014 showed that FABP was reduced when NEC inflamed intestine was augmented with *L. acidophilus*.

The sterol transporter NPC1L1 was downregulated in the presence of live *Lactobacillus rhamnosus.* Caco2 treated with bacterial cytoplasmic or its filtered spent broth also showed no significant change in NPC1L1 expression, heat inactivated bacteria did not alter NPC1L1 expression. Huang and Zheng's study in 2010 on elucidating the possible underlying mechanism behind NPC1L1 inhibition by probiotic *L. acidophilus* during bacteria-cell interaction reported that heat inactivated *L. acidophilus* did not significantly reduce NPC1L1 expression. They further stressed that bacteria culture supernatant caused reduction in NPC1L1 expression (Huang and Zheng 2010), these findings are however consistent with our results. Although there was no significant change in *NPC1L1* expression in FSB treated in all protocols relative to control, nevertheless, there was a significant decrease in *NPC1L1* expression in FSB treated cell according to protocol 1 (overnight co-cultured cells overlaid with lipids without washing) compared to protocol 3 (overnight co-cultured cells washed and treated with lipids only).

Gorenjak et al*.* in 2014 revealed a contrary result to that of Huang and Zheng when he stated that *Lactobacillus* GG did not downregulate NPC1L1 expression in Human intestinal epithelia cell line (HIEC), while Huang and Zheng reported that *L. rhamnosus* GG significantly reduced NPC1L1 in Caco-2 cell line. Gorenjak suggested that the possibilities in the different results might be attributable to different cell lines used and discrepancies in the qPCR layout. Stancu et al (2014) concluded that administration of probiotic mix of *Lactobacillus acidophilus* and *Bifidobacterium animalis* led to an overall decrease in expression on NPC1L1 and *MTP* in the gut with a corresponding decrease in plasma lipid and total cholesterol. Mice

expressing loss of intestinal NPC1L1 have also been shown to display reduced intestinal cholesterol absorption and likely to be less prone to hypercholesteremia (Eddy and Masuku 2014). This finding was corroborated by Lim et al. in 2016 when he concluded that prolonged exposure of HT29 cells treated with probiotics led to downregulation of NPC1L1.

In conclusion, intracellular triglyceride level (TG) of Caco2 cells was significantly reduced by live *Lactobacillus rhamnosus* (LB) compared with other bacterial products indicating that presence and interaction with the whole *Lactobacillus rhamnosus* might be required to inhibit lipid absorption. *MTP* expression in Caco2 cells was minimally reduced in live *L. rhamnosus* (LB) treated Caco2 cells. However, the expression level GPAT3, I-FABP and NPC1L1 was reduced in Caco2 cells treated with live bacteria. *Lactobacillus rhamnosus* MRS6AN may reduce lipid uptake and accumulation perhaps via modulation of GPAT3, I-FABP and NPC1L1.

Future Research

Future study designed to investigate the effect of probiotics on mRNA level of various genes involved in G3P pathway in lipid absorption is highly recommended. This is because with respect to the results obtained from this study, there might be transcriptional regulation of some of the genes at mRNA level. It is also essential to determine the specific receptor on the bacterial cell responsible for interaction with Caco2 cells during lipid absorption. This study focused on effect of probiotics on lipid uptake and accumulation in Caco2 cells, subsequent study can possibly look into their effect on secretion of triacylglycerol (TAG). These might help in designing therapeutic and novel drugs that will help in reducing intestinal absorption and accumulation of dietary lipids. This study should also be carried out in mice models fed with high fat diet alongside with probiotics and investigate its effect on intestinal G3P pathway

expression, this might help in establishing the effect of probiotics on GPAT3 mediated pathway in lipid uptake.

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VITA

AYODEJI O. IPINMOROTI

Education: BTECH Microbiology, Federal University of Technology Akure, Ondo state, Nigeria 2011.

MS Biology, East Tennessee State University, Johnson City, Tennessee 2017.

Professional Experience: Tutor, Evangel Nursery/Primary School, Akure, Ondo state, Nigeria 2001-2002.

Teaching Assistant, Department of Health Science, College of Public Health, East Tennessee State University, Johnson City Tennessee 2015-2017.

Affiliations:

- Member of the Graduate Professional Student Association (GPSA) ETSU chapter (2016 till date).
- Member of the American Physiology Society (APS) (2017 till date).
- Member of American Society of Biochemistry and Molecular Biology (ASBMB) (2017 till date).
- Member National Society of Black Engineers (NSBE) (2016 till date).
- Member Junior Chambers International (JCI) 2009-2011

Conferences Attended:

- Appalachian Student Research Forum (ASRF) - April 2017.
- Experimental Biology Conference (EB) - April 2017.

Honors and Awards: Award of Recognition “Gifted Hands” of the year, Department of Industrial Design, Federal University of Technology Akure, Ondo state Nigeria 2011.