Bile Salt Hydrolyzing *Lactobacillus reuteri* (NCIMB 30242) for the reduction of markers of metabolic disease

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TO MY FAMILY AND LOVED ONES
FOR THEIR UNDYING SUPPORT

Abstract

Metabolic disease is a global epidemic affecting millions of individuals. Downstream symptoms include diabetes, atherosclerosis and fatty liver disease. While drugs exist to alleviate these symptoms, they retain numerous side-effects. Probiotics, beneficial bacteria, have been the source of interest in recent years due to their varying enzymatic functions. One such function is the capability of some probiotics to hydrolyze bile salts via an enzyme called BSH (bile salt hydrolase). As a result of this, BSH-active bacteria have been investigated for their potential in lowering hypercholesterolemia and hyperlipidemia. However, survival through the stomach has been a major limitation of probiotic therapy. Microencapsulation of probiotics has been shown to increase their viability during their passage through the stomach. This opens the possibility for enhanced reduction of factors for metabolic disease in an animal model via oral delivery of microencapsulated BSH-active probiotics.

In this thesis, a BSH-activity assay was developed to identify a highly active *Lactobacillus reuteri* strain and was adapted to probiotics encapsulated in APA-microcapsules (alginate-poly-L-lysine-alginate). The stability of the APA-microcapsule doses was determined. Finally, to determine the effect of this treatment on markers for metabolic disease, Bio F1B hamsters fed a high-fat diet were administered APA-microcapsules containing the active probiotic. Physical, serum, and tissue factors linked to metabolic diseases were monitored.

Results showed that the developed assay was an effective method of determining BSH-activity in free and microencapsulated probiotics. A *L.reuteri* strain was selected and its therapeutic stability was shown to remain high for an extended period of time following APA-microencapsulation. The animal study demonstrated that the probiotic treatment improved physical, blood and tissue markers of metabolic disease in Bio F1B hamsters induced with a high-fat diet.

Résumé

Les problèmes métaboliques affectent des millions d'individus dans le monde. Ses symptômes mènent à des syndromes métaboliques comme le diabète, l'athérosclérose et des maladies de foie. Malgré l'existence de médicaments qui allègent ces symptômes, ces derniers portent de nombreux effets secondaires. Les bactéries probiotiques, des bactéries bénéfiques, furent récemment une source d'intérêt grâce à leurs capacités enzymatiques extrêmement variées. Parmi d'autres, une tel enzyme s'appelant hydrolase de sels biliaire (HSB) est exprimé dans plusieurs bactéries. En conséquence, les bactéries exprimant l'enzyme HSB furent le sujet de recherche à cause de leur potentiel à réduire les niveaux physiologiques de cholestérol and lipides. Néanmoins, la survie des bactéries probiotiques pendant leur transport via l'estomac a toujours été une limitation majeure d'une telle thérapie. Par contre, les recherches ont démontré que ceci peut être amélioré en enveloppant les bactéries probiotiques dans des microcapsules protectrices. La microencapsulation de bactéries probiotiques exprimant l'enzyme HSB ouvre la porte pour de nombreuses possibilités à réduire plus efficacement les facteurs associés aux problèmes metaboliques.

Dans cette thèse, une essai fut développé pour quantifier le niveau d'activité enzymatique de HSB et adapté pour les microcapsules APA (alginate-poly-L-lysine-alginate). Avec l'aide de cet essai, une bactérie *Lactobacillus reuteri* fut sélectionnée. La stabilité des doses de microcapsules APA fut déterminée. Finalement, pour déterminer l'effet de cette thérapie sur les facteurs metaboliques, des hamsters Bio F1B (nourri avec un régime alimentaire riche en lipides) furent donnés microcapsules APA contenant les bactéries probiotiques actives. Des facteurs physiques, sanguins, et tissulaires furent suivis de près.

Les résultats ont montré que l'essai développé est une méthode efficace de déterminer l'activité de l'enzyme HSB dans les bactéries probiotiques libres et microencapsulés. Une souche bactérienne de *L.reuteri* a été sélectionnée. Une étude sur sa stabilité enzymatique a démontré que sa viabilité et activité ont demeuré hautes suivant leur microencapsulation. Une étude animale a démontré que cette thérapie probiotiques améliore les facteurs physiques, sanguins and tissulaires liés aux problèmes métaboliques dans les hamsters Bio F1B (nourri avec un régime alimentaire riche en lipides)

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Preface

In accordance with the McGill thesis preparation and submission guidelines, I have decided to write this thesis as a compilation of original papers. This section is provided in the McGill University Thesis Preparation and Submission Guidelines, which reads as follows:

"As an alternative to the traditional thesis style, the research may be presented as a collection of papers of which the student is the author or co-author (i.e., the text of one or more manuscripts, submitted or to be submitted for publication, and/or published articles (not as reprints) but reformatted according to thesis requirements as described below). These papers must have a cohesive, unitary character making them a report of a single program of research."

The research articles presented in this thesis (chapters 3, 4 and 5) are divided into the following sections: Abstract, Introduction, Materials and Methods, Results and Discussion, and Conclusions. This thesis also includes a common Abstract, General Introduction, Literature Review, Summary of Results, Discussion, Conclusions and future recommendations and thesis cited references.

List of Abbreviations

ANOVA - analysis of variance

APA - alginate-poly-l-lysine-alginate

BSH - bile salt hydrolase

CRP – C-reactive protein

FFA – free fatty acids

GI – gastrointestinal

HDL-c – High-density lipoprotein cholesterol

IL-6 – Interleukin-6

IL-10 – Interleukin-10

LDL-c – Low-density lipoprotein cholesterol

MRS - de Man, Rogosa and Sharpe media

SD - standard deviation

SEM – standard error of the mean

TNF-α – Tumor necrosis factor alpha

Units

CFU - colony forming units

ml - milliliters

µl – microliters

µm - micrometer

µM - micromolar

mM - millimolar

g - grams

mg - milligrams

µg – micrograms

pg - picograms

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CHAPTER I: General Introduction

Metabolic diseases affect millions of individuals worldwide. They include problems such as diabetes, fatty liver and heart disease which account for the majority of deaths in the Western world [1]. Many are marked by an increased state of low-grade inflammation. This inflammatory state originates from the adipose tissue and immune cells of affected individuals, but eventually has a systemic effect on the entire physiology of the body [2, 3]. Many drugs exist to treat the symptoms of this systemic problem (e.g statins for high cholesterol, medicine for high blood pressure, NSAIDs), however these solutions retain a number of side-effects. Furthermore, they do not treat the root cause of the problem. [4, 5]. In the Western world, hyperalimentation and consumption of calorie-dense fatty food is a notably documented problem. Compounded by low levels of physical activity, this leads to downstream metabolic problems. While treatment solutions exist to reduce absorption of nutrients in the GI tract (such as lipase inhibitors), these also have negative side-effects [6].

The gut microflora is a bacterial ecosystem lining the intestinal tract. Probiotics are bacteria capable of exhibiting a positive enzymatic effect on the host health when introduced in the GI tract. Due to their extremely varying enzymatic functions, interest has developed in using probiotics for treatment purposes [7]. One such enzymatic function is the capability of some probiotics to hydrolyze bile salts via an enzyme called bile salt hydrolase (BSH). BSH activity has been documented in several different bacterial strains [8]. The enzyme cleaves bile salts, rendering inactive and promoting their excretion via the feces. Bile salts are amphipathic molecules found in bile (which is secreted by the liver in the duodenum) which facilitate the emulsification and subsequent absorption of dietary fats [9, 10]. As a result, it is theorized that

BSH-active probiotics may alleviate hyperlipidemia by interfering with lipid absorption. In addition, these probiotics have been studied for their potential cholesterol-lowering capabilities. Since cholesterol is a precursor to bile salts, increased bile salt excretion would result in lower cholesterol levels [9, 11].

While there is a growing interest in probiotics for their therapeutic effects, their oral delivery faces one major limitation: survival through gastric transit. The stomach is a low-pH, degradative environment high inhospitable to bacteria. When attempting to cross to the intestinal tract, ~99% of bacteria do not survive through this hazardous compartment. Microencapsulation has been explored as a method to increase the viability during gastric transit. For probiotics, encapsulation within APA (alginate-poly-L-lysine-alginate) microcapsules has seen good success in delivery to the GI tract [10, 12, 13]. Therefore, treatment of metabolic problems via microencapsulated BSH-active probiotics holds viable potential. However, several factors need to be considered and resolved prior to testing such a treatment in vivo. Firstly, a spectrophotometric assay capable of effectively and easily quantifying BSH-activity in microencapsulated probiotics must be developed in order to select a BSH-active strain and monitor dose activity. Furthermore, treatment formulations of the probiotic microcapsules must have their stability well characterized prior to in vivo testing. Finally, an appropriate animal model must be chosen to evaluate the effect of the microencapsulated probiotic doses on metabolic disease. This thesis bares these main goals in mind to develop the following research objectives.

1.1 Hypothesis

This thesis hypothesizes that a spectrophotometric assay can be developed for the quantification of BSH-activity of probiotics in simple and complex media, as well as in microencapsulated form. This study also postulates that a stable treatment dose of APA-microencapsulated BSH-active probiotics can be produced that is capable of improving markers for metabolic disease in an animal model.

1.2 Research Objectives

The primary objectives of the current study can be separated into a series of objectives:

1) To develop an assay for the determination of BSH-activity in free and APA-microencapsulated probiotics.

2) To select a BSH-active probiotic by using the developed assay.

3) To evaluate the optimal activity conditions of the selected probiotic.

4) To determine the stability of the treatment doses of microencapsulated probiotic.

5) To evaluate the effect of APA-microcapsules containing BSH-active probiotic on improving markers for metabolic disease.

CHAPTER II: Literature Review

2.1 Bile salt hydrolyzing probiotics as a therapeutic solution to metabolic disease

2.1.1 Probiotics for treatment of diseases

Probiotics are defined as microorganisms which, when administered in adequate amounts, can confer beneficial effects on the host's health [14]. Significant interest has existed in probiotics for treatment of several diseases. Inflammatory intestinal diseases have been shown to be strongly influenced by the host's gut microflora, as well as stimulation of pro-inflammatory immune pathways. Studies have demonstrated the beneficial effect of probiotics on such conditons. Such a study by Caplan et al [1999] has shown that supplementation of *Bifidobacterium* reduces the incidence of necrotizing enterocolitis neonatal rats [15]. Effects on colitis have similarly been shown using a *Lactobacillus* strain [16, 17]. Probiotics have been additionally shown to help with inflammatory bowel diseases, including Crohn's disease [18, 19]. Non-alcoholic fatty liver disease has also demonstrated improvement via probiotics [20]. Much research must still be conducted for these probiotic therapies to become viable treatment options in the clinic. [7] Finally, of notable interest, research on probiotics expressing an enzyme called bile salt hydrolase (BSH) has reported that treatment via these bacteria has beneficial effect on both hyperlipidemia and hypercholesterolemia [21, 22]. The mechanism of the enzyme requires an understanding of the biological interactions at its target site, which will now be treated.

2.1.2 Cholesterol and its connection to bile salts

Among its many functions (export to extrahepatic cells for membrane integrity, hormone production), cholesterol is used during the production of bile in the liver [23]. Bile (or gall) is a green/yellowish fluid mixture that is produced by hepatocytes and cholangiocytes. While constituting mostly of water (85%), the primary components of bile are amphipathic molecules called bile salts (also known as bile acids) (10%). The biosynthesis of most types of bile salts first involves the addition of a hydroxyl group at the C7 position (2^{nd} ring of cholesterol) via an enzyme called 7α-hydroxylase. Following this, most of the bile salts (>99%) are conjugated an amino acid; either glycine or taurine [24] Various types of bile salts exist in bile, which differ by their isometry, the presence/lack of a hydroxyl group at the C7 position, and their amino acid side-chain. These include cholic acid (CA), chenodeoxycholic acid (CDCA), glycocholic acid (GCA), glycodeoxycholic acid (GDCA) and taurodeoxycholic acid (TDCA) [24, 25]. These molecules are amphipathic and can easily form micelles; the bile salts arrange themselves to orient their hydrophobic cholesterol moieties towards the inside of the micelle, while pointing their hydrophilic glycine/taurine moieties towards the outside. The liver stores these micelles into the gall bladder (with other amphipathic molecules such as phosphatidylcholine) until their eventual release into the upper intestinal tract [25, 26].

2.1.3 Bile salt function and circulation

Bile salts fulfill the primary function of bile; the absorption of dietary fats and liposoluble vitamins. To achieve this, bile stored in the gall bladder is secreted in a portion of the upper intestinal tract called the duodenum. This usually occurs postprandially as a preemption to digestion. [26] Dietary fats enter the duodenum and tend to clump into large lipid aggregates.

Lipases secreted into the intestinal tract by the pancreas would be unable to effectively cleave the fats due to the limited access caused by low surface area of the lipid aggregate. In order to alleviate this problem, bile salts are secreted into the duodenum, and emulsify the large lipid clump into small droplets (~1μm diameter) [27]. This is achieved first by intestinal mixing which temporarily breaks the large aggregate into smaller droplets. Then, bile salts absorb onto the surface of the large aggregate via their hydrophobic head group, leaving their hydrophilic amino acid outside. The bile salts thus surround the droplet with a water-soluble layer. This layer is negatively charged due to the outer amino acid side-chains, and thus causes an electrostatic repulsion between the droplets which ensures that they remain separated. The final result is an increase the surface area of the dietary fats clumps; which allows them to be efficiently digested by pancreatic lipases [23, 27]. Bile salts are therefore essential for lipid absorption [23]. Following their mechanism of action, 85-95% of all bile salts are re-absorbed back to the liver (via the hepatic portal) in a section of the digestive tract called the terminal ileum [24, 27] . Only about 5-15% of total bile salts are lost in the feces. The liver only resynthesizes this 5-15% in order to maintain homeostasis, before secreting it back into the duodenum via the bile duct [27]. This bile salt recycling pathway is known at the enterohepatic circulation. It ensures minimal loss of bile salts by excretion, and is under the regulation of a negative-feedback system which ensures steady level of available bile salts in the system. [24, 26, 27]. This recirculation is illustrated in the figure below.

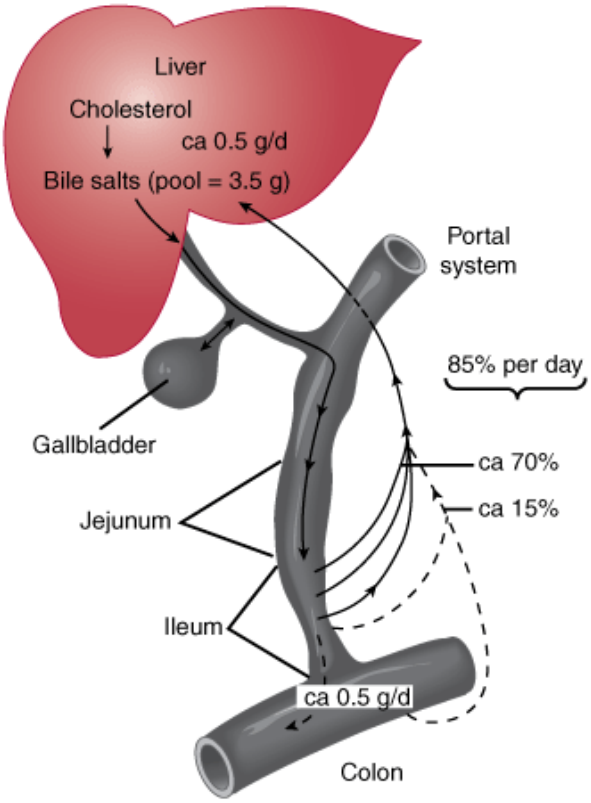

Figure 2.1 Enterohepatic circulation of bile salts[28]

2.1.4 The Bile Salt Hydrolase (BSH) enzyme

Bile salt hydrolase (BSH) is a bacterial protein belonging to the choloylglycine hydrolase family of enzymes. The enzyme was generally found to be mostly cytosolic and have an activity peak at slightly acidic pHs (<6.0) [29, 30]. BSH catalyzes the hydrolysis of bile salts at their amino acid/cholesterol junction. Protein sequence alignments using CLUSTAL software have shown that shown several conserved amino acids in its sequence, the most crucial of which is an N-terminal catalytic cysteine residue. [9, 31] BSH active sites recognize the amino acid moieties of bile salts, and have higher efficiency at deconjugating glycine bile salts than taurine bile salts.[32, 33] Research is still ongoing to discern the exact function of bacterial BSH enzymes, however a number of proposed theories have been hypothesized. One theory suggests that the

amino acids released from the hydrolysis may be used as energy sources for the bacteria. It has been observed that taurine released from bile salts can be utilized as an electrons acceptors and improve growth in *Clostridium* bacterial strains [34, 35]. Another theory proposes that BSH is expressed in intestinal bacteria to facilitate the incorporation of freed cholesterol into their membranes. This could offer protection and stability by improving the tensile strength and fluidity of the membrane. [36, 37] Finally, the most supported theory suggests that BSHs enzymes are expressed in intestinal bacteria to protect from the damaging effects of bile. [38] All theories have valid justifications, and it has become widespread belief that BSH serves all these functions to some degree in bacteria.

2.2 Systemic metabolic syndrome as a pro-inflammatory state

In recent years, research has shown that metabolic diseases such as obesity and diabetes are strongly associated with a chronic and systemic state of inflammation. This inflammatory state has been evidenced by changes in secretory cells and circulating levels of biochemical markers. Both adipose (fat) tissue and the liver play important roles in the pathophysiological development of metabolic problems.

2.2.1 Physiological role of adipocytes

Adipose tissue (or fat tissue) is used by the body for thermal insulation and cushioning against shock, as well as serving as the primary location of long-term energy storage. It is composed of adipocytes, connective tissue, and a number of immune cells (primarily macrophages). The tissue is heavily vascularized and riddled with nerve fibers [39, 40]. Adipocytes mature from pre-adipocytes that originate from mesodermal multi-potent stem cells

that are perpetually generated throughout human life [41]. Mammals have evolved effective methods for storage of energy during periods of caloric excess, which allow helps ensure survival during periods of famine. Adipocytes in white adipose tissue (WAT) play the most predominant role in this function [42]. The cells store triglycerides in a large lipid droplet which comprises the majority of their volume. During caloric deficiency, these triglycerides are hydrolyzed by hormone sensitive lipase (HSL) which is down-regulated mainly by insulin [2]. The hydrolysis of triglycerides results in free fatty acids that are released into the bloodstream by the adipocytes to satisfy the energy requirements in other tissues [2]. In recent years, WAT adipocytes have been shown to not only shown serve as passive deposits of energy, but as key secretory cells which play important endocrine functions in the body [40, 43-45]. Adipocytes were found to produce a variety of hormones and cytokines that regulate metabolic homeostasis in many organs and tissues including muscle, liver, blood vessels and the central nervous system. These metabolic function are involved with glucose and lipid metabolism, inflammation, coagulation, blood pressure, and feeding behaviour [40, 43-45]. In summary, adipose tissue can be considered a complex secretory and regulatory organ which is connected to metabolic function at the physiological level via numerous biochemical pathways. Therefore, its dysfunction has severe repercussions on the metabolic and inflammatory homeostasis of the body.

2.2.2 Physiological role of liver

The liver is a vital organ with a wide range of functions in detoxification, metabolism, and immune response. It is comprised of many diverse cells types which include lymphocytes, biliary epithelial, sinusoidal endothelial, Kupffer, stellate and dendritic cells. Approximately two

thirds of its total cells are hepatocytes, which act as the main enzymatic work-horses involved in metabolism [46]. The other cells hold specialized immune functions. Sinusoidal endothelial cells form a sieve-like epithelium involved in antigen presentation [42]. Kupffer cells phagocytose foreign microorganisms and debris which travel through the sinusoidal space. Liver lymphocytes are comprised of T cells, natural killer cells and, to a minor degree, B cells [46].Biliary epithelial cells (or cholangiocytes) primarily regulate ductal bile secretion [47]. Amongst many enzymatic functions, hepatocytes are responsible for the biosynthesis of cholesterol. Cholesterol is an essential molecule required in all cells for integrity of their outer lipid membrane. It is synthesized from lanosterol in a 19-step enzymatic process. Post-synthesis, cholesterol is esterified in hepatocytes by acyl-Coa:cholesterol acyltranferase (ACAT) to form cholesterol esters. These are secreted in circulation as complexes called VLDLs (very-low-density lipoprotein). Muscle cells and adipocytes uptake and convert VLDL to LDL (low-density lipoprotein) which then travel to all peripheral tissues where they are uptaken via receptor-mediated endocytosis [23, 48].

2.2.3 Development and pathophysiology of metabolic disease via hyperalimentation

The development of metabolic disease via hyperalimentation, and its downstream complications, is a progressive problem initially involving deregulation of adipocyte-macrophage interactions. Since adipose is vascularized, this dysfunction can then spread systemically, causing secondary negative effects on liver function, glucose uptake in tissues and the cardiovascular system. The development of diet-induced metabolic problems is initiated with hyperalimentation. This leads to an accumulation of lipids in adipocytes, and subsequent expansion of the cells within the tissue [2, 3, 42]. These enlarged adipocytes have comprised

triglyceride uptake, and tend to release saturate free fatty acids (FFAs). FFAs bind to toll-like receptors (TLR-4) found on the surface of macrophages that are imbedded within the adipose tissue. This results in the production of TNF-α (an inflammatory cytokine) by the proximal macrophages through the activation of the NF-κB pathway [49, 50]. This secreted cytokine in turn increases lipolysis and free fatty acid release from adipocytes. Adipocytes also react to TNF-α by activating a variety of genes which include Interleukin-6 (an inflammatory cytokine), intracellular adhesion molecule-1 (ICAM-1), and macrophage chemo attractant protein-1 (MCP-1) [51, 52]. MCP-1 and ICAM-1 promote the recruitment of monocytes into the adipose tissue and their differentiation into macrophages. The final result is the development of a local positive-feedback loop involving the paracrine interaction of adipocyte-secreted FFAs and macrophage-secreted TNF-α [2]. As adipocytes enlarge, local hypoxia occurs because the diameter of the cells surpass the diffusion distance of oxygen (which is approximately 100 μm) [53]. Adipocytes respond to these hypoxic conditions by secreting angiogenic factors which include VEGF, hepatocyte growth factor and PAL-1. This in turn results in the creation of blood vessels that further vascularize the adipose tissue, in an attempt to counter these hypoxic conditions [54, 55]. However, the unfortunate side-effect of this angiogenic response is that the local FFA/TNF-α paracrine loop is now more heavily irrigated with blood vessels. Therefore, this predominantly local loop can now become systemic. Mediators of metabolic diseases are therefore secreted from the adipose tissue and travel to a variety of tissues in the body where they can cause metabolic diseases [2, 42]. The secretory changes are illustrated in the figure below.

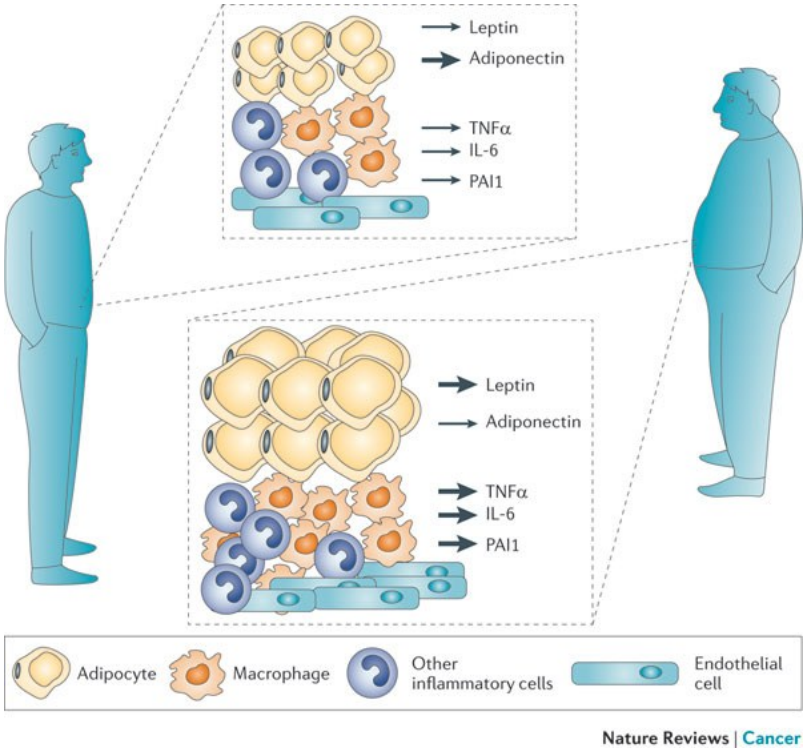

Figure 2.2 Secretory changes in adipose tissue via hyperalimentation [56]

The mediators, and their resulting effects at the systemic level, will now be discussed in further detail.

2.2.4 Mediators and markers of metabolic diseases

2.2.4.1 Free fatty acids (FFAs)

The evidence linking elevated levels of plasma FFAs to an increase in the incidence of type II diabetes has been growing over the last several years [3, 57]. Diabetes is defined as the system's inability for cellular glucose uptake due either lack of insulin secretion (type I) or a ineffective response to insulin (type II). Free fatty acids released in the bloodstream from enlarged adipocytes have been shown to act on many biochemical pathways involved in insulin

secretion and response. In skeletal muscle, increased concentrations of plasma FFAs have been shown to block insulin signaling by inhibiting tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), the interaction between the p85 subunit of phosphoinositol 3-kinase (PI3K) with IRS-1, and activation of PI3K. [58-60]. In addition, they have been shown to affect glucose metabolism, glucose phosphorylation and glycogen synthase [61-63]. Insulin is synthesized and secreted into the bloodstream by β-cells located in the pancreas. FFAs have been shown to inhibit insulin secretion through the opening of β-cell potassium channels, the reduction of ATP production in β-cells, and induction of β-cell apoptosis through a stress response pathway involving Bcl-2 [64]. In turn, reduced levels of insulin (the primary regulator of lipolysis) result in increased release of free fatty acids from adipocytes. In the liver, FFAs have been shown to increase lipid accumulation [2]. Finally, FFAs have been shown to increase cardiovascular risk [65], primarily through an increased synthesis of LDL-cholesterol in the liver and their contribution to epithelial dysfunction [2].

2.2.4.2 Interleukin-6 (IL-6)

Interleukin-6 (IL-6) is a major pro-inflammatory cytokine. As previously stated, it is secreted in the bloodstream by enlarged adipocytes in a state of low-grade inflammation. Like FFAs, high circulating levels of IL-6 have been implicated with both insulin resistance and risk of cardiovascular disease [66, 67]. As with FFAs, IL-6 contributes to the development of diabetes by both inhibiting autophosphorylation of the insulin receptor [68], and contributing to β-cell failure in the pancreas [69]. In the liver, evidence suggest that IL-6 is an inducer of synthesis of C-reactive proteins (CRPs), which are strong contributors to cardiovascular risk [70]. Through the action of CRP synthesis, as well as promoting transformation of macrophages

into foam cells on arterial walls [71], IL-6 contributes heavily to cardiovascular disease (atherogenesis).

2.2.4.3 Tumor necrosis factor alpha (TNF-α)

Tumor necrosis factor alpha TNF-α, is a pro-inflammatory cytokine found locally in high concentrations in white adipose tissue during obesity and metabolic disease. However, like IL-6, elevated circulating levels of TNF-α contribute to insulin resistance via IRS-1 inhibition [72], and to cardiovascular risk via atherogenesis [43]. It is a large contributor to the promotion of lipolysis in adipocytes, which promotes strong release of FFAs into the bloodstream [2].

2.2.4.4 Cholesterol, HDL-c, LDL-c

Cholesterol is an essential molecule synthesized in the liver. As described previously, hepocytes deliver cholesterol to other tissues by complexing it into VLDL-c. This is converted to LDL-c by adipocytes, secreted into the bloodstream, and uptaken by the target cells. To return cholesterol to the liver, it is complexed by the extrahepatic cells into HDL-c (high-density lipoprotein cholesterol), secreted into the bloodstream, and uptaken by the liver [23]. Under conditions of high concentration hepatic free fatty acids, the production and secretion of LDL-c particles are increased. In addition, enlarged adipocytes which are under a state of low-grade inflammation secrete to a larger extent a plasma protein called cholesteryl ester transfer protein (CETP) [2]. During metabolic disease, CETP converts HDL-c into triglyceride-rich HDL particles (TG-HDLc) which are rapidly hydrolyzed and cleared from circulation [2, 73]. Both these factors result a relative larger concentration of plasma LDL-c over HDL-c [2]. Elevated

levels of LDL-c are an extremely large risk factor for atherosclerosis, the blocking of arterial blood vessels via the accumulation of fatty materials along its walls. Circulating LDL-c is readily oxidized in the blood, and tends to aggregate along the arterial wall. The resulting aggregate forms a "plaque", which attracts monocytes. The monocytes attempt to phagocytose the oxidized LDL-c, however fail and are converted into foam cells which become trapped within the plaque. This cycle continues, as monocytes keep invading, and the plaque grows until it results in blockage [74]. Under conditions of elevated inflammation, the plaque can burst, dislodge and cause blockage in a downstream location of the blood vessel. The resulting interruption of blood flow can cause permanent damage to the location in question, or death if it affects physiologically vital area [74]. Therefore, high circulating levels of LDL-c, low levels of HDL-c, and a high atherogenic index (defined as log [TG/HDL-c]) are strong markers for risk of cardiovascular disease during a state of metabolic syndrome.

2.2.4.5 C-reactive proteins (CRPs)

C-reactive proteins (CRPs) are predominantly synthesized in the liver in response to elevated IL-6 [42, 70].They are acute-phase proteins which are considered markers for cardiovascular disease, and to a lesser degree, insulin resistance [66, 75-77]. CRPs have been shown to be one of the strongest univariate predictors of cardiovascular events between 12 other common markers [78, 79]. Its precise mechanism of action is still being studied.

2.3 Animal model for metabolic disease

2.3.1 Bio F1B Hamster as an animal model for diet-induced metabolic disease

The Bio F1B hamster is an extremely useful animal for studies of metabolic diseases. In research, its applications are in studies concerning hyperlipidemia, hypercholesterolemia, atherosclerosis, diabetes, inhalation toxicology, gait and electrocardiographic abnormalities. The Bio F1B model is a genetically defined hamster that is the result of cross-breeding between two other hamster lines; Bio 87.20 female with a Bio 1.5 male [80]. Hamsters have been shown to have blood lipid profiles similar to humans. Spady & Dietschy showed that hamsters have circulating LDL-cholesterol levels that respond almost identically to dietary lipids as humans [81]. Both hamsters and humans were shown to have the same difficulty in adapting to cholesterol flux in the liver, as compared to mouse and rat models [82]. The Bio F1B hamster is of particular interest for metabolic problems because they have been shown to develop atherosclerotic lesions in the aorta at significantly lower blood cholesterol concentrations [83, 84] The animal model also has plasma CETP activity comparable to humans, unlike pig, rat and mouse models which have little to no activity of this protein in the blood [85, 86]. The Bio F1B hamster has been a useful model in studies on hyperlipidemia [87-89]. Finally, Bio F1B hamsters easily incorporate fat and develop a relatively high body weight. They can survive long (50% survival rate after 120 weeks) despite conditions of hyperlipidemia and high-risk levels of atherogenic factors [90]. Given all this evidence, the Bio F1B hamster is an excellent candidate for studies in metabolic diseases.

2.3.2 Alternative animals models for metabolic disease

Aside from Bio F1B Golden Syrian hamsters, other rodent animal models exist to study metabolic diseases. Obese *(ob/ob)* mice are a popular choice for studying obesity and metabolic problems [91, 92]. The model is the result of a knockout mutation on the *ob* gene which codes for leptin, a protein which down-regulating appetites and controls other metabolic functions *Ob/ob* mice are incapable of producing functional leptin. This leads to uncontrolled appetite which causes accumulation of adipose tissue, high blood sugar and increased levels of insulin [93, 94]. Studies have also made use of C57BL/6C mice. C57BL/6C mice displays high leptin levels, glucose intolerance (leading to development of diabetes) [95, 96]. Currently, *ob/ob* and C57BL/6C strains are standard for metabolic syndrome studies in mice [97], however they do not have blood lipid profiles as similar to humans as Bio F1B hamsters [82]. In addition to mice, several rat models exist for metabolic syndrome studies. OLETF (Otsuka Long Evans Tokushima Fatty) rats are a polygenic diabetes model that develop hyperinsulinemia, hyperglycemia, insulin resitance, hypertriglycemia and mild obesity.[98, 99] Wistar rats show obesity responses for both leptin and insulin under a high-fat diet. Female hooded Wistar rats fed a high-fat diet have been shown to have significantly lower plasma leptin concentrations than rats fed low-fat diets [100, 101]. This rat model was also shown to have a compromised insulin response under a high-fat diet [101]. Fat deposition was found to be significantly increased in Wistar rats [102]. Male Sprague-Dawley rats are a model with useful metabolic parameters for studies on metabolic diseases. These rats were shown to become insensitive to plasma leptin following 5-week induction with high-fat diet [101] They also showed a tendency towards hypertrophic adipocyte accumulation when fed a high-fat diet rich in long-chain fatty acids.[103, 104] Consequently, under a high-fat diet, these rats have shown a fast rate of weight gain [105].

This weight gain can be maintained despite a dietary decrease in energy intake, indicating resistance towards lipolysis [106]. Finally, the use of Zucker rats is very common for studies in metabolic diseases. These animals show high weight gain when fed *ad libitum* [107]. Similar to *ob/ob* mice, obese Zucker rats are a genetic model expressing a homozygous recessive trait (*fa/fa*) on the leptin receptor [108]. This model is marked by several metabolic characteristics which include heavy weight gain [109, 110], insulin-resistance [111, 112], hyperphagia [113, 114], hyperlipidemia in adipose tissue and liver [115, 116], and adipocyte hypertrophy [117]. While Zucker rats are a useful tool for these conditions, their primary disease mechanism is uncontrolled hyperalimentation due to a deficiency in the leptin receptor regulating appetite. Induction of metabolic diseases in this model is therefore independent of diet type. Treatment via BSH administration in the model would be swamped by excessive dietary intake. In summary, all these rodent models have differing, yet useful applications in studies for metabolic disease.

2.4 APA-microencapsulation as a technique for oral delivery of probiotics

One of the largest limitations of oral probiotic delivery is the survivability of bacteria through gastric transit. While the upper duodenum maintains a steady stable environment for probiotic action, the lower pH and degradative conditions (presence of pepsin, etc) in the stomach make it difficult to successfully deliver bacteria to the intestinal tract. In response such challenges, microencapsulation was developed. Microencapsulation can be defined as the "entrapment of a compound or system inside a dispersed material" [118] While several types of encapsulation exist, microencapsulation within alginate-poly-L-lyise-alginate (APA) microcapsules has seen the greatest potential for oral delivery of probiotics to the intestinal tract. This is illustrated in the figure below.

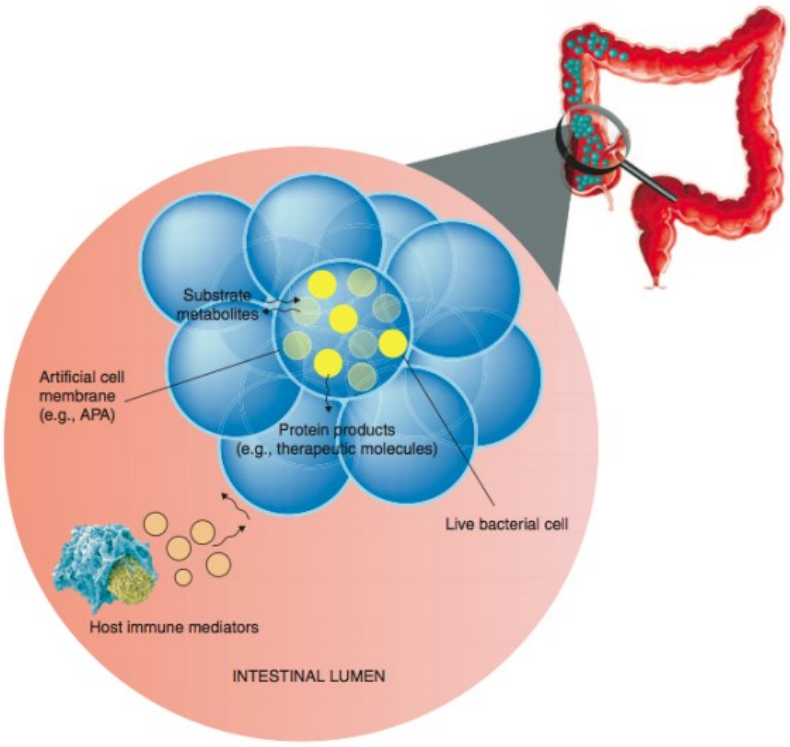

Figure 2.3 Delivery of probiotic to GI tract via encapsulation in APA-microcapsules [119]

2.4.1 APA-microcapsule structure

The APA microcapsule is a three-layered capsule of comprising of an inner core of alginate, middle layer of poly-L-lysine, and an additional outer layer of alginate [120]. This three-layered structure is illustrated below.

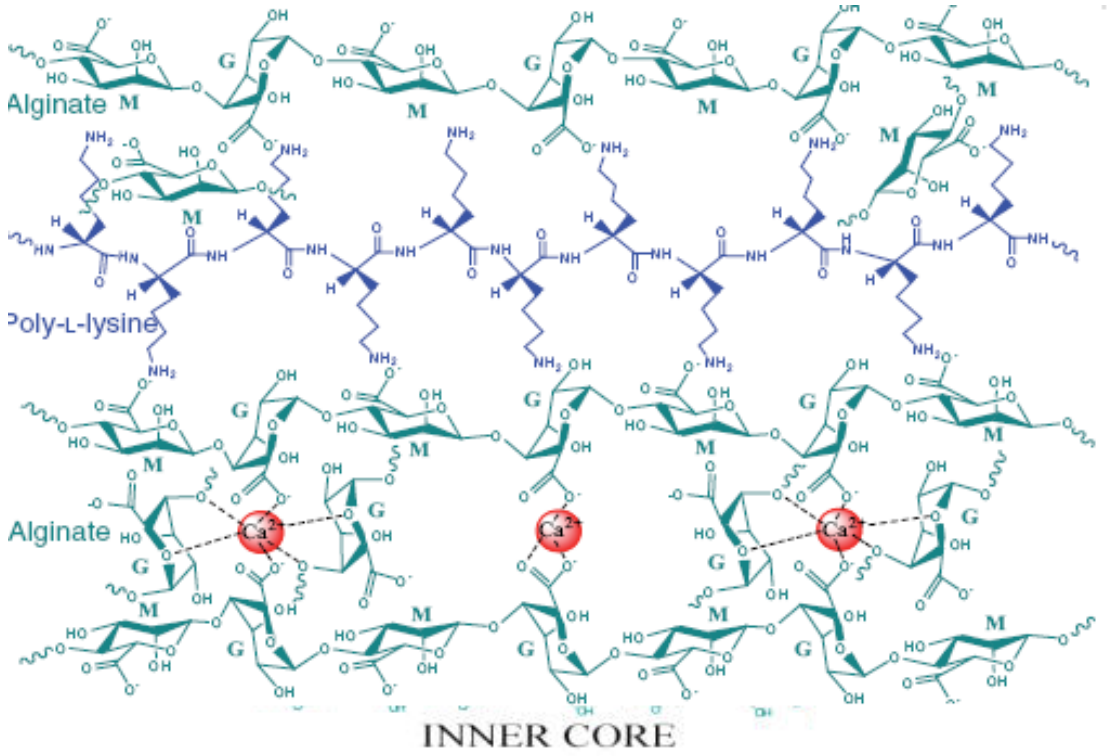

Figure 2.4 Molecular structure of the three-layered APA polymeric membrane [121]

The inner alginate core is gelated as transient polymer. Alginate is a copolymeric saccharide comprised of one of two types of monomers: mannuronic acids (the "M" monomer) and guluronic acid (the "G" monomer). Mannuronic and guluronic acids are simply diastereomers of one another differing only by the orientation of the carboxyl group at their C5 carbon position [120, 122]. Alginate polymers can have varying lengths, and can be dissolved in aqueous solution. However, in the presence of calcium ions (Ca^{2+}), the positive charges of these ions

begin to ionically interact with the negatively charged carboxyl groups of alginate. The final result is a densely-packed matrix of alginate strands which is held together by Ca^{2+} ions. Surrounding the alginate core is a layer of poly-L-lysine (PLL); long chains of lysine amino acids [122]. This coating is maintained by hydrogen-bond interactions between the amino group of lysine with the hydroxyl and carboxyl groups of the alginate molecules located on the outer limit of the core. Above this polypeptide layer lies an additional coat of alginate, held in place by similar hydrogen bond interactions. The resulting triple-layered microcapsule maintains a stable structure and has key properties that are relevant towards oral delivery [118, 120, 122].

2.4.2 Essential requirements met by APA-microcapsules

2.4.2.1 Semi-permeability and protection

Semi-permeability is an important feature of the APA microcapsule. In the case of encapsulated probiotics, semi-permeability confers protective advantages to the bacteria, while helping to maintain their natural health. The mesh-like alginate matrix provides a sieve through which small molecules can cross but large molecules cannot [123]. Essential metabolic substrates (e.g glucose) and waste products can freely pass in and out of the alginate core which contains the bacteria. However, larger molecules such as pepsin, which could damages the probiotics during gastric transit, cannot enter and do not reach the probiotics [123-125]. BSH-active bacteria can thus fulfill their catalytic function without the need to exit the capsule, since bile salts can freely enter the alginate core, and the cholesterol/glycine cleavage products can exit. The APA microcapsule thus confers a protective feature which increases the viability of encapsulated cells, while maintained their activity [126].

2.4.2.2 Mechanical strength

The middle PLL layer is the primary structural component which provides mechanical stability to the microcapsule. This inner polypeptide coating ensures that the capsule remains stable despite mechanical forces in the stomach and intestinal tract, as well as osmotic pressures, which impose shear stress. Thus, the spherical capsule better maintains a stable structure which protects its probiotic content from exposure [127].

2.4.2.3 Biocompatibility (immunogenicity and biodegradability)

The APA microcapsule's most prominent feature is its biocompatibility. This is an important factor, given its applications for oral delivery of probiotics. Epithelial cells lining the intestinal wall crosstalk with mucosal dendritic cells. This interaction ensures that the introduction of potentially hazardous foreign materials and organisms within the gut can activate an immune response, as a protective measure [128]. While only some probiotics activate this immune response, the PPL layer of the APA microcapsule is immunogenic; activating the complement immune system [129] The outer alginate layer is intended to protect the middle PLL coating from detection by macrophages, and prevent subsequent immune response. While this biocompatibility has good success, APA-microcapsules have nonetheless been shown to activate the complement system, as well as increase production of IL-1 and TNF-α by macrophages [13, 130-132]. This can however be attributed to the biodegradability of the APA-microcaspule [122] which exposes regions of the PLL coating downstream in the lower intestine and colon. Incomplete encapsulation may also explain these discrepancies, again exposing areas of the PLL layer to the outer environment.

2.4.3 APA-microencapsulation technology via electrostatic separation

Microcapsules are formed by separating a liquid stream of an aqueous alginate solution into small droplets. The alginate solution contains the sample intended for encapsulation. Electrostatic generators achieve the separation by applying a voltage across each incoming droplets. This electrostatic potential counters the natural electrostatic interactions (hydrogen-bonding interactions) of the water molecules, thus rendering the aqueous alginate solution less cohesive. The result is the formation of droplets in the range of micrometers [118]. This process is schematically illustrated below.

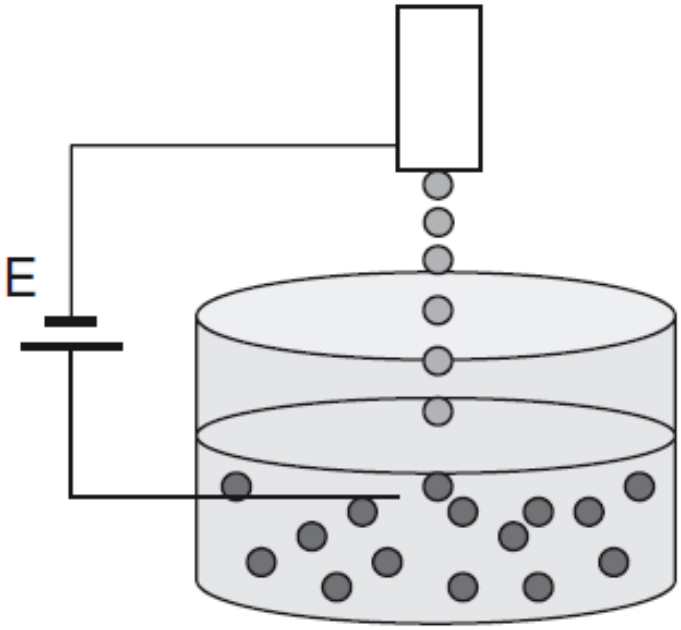

Figure 2.5 Microencapsulation technology via electrostatic separation of droplets [118]

Nozzle resonance assists in the separation by vibrating the jet of solution exiting nozzle at a specific frequency which ensures uniform droplets. Electrostatic generation and nozzle resonance work in tandem for optimal separation of aqueous solutions. For APA-

microencapsulation, these techniques are prime candidates. The liquid droplets fall into a bath of $CaCl_2$ solution, causing their gelation. Incubation of the gelated beads in a bath of PLL solution, and subsequently an alginate solution, results in a formation of APA-microcapsules containing the sample of interest. [118, 121]

2.4.4 Alternative polymeric membrane technologies for cell delivery

Alternative polymeric membranes exist for the delivery of cells for physiological target areas. HEMA-MMA (hydroxyethyl methacrylate-methyl methacrylate) is a copolymer which can serve as an effective membrane for encapsulation. HEMA-MMA microcapsules retain good stability and durability, however may not be suitable for all cell types which require anchorage within a matrix [133-135]. Chitosan microcapsules are another viable option which have even superior biocompatibility to APA membranes. However, in addition to requiring acidic conditions for its function, immunogenic properties of this membrane have shown mixed results [136-138]. Cellulose sulfate membranes have good mechanical stability, can be prepared in a single step of synthesis, and retain the possibility to adjust a number of physical and chemical parameters to suit its specific application. However, its semi-permeability for water-soluble molecules has not been explicitly demonstrated [133, 139]. Barium-based alginate microcapsules make use of Ba^{2+} ions for stability of the alginate matrix. They show both improve physical and chemical stability over calcium-based APA microcapsules, however, inhibit potassium ion channels and have mixed results with regard to immunogenicity [133, 140].

2.5 Research justification

Metabolic diseases comprised the most prominent health issues in the Western world. As illustrated in the review above, its negative physiological repercussions are systemic. Metabolic boosters and drugs (e.g statins) which target specific pathologies related to metabolic problems can alleviate symptoms, but hold a number of undesired side-effects. This thesis investigates APA-microcapsules containing BSH-active probiotic as a potential means of lowering markers for metabolic disease.

Research articles presented in Chapters III, IV and V:

1. **Daniel Marinescu**, Catherine Tomaro-Duchesneau, Meenakshi Malhotra, Mitchell L. Jones and Satya Prakash*. (2012) Determination of enzymatic bile salt hydrolase activity in free and immobilized probiotic bacterial cells: a novel spectrophotometric assay. Submitted to *Journal of Biotechnology*.

2. **Daniel Marinescu**, Catherine Tomaro-Duchesneau and Satya Prakash*. (2012) Maintenance of viability, activity and morphology of microencapsulated formulations of *Lactobacillus reuteri* (NCIMB 30242). To be submitted.

3. **Daniel Marinescu**, Catherine Tomaro-Duchesneau, Meenakshi Malhotra, Laetitia Rhodes, Imen Kahouli, Mitchell L. Jones, Satya Prakash*. (2012) Effect of orally administered APA microencapsulated BSH-active Lactobacillus reuteri NCIMB 30242 on markers of obesity: an in vivo analysis. Submitted to *Journal of Medical Microbiology*.

N.B.: All the manuscripts presented in this thesis are under preparation

Contributions Not Included in Thesis

Original research articles

1. Michael Coussa-Charley**, Daniel Marinescu**, Arghya Paul, Afshan Afsar Khan and Satya Prakash*. (2012) *Lactobacillus* probiotic can efficiently hydrolyze bile salts and displace potential pathogen in a simulated gut model. To be submitted to *Journal of Biologics: Targets and Therapy*.
2. Laetitia. Rodes, Afsah Khan, Michael Coussa-Charley, Arghya Paul, **Daniel Marinescu**, Wei Shao, Satya Prakash. (2012) In vitro screening of anti-inflammatory properties of probiotic bacteria: an in vitro study using a human colonic microbiota model and macrophage cell lines. Submitted to *Current Microbiology*.
3. Laetitia Rodes, **Daniel Marinescu**, Wei Shao, Catherine Tomaro-Duchesneau, Sana Saha, Imen Kahouli, Satya. Prakash. (2012) Alginate Poly-L-Lysine Alginate microencapsulation enhances Bifidobacterium longum subs. infantis tolerance to gastro intestinal conditions. 2012. In progress, to be submitted

Conferences

1. **Daniel Marinescu**, Hani Al-Salami, Catherine Tomaro-Duchesneau, Satya Prakash*. (2011) The stability of bile salt hydrolase producing microencapsulated *Lactobacillus* bacteria. Presented at the *Canadian Society of Pharmaceutical Sciences*

Contributions of Authors

Subsequent chapters are original research articles and I am the first author in each. I was responsible for designing studies, conducting experiments, analyzing data and finally, preparing manuscripts. Dr Satya Prakash, was reported as the last author in all my articles, is my supervisor, research advisor and corresponding author. Catherine Tomaro-Duchesneau, Laetitia Rodes, Imen Kahouli and Meenakshi Malhotra and Hani Al-Salami have all provided suggestions and assistance in designing and performing experiments. Details of each authors' contributions are described at the beginning of each chapter.

CHAPTER III:

Determination of enzymatic bile salt hydrolase activity in free and immobilized probiotic bacterial cells: a novel spectrophotometric assay

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Preface:

The present study presents the development of a novel spectrophotometric assay for the determination of bile salt hydrolase (BSH) activity in probiotics. It involves the detection of glycine release, following a cell-free incubation of the enzyme with bile salt. The assay is performed on a selection of free probiotics, on a single selected probiotic over the course of its growth curve, and on APA-microcapsules containing the selected probiotic. This study is imperative for the development of an assay to monitor the activity of probiotic doses during in vivo studies. It also serves in the selection of a BSH-active probiotic strain for these eventual animal studies. Catherine Tomaro-Duchesneau has contributed in experimental design, statistics and essential training.

3.1 Abstract

Bile salt hydrolase (BSH) catalyzes the hydrolysis of glycine- and taurine-based bile salts, resulting in their deconjugation. Research has demonstrated the use of BSH-active bacterial cells for many applications, including for lowering cholesterol levels. Microencapsulation and other immobilization techniques are currently being used for the probiotic delivery, posing a challenge when quantifying BSH activity using current methods. BSH quantification is important for the screening, selection and optimization of probiotic formulations. In this study, a spectrophotometric assay has been developed for quantifying BSH activity in free and immobilized cells. The assay was tested, developed and validated using four probiotic strains, with a ninhydrin-based detection of glycine following deconjugation of glycodeoxycholic acid. *Lactobacillus reuteri* NCIMB 30242, a known BSH-active strain was selected as the most BSH-positive strain in a validation test, with an activity of 17.09 ± 4.37 μmol of glycine/hr/g of bacteria. BSH activity of the same strain, microencapsulated, was 3.56 ± 0.24 μmol/hr/g, comparable to the free bacteria activity, validating the assay for use with immobilized cells. This study supports the use of this spectrophotometric assay for the quantification of BSH-activity in free and microencapsulated bacteria, with potential in future use for the development of a BSH active probiotic formulation.

3.2 Introduction

Recent clinical studies have demonstrated that an elevated level of serum cholesterol is strongly correlated to an increased risk of coronary heart disease, one of the leading causes of death in the Western world [22]. The interest in bile salt hydrolase (BSH)-active bacteria has expanded, in part due to their beneficial therapeutic effects on hypercholesterolemia [21, 22]. BSH is a predominantly cytosolic enzyme, found in a variety of bacterial strains, including those of *Lactobacillus*, *Clostridium*, *Bifidobacterium* and *Bacteroides* [8]. Potentially used as a salvage pathway by intestinal bacteria, BSH catalyzes the hydrolysis of bile salts, rendering them inactive [141]. (**Figure 3.1)**

Interestingly, bacteria expressing this enzyme have been shown to reduce LDL-cholesterol levels in animals [141, 142]. Further studies have demonstrated a similar beneficial effect in humans [143, 144]. However, one of the main limitations of the oral administration of probiotics is their lack of survival during the gastric transit. Microencapsulation of bacteria within ultrathin semipermeable polymer membranes has been used to improve cell viability during the gastric transit [12, 123-125, 145]. Polymeric alginate-polylysine-alginate (APA)-microcapsules are widely used due to their properties of biocompatibility and biodegradability [146]. Hence, BSH-active bacteria can be readily encapsulated within APA polymers for oral delivery, allowing for bacterial protection and maintenance of their enzymatic activity [10, 147].

A variety of methods currently exist to quantify BSH activity; such as radiochemical assays using ^{14}C-tagged taurocholic acid as a substrate and measuring radiotagged cholic acid as the product [148, 149]. Also, BSH activity can be assessed spectrophotometrically by measuring the levels of free acids and bile acid conjugates [150], by monitoring the interaction between cholic acid and a chromophore, 5-amino-2-nitro-benzoic acid [151], or via a cholesterol co-

precipitation assay [152]. Finally, an accurate high-pressure liquid chromatographic (HPLC) method can be used for the separation of either conjugated or deconjugated bile salts followed by their quantification [8, 10]. While these methods are available, many remain expensive, time-consuming, or are not conveniently adapted for the use of microencapsulated or immobilized bacteria. In this study, we made use of a modified version of the spectrophotometric assay from Kumar et al., (2006), as outlined originally by Stellwag et al. [153, 154]. *Lactobacillus* strains were tested via the method. The primary objective of this study was to demonstrate the ability of this simple modified spectrophotometric assay to detect and quantify BSH-activity in both free and APA-microencapsulated BSH+ probiotics. Given the prominent use of APA-microcapsules in cell delivery, this study aimed to show that the assay is readily compatible with bacterial cells encapsulated in biodegradable polymeric membranes such as that of APA.

3.3 Materials and methods

3.3.1 Chemicals

All chemicals 1) GDCA salt 2) de Man-Rogosa-Sharpe (MRS) broth 3) poly-L-lysine 4) alginate (low viscosity) 5) TCA 6) ninhydrin (2%), glycine) as well as all buffers were purchased from Sigma Aldrich (Oakville, Canada) and were of HPLC or standard analytical grade.

L. reuteri (NCIMB 30242), *L. reuteri* (NCIMB 702656), *L. reuteri* (NCIMB 701089), *L. reuteri* (NCIMB 11951), *Lactobacillus fermentum* (NCIMB 5221) were purchased from NCIMB (National Collection of Industrial Bacteria, Bucksburn, Aberdeen, Scotland).

3.3.2 Determination of viability in free and encapsulated bacteria

Bacterial cell counts of the samples were determined via standard measurements of colony forming units (CFU) on agar plates. Briefly, 10-fold serial dilutions in sterile 0.85% (w/v) NaCl (saline) were prepared from a bacterial suspension. From each prepared dilution, 100µl were removed and plated onto MRS agar plates. The plates were allowed to incubate overnight at 37^{o}C in a 5% CO_2 chamber. Viability was calculated as CFU/ml for free-cell bacterial suspensions.

3.3.3 Development of method for BSH-activity determination in microencapsulated bacteria

Determination of BSH-activity in microencapsulated bacteria was developed following their encapsulation within APA-microcapsules. The encapsulation procedure was as follows. Cultures grown in MRS broth (supplemented with 5mM GDCA) for 16 hours were isolated by centrifugation at 8000xg for 20 minutes at 4^{o}C. The pellets were resuspended in saline and gently added to a slowly stirred 2% (w/v) sodium alginate solution. The final bacterial concentration in the alginate solution was 8% of the total volume (w/v). The bacterial alginate solution was run through an Inotech Encapsulator IER-20 (Inotech Biosystems International Inc, Rockville, MD) with a 300 µm diameter nozzle. The resulting droplets were collected in a crystallizing dish containing a stirring sterile 0.1M $CaCl_2$ solution. The gelated beads were allowed to sit in the $CaCl_2$ solution for 10 minutes. The beads were then washed with sterile saline and transferred in an equal volume of 0.1% w/v poly-L-lysine for 10 minutes. The coated beads were washed once more with saline and transferred into a solution of 0.1% (w/v) sodium alginate for 10 minutes for the final coating step. After an additional wash in saline, the microcapsules were transferred into

two equivalent volumes of 10% MRS solution for storage at 4°C. The BSH-activity assay was adapted to these encapsulated bacteria for the determination of activity.

3.3.4 Statistical Analysis

The experimental results are expressed as means ± standard error of the mean (SEM). Statistical analysis was carried out using SPSS Version 17.0 (Statistical Product and Service Solutions, IBM Corporation, New York, NY). Statistical comparisons were carried out using the General Linear Model and Tukey's post-hoc analysis for comparison of the means. Statistical significance was set at $p < 0.05$ and p-values less than 0.01 were considered highly significant.

3.4 Results

3.4.1 Spectrophotometric assay for the determination BSH-activity in free and APA-microencapsulated probiotic strains

The BSH-activity assay was developed using a modified version of the protocol by Stellwag et al. (**Figure 3.3).** In the protocol, bacterial cultures grown in MRS broth supplemented with 5mM GDCA are pelleted by centrifugation at 10 000 x g at 4^{o}C for 10 minutes. The pellet is washed three times with 0.1M sodium phosphate pH 7.0. Following each wash, the supernatant is drained and the pellet is resuspended in 25 ml fresh buffer before being re-centrifuged at 10 000 x g for 10 minutes at 4^{o}C. Following the final wash, the pellet is resuspended in 3ml of 0.1M sodium phosphate buffer supplemented with 50mM dithiothreitol (DTT) and 1X protease inhibitor solution (Protease Inhibitor Cocktail Tablets, 25X concentrated stock solution, Sigma Aldrich). The sample is maintained on ice throughout this process. The solution is then sonicated for 7 pulses of 10 seconds, at 50% power (Misonix XL2000 Ultrasonication System), while on ice. From this, 1ml is extracted and centrifuged at 20000xg for

10 minutes at 4°C. Between 10-40 µl of sample supernatant is removed and added to 20mM GDCA in 50mM sodium acetate buffer pH 4.2, supplemented with 10 mM DTT and protease inhibitors; to a final volume of 200 µl. The cell-free bile salt hydrolysis reaction is then incubated at 37°C for 0, 30, 60 and 90 min to allow for the progression of the hydrolysis reaction of GDCA bile salt. **(Figure 3.1)** At each time point, 50 µl of the reaction mixture is removed and added to 50 µl of 15% (w/v) TCA solution to precipitate all the proteins and to cease the hydrolysis reaction. The sample is centrifuged at 10 000xg for 10 minutes at 4°C. 10µL of supernatant is removed and added to 40 µl of distilled water. To this mixture, 950 µl of ninhydrin reagent (2.5:6:1 ratio by volume of 2% (v/v) ninhydrin:glycerol:0.5M sodium citrate pH 5.5) is added. The sample is heated at 100°C for 14 minutes to initiate the ninhydrin/glycine reaction. **(Figure 3.2)** The color development is achieved via a chemical reaction involving four main steps. First, a dehydration reaction occurs in which glycine complexes to the reduced ninhydrin via its amino group. Then, under heated conditions, a decarboxylation reaction occurs in which the carboxylic acid group of glycine leaves the reaction as CO_2. The remaining methyl group is hydrolyzed and exits as methanal, leaving behind only an amino group on the ninhydrin molecule. Finally, this ninhydrin amine can react with an additional reduced ninhydrin to form a colored complex. The resulting solution appears as a blue/purple mixture which is cooled at room temperature and read at 570 nm using a PerkinElmer 1420 Multilabel Counter. The concentration is calculated using a standard curve of serial diluted glycine standards which are incubated similarly. Glycine standards are prepared at the concentrations: 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, and 0.078 mM (R^2=0.998). These are identically processed as the samples. BSH-activity is calculated as a rate of glycine release per gram of bacterial pellet according to the standard curve obtained.

3.4.2 Determination of BSH-activity in selected free bacterial strains via spectrophotometric method

The BSH-activity spectrophotometric assay was performed on free bacteria from over-night cultures (12h). BSH+ *L. reuteri* NCIMB 30242, *L. reuteri* NCIMB 702656, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 11951, *L. fermentum* NCIMB 5221 (**Figure 3.4)**. Results show highest activity *L. reuteri* NCIMB 30242 of 17.09 ± 4.37 μmol /hr/g pellet (n=3), with $p \leq 0.05$, as compared to the next highest active strain. The other tested *L. reuteri* strains NCIMB 702656, NCIMB 701089, NCIMB 1195 and *L. fermentum* NCIMB 5221 showed activities of 4.55 ± 1.89, 8.49 ± 4.14, 5.22 ± 2.61, and 0.77 ± 0.34 μmol /hr/g, respectively (n=3, data shown as mean ± SEM).

3.4.3 Time-course BSH-activity and viability in free L. reuteri NCIMB 30242

BSH-activity via the spectrophotometric assay and viability were measured on free BSH+ *L. reuteri* NCIMB 30242 at 4-hour intervals during its growth in MRS broth **(Figure 3.5).** The results demonstrate that maximal BSH-activity of BSH+ *L. reuteri* NCIMB 30242 was optimal at 16 hours of incubation with 42.82 ± 4.26 μmol of glycine/hr/g of bacterial pellet (n=3). The *L. reuteri* strain's activity decreased at the 20-hour time point. Viability showed a logarithmic increase in cell count within the first 12 hours of incubation. Results demonstrated the highest viabilities for *L. reuteri* (NCIMB 30242) at 12h and 16h, with values of $2.2 \pm 1.4 \times 10^8$ and $8.6 \pm 4.51 \times 10^7$ CFU/ml respectively (n=3, data shown as mean ± SEM).

3.4.4 Determination BSH-activity of microencapsulated bacterial strains

For the determination of BSH-activity in APA-microencapsulated bacteria, the microcapsule membrane must first be dissolved before undergoing the aforementioned procedure. **(Figure 3.6a)** This was achieved by adding 5g of APA-microencapsulated bacteria to

a solution of cold 0.1M sodium citrate at a ratio of 20:1 sodium citrate:APA-bacteria. The mixture was vortexed vigorously until complete dissolution of the microcapsules was obtained. The mixture was then centrifuged at 10 000 x g at 4^{o}C for 5 minutes to pellet the released bacteria. The supernatant was removed, and the pellet washed three times with cold 0.1M sodium phosphate buffer pH 7.0. The determination of BSH-activity was then accomplished using the same procedure as for free probiotics. BSH-activity was calculated as a rate of glycine release per gram of microcapsule, knowing that bacteria represent 8% (w/w) of the final weight of the microcapsules. The protease inhibitors are needed in this assay to block both the endogenous protease activity from the bacterial lysate and proteolytic degradation of any remnant PLL molecules from the original microcapsule's coating which may skew the results by releasing non-relevant amino acid residues in the solution.

L. reuteri and *L. fermentum* strains were encapsulated in APA microcapsules. The activity assay was performed on both of the encapsulated strains. The activity of APA-microencapsulated *L. reuteri* was measured at 3.56 ± 0.24 µmol of glycine/hr/g microcapsule. As negative controls, the assay was performed on samples of APA-*L. reuteri* with no GDCA substrate added, samples of APA-microencapsulated *L. fermentum* (not BSH active), , and samples of empty APA-microcapsules (absent of any bacteria).These negative controls revealed no significant levels of glycine release; respectively, 0.08 ± 0.05, 0.14 ± 0.06, and 0.05 ± 0.04 µmol of glycine/hr/g microcapsule for samples of APA-*L. reuteri* (NCIMB 30242) with no GDCA substrate, APA-*L. fermentum* (NCIMB 5221), and empty microcapsules (n=3 data shown as mean ± SEM for all).).(**Figure 3.6b)**

3.4 Discussion

A spectrophotometric assay for BSH-activity in free and encapsulated probiotics was developed (modified from the procedure outlined by Stellwag et al. [153]). This assay can effectively quantify BSH-activity in probiotic strains with different activities. The BSH-activity of free *L. reuteri* NCIMB 30242 was measured at 17.09 ± 4.37 μmol /hr/g pellet and was shown be have significantly higher activity ($p \leq 0.05$) as compared to all other tested strains. The time-course study over 24h (4h intervals) on the most BSH-active strain (*L. reuteri* NCIMB 30242) demonstrated the ability of the assay to quantify varying degrees of BSH-activity for a single strain. BSH-activity was shown to be optimal at 16 hours at 42.82 ± 4.26 μmol of glycine/hr/g. These results are in agreement with that of the free probiotic BSH-activity performed in other studies [10, 152].

For APA-microencapsulated *L. reuteri*, the assay was adapted for encapsulated probiotics. BSH activity in APA-*L.reuteri*, encapsulated at 16h after growth, was measured at 3.56 ± 0.24 μmol of glycine/hr/g microcapsule via the adapted spectrophotometric assay. The bacteria represent 8% (w/w) of the total mass of the microcapsule. Standardizing the BSH-activity per gram bacteria (multiplying by a factor 100/8) yielded 44.50 ± 3.00 μmol of glycine/hr/g bacteria. Comparing this standardized value to the previously measured free cell activity of 42.82 ± 4.26 μmol of glycine /hr/g demonstrated that the assay remained as accurate with APA-microencapsulated as with free probiotics. Detection was not affected by APA-microencapsulation. Negative controls eliminated the possible interfering factors of the assay. Respectively, samples of (1) APA-*L. reuteri* with no addition of GDCA and (2) empty microcapsules proved that glycine release due to endogenous proteolytic pathways and free lysine residues from PLL did not contribute to any observed activity. The assay maintained the

same sensitivity in differentiating between encapsulated *L. reuteri* and *L. fermentum* strains as for free cells. These findings support the use of the assay for effectively quantifying BSH activity in probiotics encapsulated in APA polymeric membranes or immobilized in other matrices.

3.6 Conclusion

In summary, the modified spectrophotometric method was shown to be effective for the comparative determination of BSH-activity for free and APA-microencapsulated probiotics. Compared to other chromatographic methods, this assay offers an inexpensive, simple, and quick method for BSH-activity quantification of free and microencapsulated probiotics. With the growing interest in the oral administration of BSH-active bacteria, as well as APA-microencapsulation as a means of improved delivery of probiotics, this assay has potential to become a common tool for future pre-clinical and clinical studies.

In addition, this study identifies *Lactobacillus reuteri* (NCIMB 30242) as a highly BSH-active bacterial strain. This can be utilized for therapeutic purposes in metabolic disease by oral delivery in APA-microcapsules. However, before proceeding to in vivo testing, the APA-microcapsule formulations of the selected *L.reuteri* strain should be studied for verification of the stability of their activity and viability over time.

Acknowledgements

The authors would like to acknowledge the Canadian Institute of Health Research (CIHR) Grant (MPO 64308) and grants from Micropharma Limited to Dr. S. Prakash, a Doctoral Alexander Graham Bell Canada Graduate Scholarship from NSERC to Catherine Tomaro-Duchesneau and a Doctoral FRSQ Scholarship to Meenakshi Malhotra.

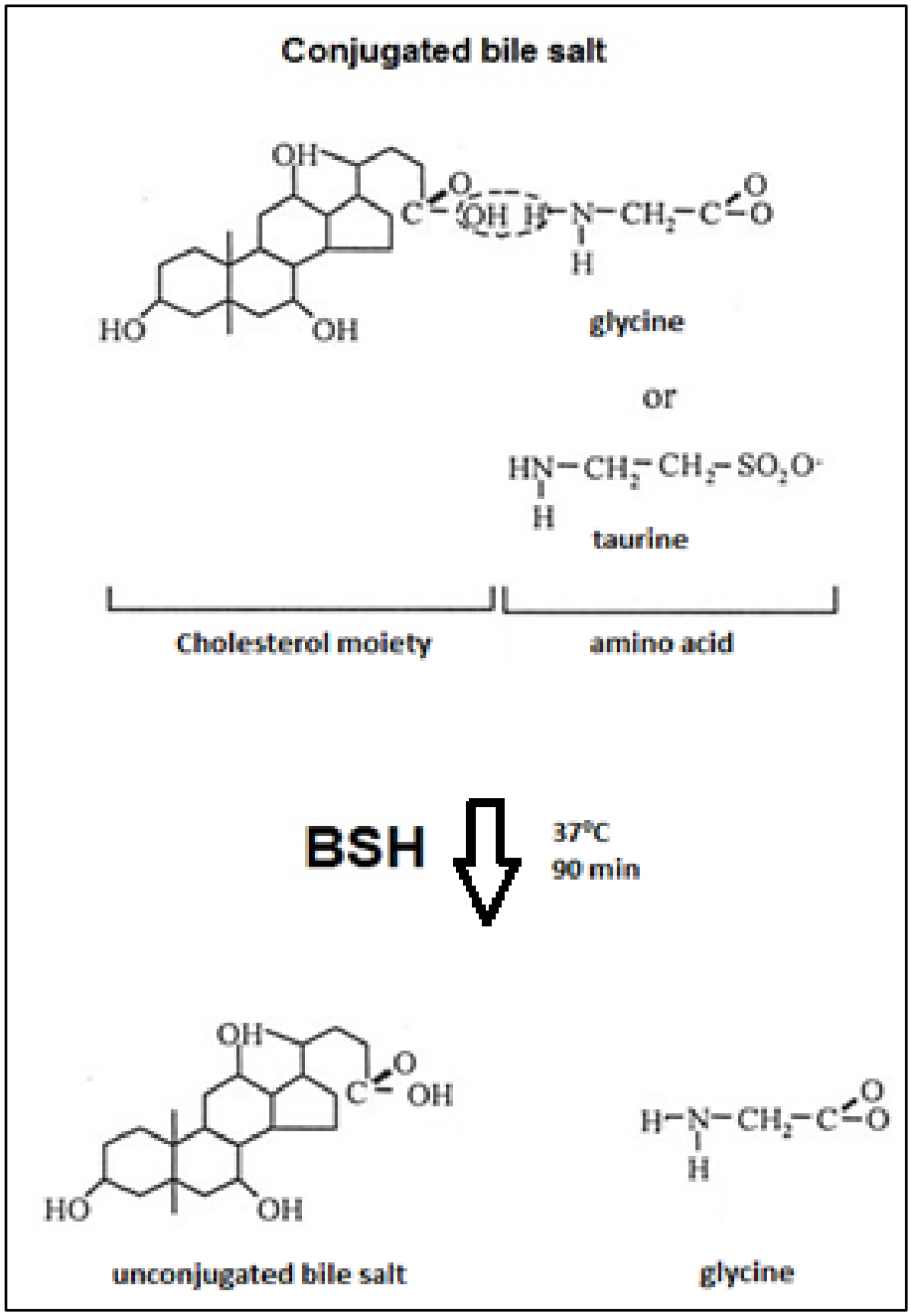

Figure 3.1: Hydrolysis of GDCA via bile salt hydrolase (BSH). Cholesterol precipitate and aqueous glycine are released.

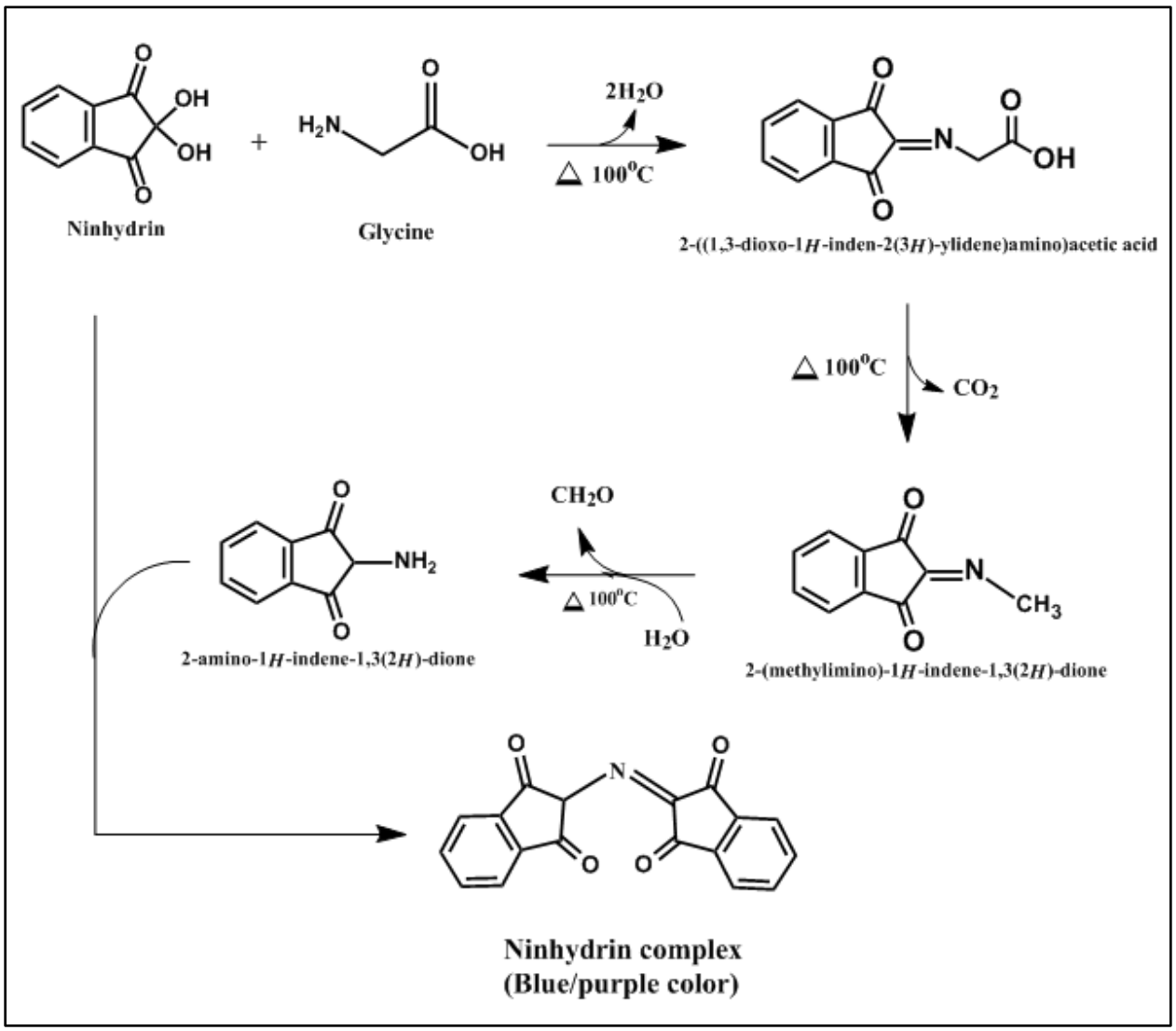

Figure 3.2: Step-by-step mechanism of ninhydrin/glycine chemical reaction. (1) Reduced ninhydrin first reacts through a dehydration reaction with glycine to create a ninhydrin/glycine complex. (2) This complex is then decarboxylated, releasing CO_2. (3) A hydrolysis releases methanol, resulting in a ninhydrin amine product. (4) The ninhydrin amine reacts with another reduced ninhydrin molecule to yield a blue/purple colored ninhydrin complex.

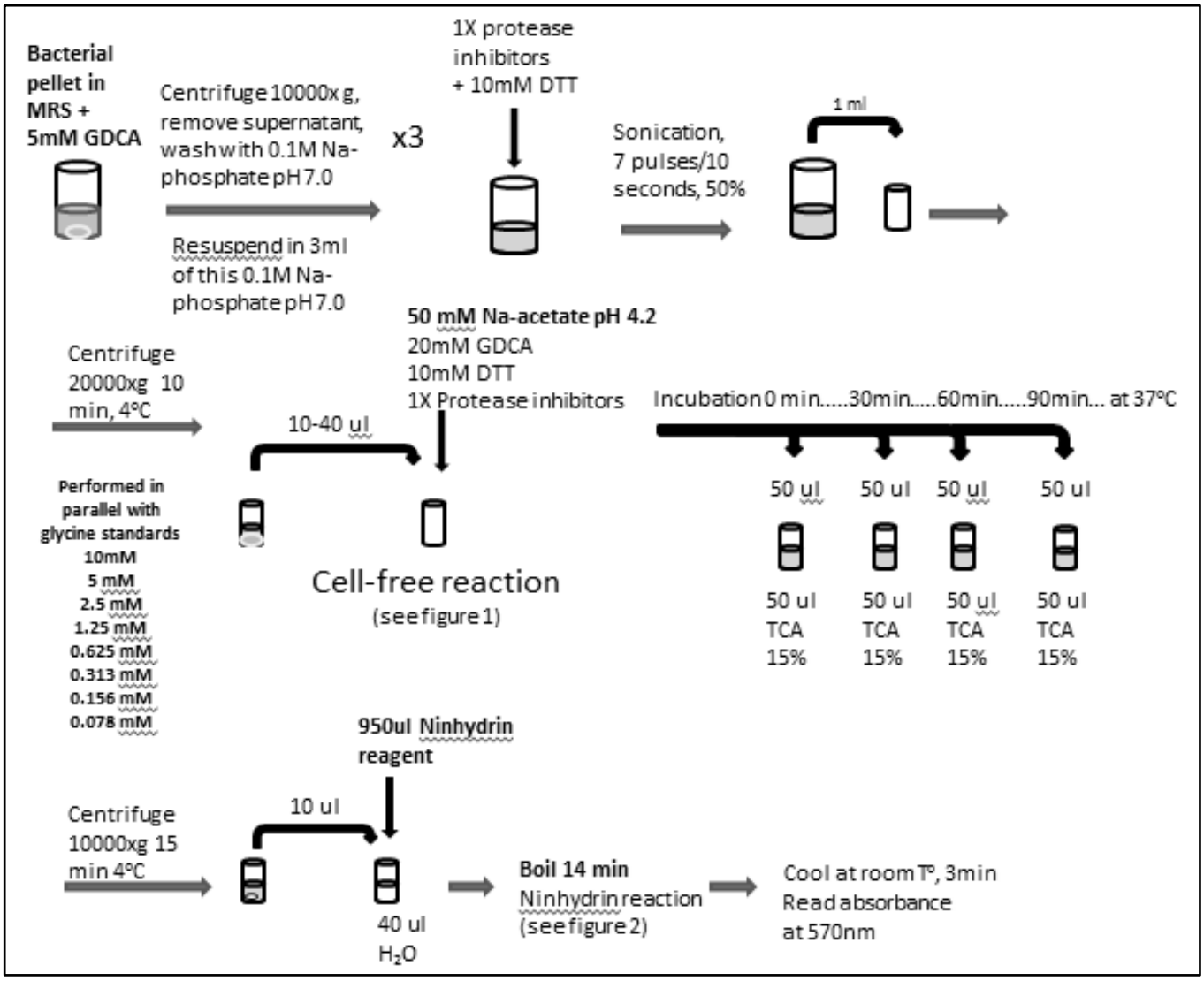

Figure 3.3 Schematic protocol of novel spectrophotometric BSH-activity assay in free probiotics.

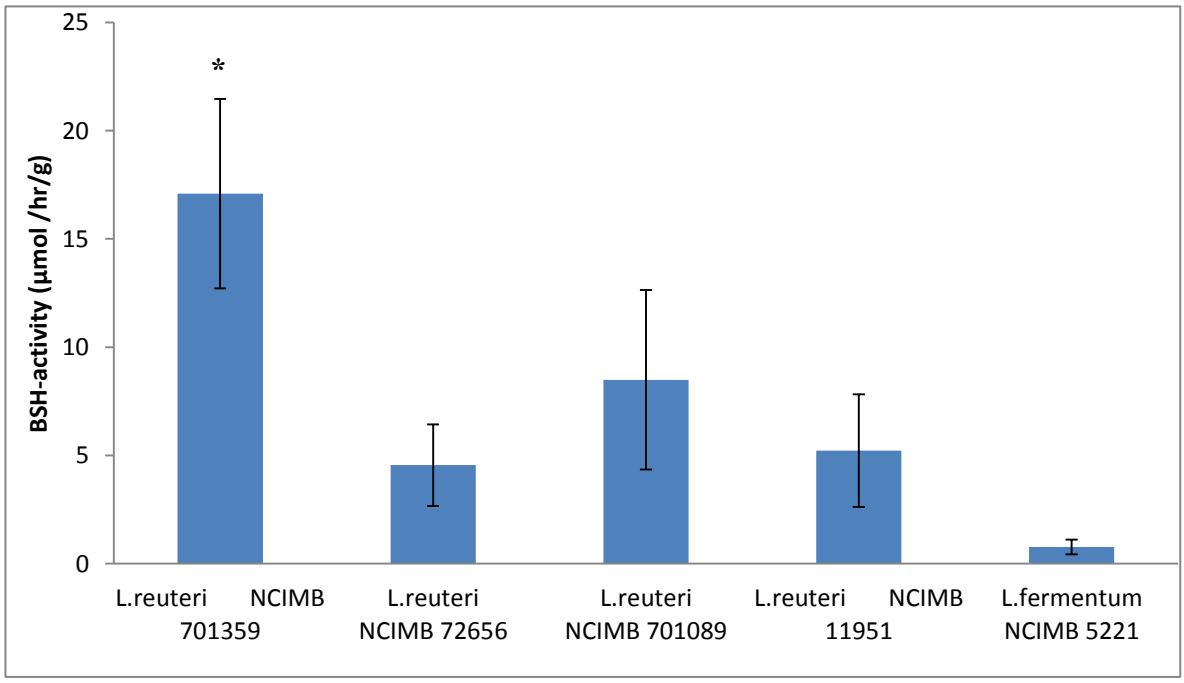

Figure 3.4: Spectrophotometric assay of multiple free *Lactobacilli* strains. Novel spectrophotometric assay was performed as per protocol on five selected free bacterial strains after 12h growth in MRS: *L. reuteri* NCIMB 30242, NCIMB 702656, NCIMB 701089, NCIMB 11951, and *L. fermentum* NCIMB 522. Results show that *L. reuteri* NCIMB 30242 has the largest activity of all the *L. reuteri* strains tested: 17.09 ± 4.37 µmol /hr/g of bacterial pellet (* $p \leq 0.05$ as compared to the next highest activity: *L. reuteri* NCIMB 701089). Error bars represent the standard error of the mean (SEM), shown for all data points. (n=3)

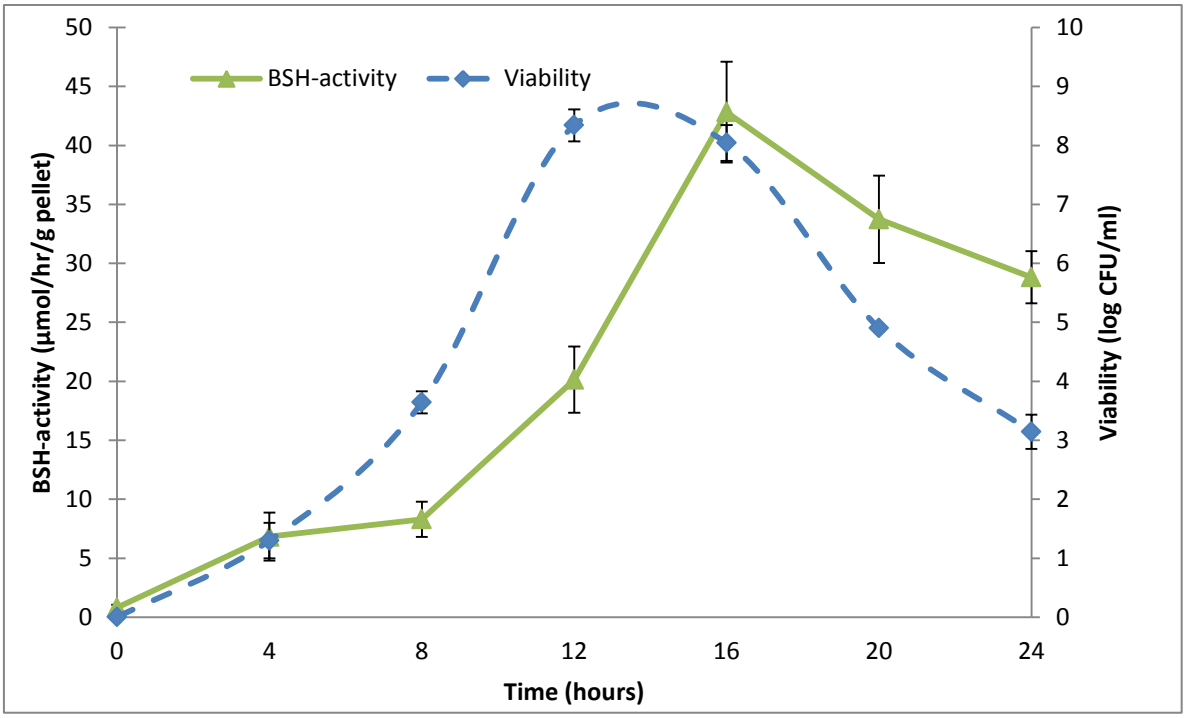

Figure 3.5: Time-course BSH activity (solid line) and viability (dashed line) of BSH-active *L. reuteri* NCIMB 30242. Cultures were grown in MRS media. Aliquots were taken were analyzed every four hours. Results show peak activity of the BSH+ *L. reuteri* NCIMB 30242 strain at 16h in its growth, with peak viability between 12-16h. Error bars representing SEM are shown for all data points. (n=3)

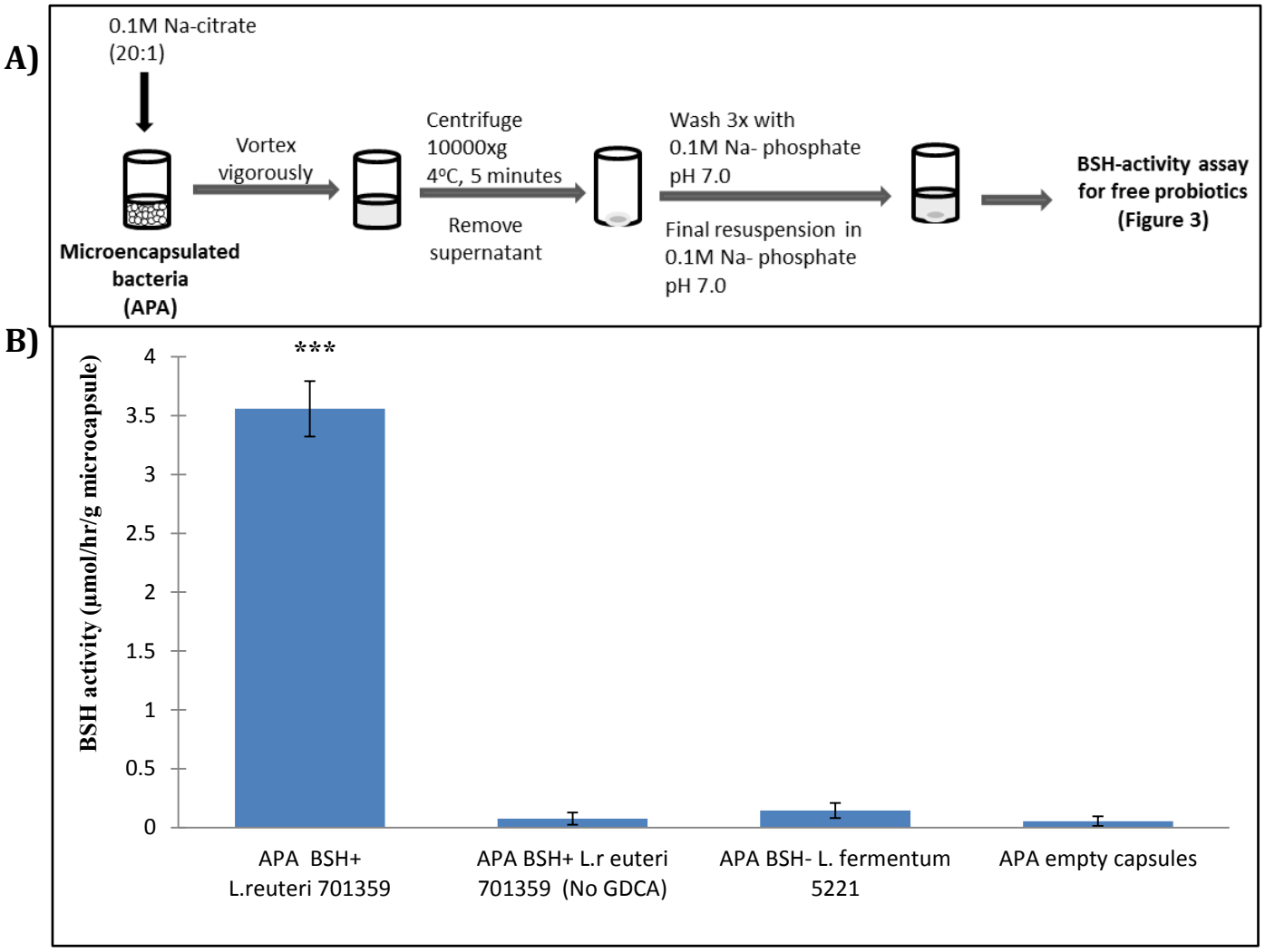

Figure 3.6 a) Schematic BSH-activity protocol for APA-microencapsulated probiotics

b) BSH-activity assay on APA-microencapsulated BSH-active *L.reuteri*, and three control samples. APA BSH+ *L. reuteri* NCIMB 70139 showed activity of 3.56 ± 0.24 in µmol/hr/g). (*** $p \leq 0.0005$, relative to negative controls) Negative controls negligible levels of activity. Error bars represent SEM. (n=3)

CHAPTER IV:

Maintenance of viability, activity and morphology of microencapsulated formulations of *Lactobacillus reuteri* (NCIMB 30242)

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Preface:

The present study applies the previously described (in chapter 3) spectrophotometric assay to study the stability of APA-microcapsules which contain the BSH-active bacterial strain *L.reuteri* (NCIMB 30242) which was selected in the previous chapter. The ability of APA-microcapsules to maintain and improve viability and BSH-activity is demonstrated, as compared to free bacteria. The morphology of the capsule, also an essential factor to the therapeutic effect is also shown to be maintained. Following this study, it is important to consider maximum maintainable time for the microencapsulated probiotic doses in order to proceed to in vivo studies. Catherine Tomaro Duchesneau has contributed in the experimental design and statistics.

4.1 Abstract

Bile salt hydrolase is a bacterial enzyme expressed in a number of probiotic strains. Studies have shown that oral treatment of BSH-producing probiotics can have beneficial effect on both hypercholesterolemia and hyperlipidemia. Microencapsulation of probiotics has been shown to improve delivery their delivery through the gastrointestinal tract. However practical applications of such encapsulated probiotic treatments are highly dependent on the stability of the formulations intended to be administered. In the case of BSH-active probiotics, maintenance of viability and BSH-activity are important for use in animal and clinical studies. In this study, viability and BSH-activity of microencapsulated *L.reuteri* (NCIMB 30242) were shown to be maintained at average values of $5.2 \pm 1.2 \times 10^9$ CFU/ml and 43.37 ± 3.34 μmol/hr/ml respectively for four weeks in 10% MRS. Comparable levels of viability and BSH-activity in free form of the bacteria were maintained for 2 days 0.9% NaCl and 2 weeks in 10% MRS. Morphology of the microcapsules containing the *L.reuteri* probiotic remained stable over its 4-week period of optimal activity and viability. This study shows the potential of APA-microencapsulation in both maintaining and improving the stability of probiotic formulations and opens the possibility for use of such a treatment in animal or clinical trials.

4.2 Introduction

Bile salt hydrolase (BSH) is a bacterial enzyme expressed in a number of probiotic strains. It is responsible for the deconjugation of bile salts via the hydrolysis of the bond linking its cholesterol moiety with its amino acid side-chain [155, 156]. Deconjugation of bile salts renders them incapable of emulsifying lipids in the intestinal tract for absorption, and results in increased cholesterol excretion in feces. As a result, there has been a growing interest in using BSH enzyme and BSH-active probiotics for beneficial metabolic effects such as lowering cholesterol and triglyceride levels [11, 157-159]. Low survival of bacteria during gastric transit is the primary limitation of delivering BSH-active probiotic to their target site [160, 161]. Encapsulation of probiotics within microcapsules has been shown to improve their viability in high pH and harsh degradative conditions of the stomach; thus assisting with delivery to their intestinal site of action [162]. Among these types of microcapsules, APA (alginate-polyL-lysine-alginate) has been shown to be a protective, biocompatible, and biodegradable polymeric membrane suitable for delivery of cells [12, 123-125, 145]. Therefore, the treatment of metabolic conditions via BSH-active probiotics has realistic potential which requires further research. However, practical application in clinical or animal studies is logistically dependent on storage factors of the probiotic formulations. These include the maintenance of viability and enzymatic activity of the bacteria for a sufficiently extended period of time. Encapsulation has been shown to improve viability in probiotic strains [163]. Encapsulation of BSH-active probiotics within APA-microcapsules may help preserve both the viability and activity of BSH-active probiotics, while maintaining the essential capsule morphology required for protection during gastric transit. This study aims to show the improved maintenance of viability and activity of a selected BSH-active probiotic strain (*Lactobacillus reuteri* NCIMB 30242) in stable APA-microcapsules. The

results of this study could open the possibility for future animal trials which utilize this APA-microencapsulated BSH-active *L.reuteri* strain in the treatment of metabolic disease.

4.3 Materials and methods

4.3.1 Chemicals

All chemicals 1) GDCA salt 2) de Man-Rogosa-Sharpe (MRS) broth 3) poly-L-lysine 4) alginate (low viscosity) 5) TCA 6) ninhydrin (2%), glycine) as well as all buffers were purchased from Sigma Aldrich (Oakville, Canada) and were of HPLC or standard analytical grade. *Lactobacillus reuteri* (NCIMB 30242) was purchased from NCIMB (National Collection of Industrial Bacteria, Bucksburn, Aberdeen, Scotland).

4.3.2 Growth and microencapsulation of BSH+ L.reuteri NCIMB 30242 within alginate-poly-lysine-alginate (APA) microcapsules

Bacterial cultures were encapsulated in APA-microcapsules following growth conditions as described in previous chapter. Briefly, *Lactobacillus reuteri* (NCIMB 30242) cultures were grown in MRS broth with 5mM GDCA for 16 hours and microencapsulated at 8% w/v of bacteria in alginate, using an Inotech Encapsulator IER-20 (Inotech Biosystems International Inc, Rockville, MD) with a 300 μm diameter nozzle. Two layers were subsequently added to the alginate beads; a coating of poly-L-lysine (PLL), followed by an additional layer of alginate. Encapsulated bacteria were stored in 10% MRS solution. Free bacteria were stored in both 10% MRS and saline (0.9% NaCl) at 4°C

4.3.3 Monitoring maintenance of viability in free and microencapsulated L.reuteri (NCIMB 30242)

Viability and BSH-activity in microencapsulated probiotic samples were monitored to ensure stability of the doses. Briefly, APA-microcapsules were dissolved in 0.1M sodium citrate at a ratio of 20:1 sodium citrate:APA-bacteria. From the supernatant of released bacteria, 100µl were removed and plated onto MRS agar plates. The plates were allowed to incubate overnight at 37^{o}C in a 5% CO_2 chamber. Bacterial cell counts of the samples were determined via standard measurements of colony forming units (CFU) on agar plates. Viability is standardized and calculated as CFU/ml of for both free and microencapsulated probiotic.

4.3.4 Monitoring maintenance of BSH-activity in free and microencapsulated L.reuteri (NCIMB 30242)

BSH-activity was determined spectrophotometrically in both free and microencapsulated bacteria using the procedures described in previous chapter. Briefly, the bacteria (following release from APA-microcapsules for encapsulated bacteria) were washed three times with cold 0.1M sodium phosphate buffer pH 7.0 supplemented with 100 µl protease inhibitor solution (Protease Inhibitor Cocktail Tablets, 25X concentrated stock solution, Sigma Aldrich). The solution was sonicated on ice at 7 pulses, 10 seconds each (Misonix XL2000 Ultrasonication System). From the supernatant, 40 µl was removed and added to 20mM GDCA in 50mM sodium acetate pH 4.2, supplemented with 10 mM DTT, 1X protease inhibitors and 20mM GDCA. The reaction was incubated at 37^{o}C. Samples were removed at time points (0, 30, 60 and 90 min) and stopped with 15% (w/v) TCA. Samples were analyzed for glycine content by addition to ninhydrin solution (2.5:6:1 ratio by volume of 2% (v/v) ninhydrin:glycerol:0.5M sodium citrate

pH 5.5), followed by 14 minutes of heating at 100°C, cooling, and absorbance measurement at 570nm. Activity for microencapsulated bacteria was standardized by a multiplying factor of 100/8 for quantitative comparison with free bacteria. Both free and encapsulated activities were calculated as the rate of glycine release per ml solution using a glycine standard curve.

4.3.5 Monitoring maintenance of morphology in free and microencapsulated L.reuteri (NCIMB 30242)

Morphology was monitored visually using a Leica ICC50 (Leica Microsystems, Wetzlar, Germany) with a DM500 attachment. Images were taken of samples at 40x and 100x magnifications. Microcapsule diameters were determined via juxtaposed images of measurement standards, and using ImageJ 1.44p (National Institutes of Health, USA).

4.3.6 Statistical analysis

The experimental results are expressed as means ± standard errror of the mean (SEM). Statistical analysis was carried out using SPSS Version 17.0 (Statistical Product and Service Solutions, IBM Corporation, New York, NY).

4.4 Results

4.4.1 Maintenance of viability of free and APA-microencapsulated L.reuteri (NCIMB 30242)

Viability was monitored in both free and microencapsulated *L.reuteri* (NCIMB 30242) over a 6-week period (**Figure 4.1)**. Microencapsulated *L.reuteri* (NCIMB 30242) maintained an average viability of 5.2 ± 1.2 x 10^9 CFU/ml for four weeks, decreasing only by week 5 to 1.1 ± 0.81 x 10^6. By week 6, its viability was negligible (~0). Free *L.reuteri* maintained an average

viability of 4.3 ± 1.2 x 10^9 CFU/ml for two days in saline (0.9% NaCl), and at an average value of 4.1 ± 1.1 x 10^9 CFU/ml for two weeks in 10% MRS. Respectively, viability decreased to 9.6 ± 4.0 x 10^1 and 5.7 ± 1.8 x 10^1 for free *L.reuteri* in saline by day 4, and free *L.reuteri* in 10% MRS by week 3.

4.4.2 Maintenance of BSH-activity of free and APA-microencapsulated L.reuteri (NCIMB 30242)

BSH-activity was monitored in free and microencapsulated *L.reuteri* in 10% MRS, and free bacteria in both saline **(Figure 4.2)**. BSH-activity in microencapsulated *L.reuteri* NCIMB 30242 was maintained in 10% MRS for 4 weeks at an average value of 43.37 ± 3.34 μmol/hr/ml. By week 5, its activity decreased to 6.45 ± 3.79 μmol/hr/ml. Free bacteria activity dropped from an average steady value of 44.52 ± 2.64 to 6.05 ± 2.29 μmol/hr/ml by day 4 in saline, and from an average value of 43.83 ± 2.07 to 1.58 ± 0.32 μmol/hr/ml by week 3 in 10% MRS.

4.4.3 Maintenance of morphology of APA-microencapsulated L.reuteri (NCIMB 30242)

Morphology of APA-microcapsules containing *L.reuteri* (NCIMB 30242) was observed at week 0 (following encapsulation) and week 4 **(Table 4.3)**. Week 0 images show microcapsules with average diameter of 541 ± 55 μm and stable spherical structure of the polymeric membrane. Week 4 images show microcapsules with average diameter of 523 ± 61 μm and mildly oblong spherical structure.

4.5 Discussion

In the present study, the improved maintenance of viability and BSH-activity were demonstrated in *L.reuteri* (NCIMB 30242) through the use of APA-microcapsules. Following microencapsulation, maintenance of viability of *L.reuteri* (NCIMB 30242) was greatly improved as compared to viability in the free bacteria. Free bacteria decreased quickly in viability from 4.3 ± 1.2 x 10^9 to 9.6 x 10^1 CFU/ml by day 4 in saline and from 4.1 ± 1.1 x 10^9 to 5.7 ± 1.8 x 10^1 CFU/ml in 10% MRS. However, microencapsulated *L.reuteri* (NCIMB 30242) maintained an average viability of 5.2 ± 1.2 x 10^9 CFU/ml for four weeks in 10% MRS, decreasing only by week 5 to 1.1 ± 0.81 x 10^6. Similarly, viability has been shown in a separate study to be maintained up to 28 days in HEMA-MMA microcapsules supplemented with agarose for cells [164]. These results demonstrate the ability for APA-microencapsulation to increase the maintenance of viability in *L.reuteri* (NCIMB 30242).

The maintenance of BSH-activity was also improved via microencapsulation: an average activity of 43.37 ± 3.34 μmol/hr/ml was maintained for four weeks in the encapsulated bacteria, dropping only by week 5 at a value of 6.45 ± 3.79 μmol/hr/ml. Comparatively, free bacteria maintained similar activities for only 2 days in saline and 2 weeks in MRS, respectively dropping to 6.05 ± 2.29 μmol/hr/ml (at day 4) and 1.58 ± 0.32 μmol/hr/ml by week 3. In a study by on BSH-activity by Corzo et al [8], *Lactobacillus* was shown to be maintained for a similar time span in free form at 5^{o}C. Interestingly, BSH-activity was maintained at -20^{o}C for periods of time which are comparable to microencapsulated bacteria in this present study. Therefore, APA-microencapsulation of *L.reuteri* (NCIMB 30242) also helps the maintenance of BSH-activity for a longer period of time without the need for cold (below 4^{o}C) storage conditions.

Finally, microscopy images weeks of microencapsulated bacteria revealed that morphology of the microcapsules remains stable for the initial 4-week period following encapsulation. Slight variation in diameter was observed from week 0 to week 4; from 541 ± 55 to 523 ± 61 μm. This variation is presumably connected to the mildly oblong morphology observed at week 4, which is likely due to osmotic transfer of solvent between the gelated beads and the outside MRS solution. However, the polymeric membrane of the microcapsules remained stable and unruptured by week 4. This ensures proper protection of the viable and active probiotics even at week 4. In summary, this study demonstrates that the BSH-activity, viability and morphology of APA-microcapsules containing *L.reuteri* are stable for a 4-week period. This evidence opens the potential for storage and preparation of APA-microcapsule formulations for probiotic delivery in animal and clinical trials.

4.6 Conclusion

The stability and maintenance of probiotic dose is an essential factor to consider prior to conducting animal studies. For BSH-active probiotics, this dose is dependent on their viability and activity. In order to determine the time length for which these factors remain stable in free and microencapsulated probiotics, they are monitored over time. The present study supports the improvement of dose stability via APA-microcapsulation, as well as establishing a maximal time limit for dosage stability under varying conditions. With the dose stability established for this microencapsulated BSH-active probiotic strain, in vivo studies in an appropriate animal model can be performed to assess the therapeutic effect of the formulations on markers of metabolic disease.

Acknowledgements

This research was supported by the Biomedical Technology and Cell Therapy Research Laboratory, under the direction of Dr. Satya Prakash, in the Department of Biomedical Engineering of McGill University. Financial support was provided by Micropharma and is greatly acknowledged.

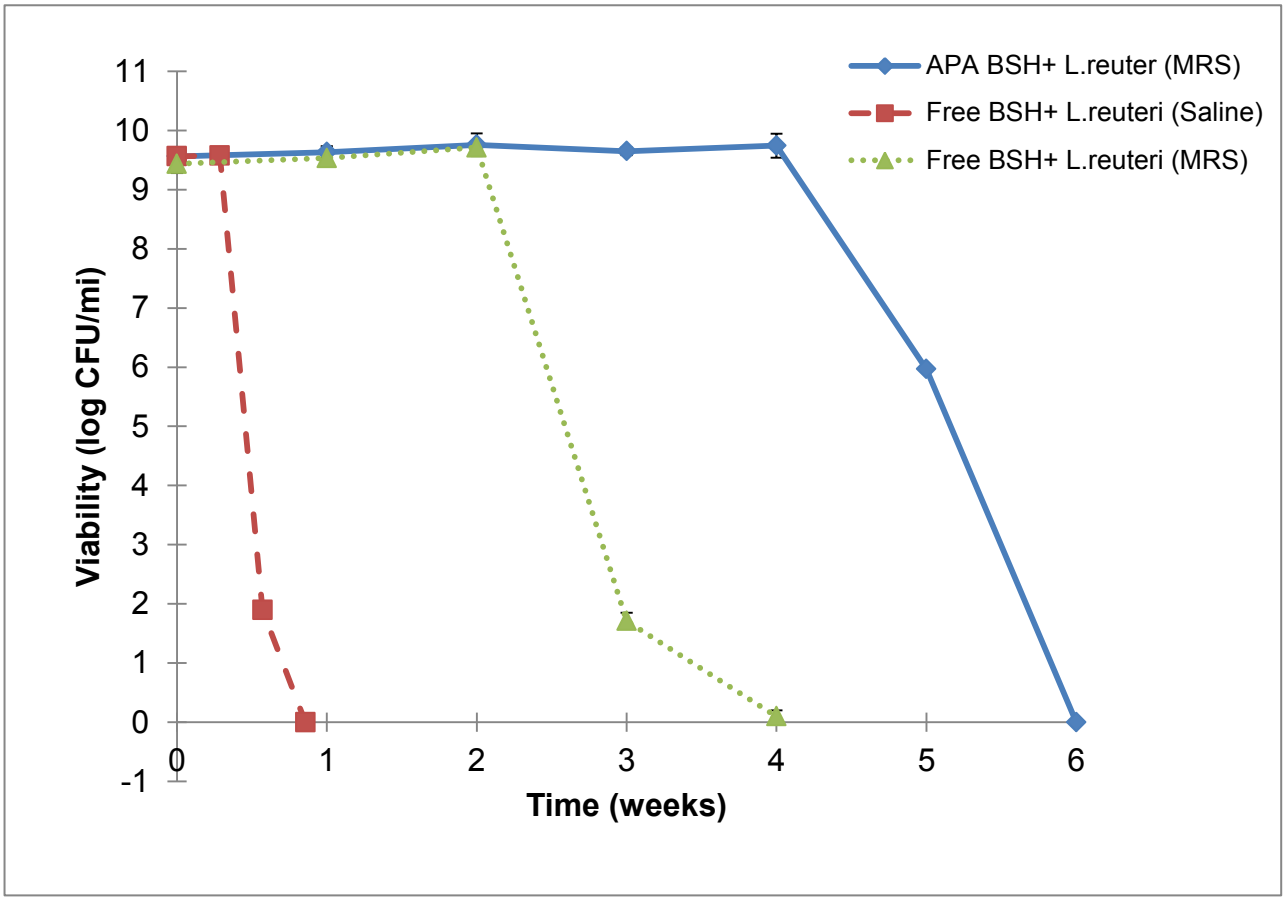


Figure 4.1: Maintaince of viability of *L.reuteri* (NCIMB 30242) under three conditions; APA-microencapsulated form in 10% MRS, free form in saline, and free form in 10% MRS. *L.reuteri* (NCIMB 30242) was grown over-night at 37°C in MRS broth. After 16h within their growth, aliquots were put at 4°C in saline (squares) and 10% MRS (triangles). Additionally, bacteria was microencapsulated bacteria and placed in 10% MRS. Viability was monitored over time for all three. Microencapsulated bacteria maintained an average viability of 5.2 ± 1.2 x 10^9 CFU/ml for four weeks. Free *L.reuteri* maintained a comparable viability for two days in saline (0.9% NaCl) and 2 weeks in 10% MRS.

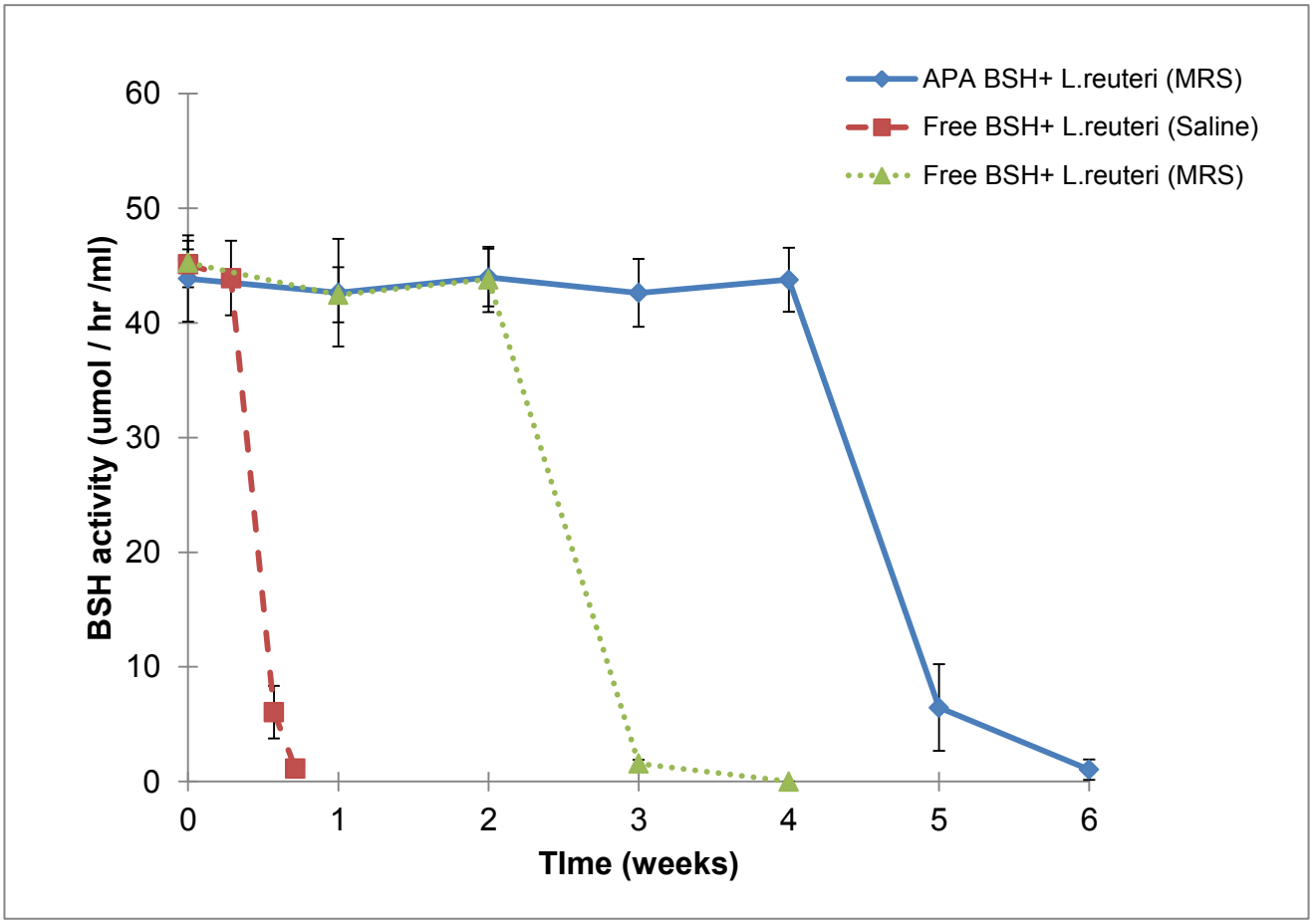


Figure 4.2: Maintaince of BSH-activity of *L.reuteri* (NCIMB 30242) under three conditions; APA-microencapsulated form in 10% MRS, free form in saline, and free form in 10% MRS. *L.reuteri* (NCIMB 30242) was grown over-night at 37°C in MRS broth. After 16h within their growth, aliquots were put at 4°C in saline (squares) and 10% MRS (triangles). Additionally, bacteria was microencapsulated bacteria and placed in 10% MRS. Viability was monitored over time for all three. Microencapsulated bacteria maintained an average activity of 43.37 ± 3.34 µmol/hr/ml for four weeks. Free *L.reuteri* maintained a comparable activity for two days in saline (0.9% NaCl) and 2 weeks in 10% MRS.

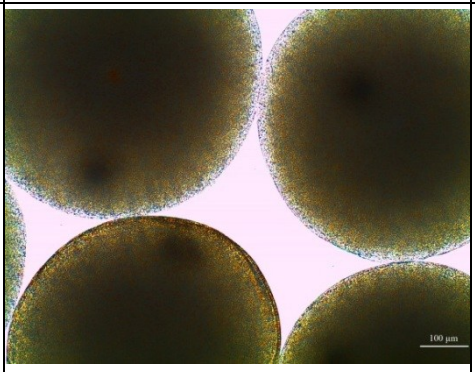
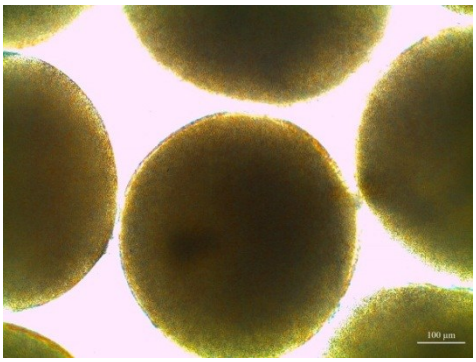
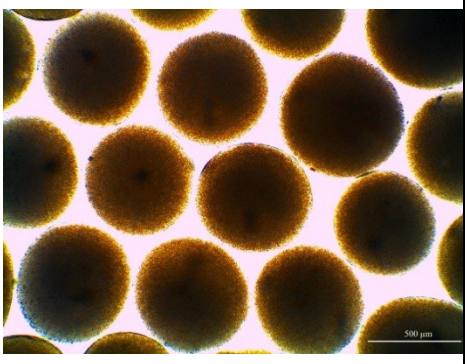
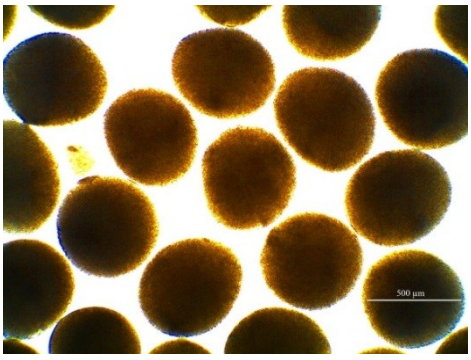
	Week 0	Week 4
100x		
40x		
Microcapsule diameter (µm)	541 ± 55	523 ± 61
Morphology	Stable, spherical structure	Mild deformation, but intact membrane

Table 4.1 Light microscopy images of microencapsulated BSH+ *L.reuteri* NCIMB 30242 at magnifications 100x and 40x. Microcapsules initially (week 0) showed a stable, spherical structure with a diameter of 541 ± 55 µm. Following a 4-week period incubation in 10% MRS at 4^{o}C, the microcapsules showed only mild deformation and shrinkage. However, a stable and intact polymeric membrane was still observed by week 4.

CHAPTER V:

Effect of orally administered APA microencapsulated BSH-active Lactobacillus reuteri NCIMB 30242 on markers of obesity: an in vivo analysis

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Preface:

Chapter 3 developed a spectrophotometric assay which developed and used for the selection of a BSH-active probiotic: *Lactobacillus reuteri* (NCIMB 30242). In chapter 4, the stability of microencapsulated formulations of this probiotic was studied using the assay, in preparation for in vivo studies. In this the present study, APA-microcapsules containing the probiotic were orally delivered in Bio F1B hamsters to determine their effect on factors of metabolic disease. Doses were prepared every four weeks as per the results in chapter 4, and monitored for activity weekly as per the assay developed in chapter 3. The effect of the probiotic treatment was determined via analysis of physical, serum, tissue and fecal markers in the animal groups. Co-authors have contributed in experimental execution (Laetitia Rhodes, Imen Kahouli, Hani Al Salami), analysis of markers (Meenakshi Malhotra), and experimental design as well as statistics (Catherine Tomaro-Duchesneau).

5.1 Abstract

Metabolic diseases affect millions of individuals in the developed world. It is marked by low-grade systemic inflammation which emerges from dysfunctional adipocyte/macrophage interactions. This can lead to several other metabolic diseases. Hyperalimentation, which leads to elelvated levels of circulating lipids, is the root cause of diet-induced metabolic problems. Bile salt hydrolase (BSH) is a bacteria enzyme which has been shown to be effective in cleaving bile salts necessary for lipid absorption in the intestinal tract. Microencapsulation has been shown to improve delivery of probiotic bacteria to the intestinal tract. In the present, Bio F1B Golden Syrian hamsters fed a high-fat diet have been orally administered APA-microcapsules containing BSH-active *Lactobacillus reuteri* (NCIMB 30242) probiotics. Viability and BSH-activity of probiotic formulations were maintained at steadily at average values of 5.42±1.39 x 10^9 CFU/g and 3.48±0.32 μmol/hr/g microcapsule throughout the 12-week period. After the end of the 12-week period, the treated hamsters showed reduction in liver weight (12.6±1.6%), liver fat content (9.9±1.8%), body weight (5.1±1.9%), and liver and blood levels of free fatty acids (7.5±1.3% and 7.7±2.1 %) with respect to untreated hamsters. From the tested inflammatory markers, C-reactive proteins (CRPs) elicited the largest absolute reduction (24.6±3.2%), while both circulating TNF-α and IL-6 also showed statistically significant ($p \leq 0.05$) decrease. However, TNF-α and IL-6 cytokines in mesenteric adipose tissue showed larger reductions of 17.2±2.9 and 16.6±5.9% respectively. Finally, fecal lipids in treatment group showed a 15.1±2.9% increase as compared to the untreated high-fat diet group, as well as serum lipids. The results presented in this study supports the use of APA-microcapsules containing *Lactobacillus reuteri* (NCIMB 30242) as a potential treatment option in the reduction of factors for metabolic diseases.

5.2 Introduction

In recent years, metabolic diseases have become a worldwide epidemic representing the fifth leading risk factor for death. Worldwide obesity and metabolic syndrome has more than doubled since 1980. In 2008, 1,5 billion adults were overweight and, of these, over 200 million men and nearly 300 million women were obese. The Western world is particularly affected by this problem [1]. Metabolic diseases, such as obesity and diabetes, are marked by an increased state of low-grade systemic inflammation. This inflammation is caused by a dysfunction in adipocyte-macrophage cytokine interactions, which is induced by hyperalimentation-induced lipidemia [2, 39, 42]. This systemic inflammation results in syndromes affecting other tissues in the body. As a result, life-threatening metabolic disorders develop, such as type 2 diabetes, insulin resistance, fatty liver disease, atherosclerosis, hypertension and several forms of cancer [165]. A search for effective therapies is still ongoing.

Bile salt hydrolase (BSH) is an enzyme expressed in several bacterial strains including *Lactobacillus* [8]. The BSH enzyme hydrolyzes bile salts in the digestive tract. While interest has existed due to its potential cholesterol-lowering effects [141, 143, 144], its mechanism of action maintains an exploitable potential for hypolipidemic effects by interfering with bile salt-dependent lipid absorption. Treatment via BSH-active probiotics is a therefore potentially viable solution to lower factors of metabolic disorders. However, its effectiveness is heavily dependent upon successful delivery of the viable and active probiotics to the upper duodenum, the intended target site [124]. APA-microencapsulation has seen success as a biocompatible and biodegradable cell delivery system to increase viability through gastric transit [123-125, 145, 146]. Delivery of encapsulated BSH-active probiotics has therefore good potential for lowering inflammation which results for the downstream effects of hyperlipidemia. While several animal

models exist, the Bio F1B hamster presents an excellent candidate for studying metabolic syndrome, due to its similar lipid metabolic profile to humans [87-89]. Given the prominence of metabolic disorders in the developed world, treatment via BSH-active probiotics deserves further investigation, and maintains potential for future clinical studies.

5.3 Materials and Methods

5.3.1 Chemicals

All reagents (GDCA and TDCA salts, MRS, poly-L-lysine, alginate (low viscosity), TCA, ninhydrin (2%), glycine) as well as all buffers were purchased from Sigma Aldrich (Oakville, Canada) and were of HPLC or standard analytical grade. *Lactobacillus. reuteri* (NCIMB 30242), were purchased from NCIMB (National Collection of Industrial Bacteria, Bucksburn, Aberdeen, Scotland). ELISA kits for inflammatory markers were purchased from eBioscience (San Diego, USA). Enzymatic kits for quantification of metabolic factors were purchased from BioAssay Systems (Hayward, USA).

5.3.2 Animals, experimental design, and treatment

Thirty six Bio F1B Golden Syrian hamsters (BioBreeders, Watertown, MA) were purchased and used for the study (7-10 weeks old/ 90-100 g). The hamsters were housed two per cage in a temperature and humidity controlled room (23^{o}C and 50% respectively), on a reversed 12h light/dark cycle (light from 7pm to 7am). They were provided excess food throughout the trial. All the hamsters were initially fed a normal unpurified diet (Lab Diet Rodent Laboratory Chow 5001, Purina, St. Louis, MO) for one week, in order to acclimatize them to the animal facility. (Figure 1) Following the acclimatization period, all animals were randomized into three

groups (n=12): a high-fat diet (HFD) group, a treatment group and a normal-fat diet group (NFD). The HFD group received a purified high-fat diet consisting of 45% fat, 19% protein and 36% carbohydrate, as a percentage of total kcal (TestDiet Basal Purified Diet w/45% Energy from Fat 58G8) and served as a positive control for induction of metabolic disease. (Figure 1) The NFD group received a purified regular-fat diet consisting of 12% fat, 19% protein, 69% carbohydrate as a percentage of total kcal (TestDiet Basal Purified Diet w/ 12% Energy from Fat 58G7) and served as a negative control for metabolic disease induction. (Figure 1) The treatment group was fed both the high-fat diet and were gavaged two daily oral administrations of 1.5g of APA-microencapsulated *Lactobacillus reuteri* (NCIMB 30242) resuspended in 2ml of 0.9% NaCl. Upon the start of the trial and initiation of the experimental diets, all hamsters were bled via saphenous vein for blood collection following an overnight fast of 16h. Blood samples were collected after weeks 2, 4, 6, 8, 10 during the experimental period. A final blood collection was performed after 12 weeks, when the animals were sacrificed via CO_2 and cardiac puncture. Livers and white adipose tissue (abdominal and mesenteric) were collected from all animals for further analysis. All animal maintenance and experimental procedures were performed according to the guidelines of McGill University's Animal Care Committee.

5.3.3 Microencapsulation of BSH+ L.reuteri NCIMB 30242 within alginate-poly-lysine-alginate (APA) microcapsules

Bacterial cultures were encapsulated in APA-microcapsules. Briefly, *Lactobacillus reuteri* (NCIMB 30242) cultures were grown in MRS broth with 5mM GDCA for 16 hours and microencapsulated at 8% w/v of bacteria in alginate, using an Inotech Encapsulator IER-20 (Inotech Biosystems International Inc, Rockville, MD) with a 300 μm diameter nozzle. Two

layers were subsequently added to the alginate beads; a coating of poly-L-lysine (PLL), followed by an additional layer of alginate. Microcapsules were stored in 10% MRS solution at 4°C. New microencapsulated doses were prepared every four weeks of the animal trial.

5.3.4 Monitoring maintenance of viability and BSH-activity in probiotic doses

Viability and BSH-activity in microencapsulated probiotic samples were monitored to ensure stability of the doses. Briefly, APA-microcapsules were dissolved in 0.1M sodium citrate at a ratio of 20:1 sodium citrate:APA-bacteria. From the supernatant of released bacteria, 100µl were removed and plated onto MRS agar plates. The plates were allowed to incubate overnight at 37^{o}C in a 5% CO_2 chamber. Bacterial cell counts of the samples were determined via standard measurements of colony forming units (CFU) on agar plates. Viability as calculated as CFU/g of microcapsule.

Using the same released bacterial supernatant, a spectrophotometric BSH-activity assay was performed. Briefly, the bacteria was washed three times with cold 0.1M sodium phosphate buffer pH 7.0 supplemented with 100 µl protease inhibitor solution (Protease Inhibitor Cocktail Tablets, 25X concentrated stock solution, Sigma Aldrich). The solution was sonicated on ice at 7 pulses, 10 seconds each (Misonix XL2000 Ultrasonication System). From the supernatant, 40 µl was removed and added to 20mM GDCA in 50mM sodium acetate pH 4.2, supplemented with 10 mM DTT, 1X protease inhibitors and 20mM GDCA. The reaction was incubated at 37^{o}C. Samples were removed at time points (0, 30, 60 and 90 min) and stopped with 15% (w/v) TCA. Samples were analyzed for glycine content by addition to ninhydrin solution (2.5:6:1 ratio by volume of 2% (v/v) ninhydrin:glycerol:0.5M sodium citrate pH 5.5), followed by 14 minutes of

heating at 100°C, cooling, and absorbance measurement at 570nm. Activity was calculated as the rate of glycine release per gram of microcapsule using a glycine standard curve.

5.3.5 Diet intake and weight gains

Body weights of hamsters were recorded weekly for all three experimental groups (N=12). Food weight was measured weekly for cages of all three groups (N=6), and reported as food intake in units kcal/animal/day. At the end of the experimental period (week 12), livers were excised from the euthanized animals and weighed. The liver:body weight ratio was calculated from these endpoint measurements.

5.3.6 Determination of serum cytokines, C-reactive proteins and free fatty acids

Serum samples from fasted animals were isolated via centrifugation in serum separator tubes (BD) at 2000xg for 20 minutes at 4^{o}C and sorted at -80^{o}C until analysis. Serum C-reactive proteins were measured enzymatically using Roche Diagnostic kits on a Hitachi 911 Clonical Chemistry Analyzer. Free fatty acids were quantified using EnzyChrom assay kits (BioAssay Systems, Hayward, USA). Serum TNF-α, IL-6 were analyzed using Rat TNF-α and IL-6 Platinum ELISA kits from eBioscience (San Diego, USA). Pre-coated microwells were incubated with serum samples for binding to immobilized anti-IL6 anti-TNF-α coating. Respectively, biotin-conjugated anti-IL6 and anti-TNF- α antibody was added, followed by incubation with Streptavidin-HRP secondary antibody. Samples were read for absorbance at 450nm following addition of tetramethylbenzidine substrate solution. Serum TNF-α and IL-6 were determined from duplicate standards prepared at serial concentrations of 2500, 1250, 625, 312.5, 156.3, 78.1, 39.1 pg/ml for TNF-α and 125, 62.5, 31.3, and 15.6 pg/ml for IL-6.

5.3.7 Determination of fecal lipids, liver lipids, and hepatic free fatty acids

Fecal pellets were collected every two weeks from cages of all three groups (N=6), and stored at -80^{o}C until analysis. Fecal lipids were determined by the extraction method described by Folch et al [166]. The frozen samples were thawed, dried via dessication. Pellets were mechanically homogenized and resuspended in 25 volumes of 1:1 mixture of chloroform /methanol (v/v). The homogenate was then filtered, collected and brought to a final solvent ratio of 2:1 chloroform/methanol (v/v). The sample was centrifuged at 400xg for 10 minutes, and the lower organic phase was collected by pipet. The lipid mixture was dried under a gentle stream of N_2. Lipid precipitate was quantified gravimetrically. For determination of hepatic lipids, tissue samples were taken from excised livers following euthanasia of all three animal groups (N=12) at the 12-week endpoint. The liver samples were homogenized in a 1:1 ratio of chloroform/methanol. The homogenate was added to 50 volumes of 1:2 chloroform/methanol solvent and centrifuged at 400xg for 10 minutes. The lower phase was collected and dried under a gentle stream of N_2. The precipitated lipids were quantified gravimetrically. To the lipids, 500 µL of deionized water was added. The mixture was shaken at 250 rpm, 37^{o}C for 40 min to solubilize the lipids. Free fatty acids were quantified enzymatically using EnzyChrom commercial kits (BioAssay Systems, Hayward, USA).

5.3.8 Determination of serum lipids

Serum samples from fasted animals at week 0 and week 12 were isolated via centrifugation in serum separator tubes (BD) at 2000xg for 20 minutes at 4^{o}C and sorted at -80^{o}C until analysis. Serum triglycerides, total cholesterol, HDL-cholesterol were measured enzymatically using Roche Diagnostic kits on a Hitachi 911 Clonical Chemistry Analyzer and

EnzyChrom assay kits (BioAssay Systems, Hayward, USA) . LDL- cholesterol was determined using the Friedewald formula [LDLC = TC – HDL – (TG/2.2)]. Atherogenic index (AI) was determined by the equation [AI = log (TG/HDLC)] according to Dobiasova et al. [143].

5.3.9 Determination of TNF-α, Interleukin-6 and Interleukin-10 in mesenteric adipose tissue

TNF-α, IL-10, IL-6 cytokines in mesenteric adipose were quantified using the method described by Lira et al [167]. Adipose tissue samples were immediately rinsed in ice-cold 0.9% NaCl post-excision to remove blood contaminants, and stored at -80^{o}C until analysis. Between 0-1-0.3 g of frozen tissue samples were homogenized in RIPA buffer (0.625% P-40, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate, and 1mM ethylenediamine tetraacetic acid at pH 7.4) treated with 10 µg/ml protease inhibitor cocktail (Sigma Aldrich, Canada). The resulting homogenates were centrifuged at 12000xg for 10 minutes at 4^{o}C and the supernatant was collected. Aliquots of the supernatant were taken and used to determine total protein concentration via Bradford assay (Bio-Rad, CA) using a standard curve generated with known concentrations of bovine serum albumin (BSA). Protein concentration was calculated with units ug/ml. Subsequently, the homogenate supernatant was analyzed for TNF-α, IL-6 and IL-10 using Platinum ELISA kits from eBioscience (San Diego, USA) with sensitivities of 10 pg/ml. Relative cytokine expression was calculated as the ratio of concentrations of cytokine over total protein, and was reported as with units of pg/ ug protein.

5.3.11 Histology of liver tissue

Liver sections were excised and immersed in 10% buffered formalin phosphate solution for fixation. The tissue samples were ultrasectioned at a thickness of 5 μm, stained with hematoxylin and eosin (H&E) and examined using a light microscope at 400x magnification. Fat deposit density was observed, quantified and graphed.

5.3.11 Statistical analysis

The experimental results are expressed as means ± standard errror of the mean (SEM). Statistical analysis was carried out using SPSS Version 17.0 (Statistical Product and Service Solutions, IBM Corporation, New York, NY). Statistical comparisons were carried out using the General Linear Model and Tukey's post-hoc analysis for comparison of the means. Statistical significance was set at $p < 0.05$ and p-values less than 0.01 were considered highly significant.

5.4 RESULTS

5.4.1 Maintenance of viability and BSH-activity of probiotics doses

Probiotic doses of encapsulated *L.reuteri* (NCIMB 30242) were prepared every four weeks during the animal trial. Viability and BSH-activity of the prepared doses were measured weekly. Results show that viability is maintained throughout the 12-week period at an average value of 5.42±1.39 x 10^9 CFU/g of microcapsule. Similarly, BSH-activity of the encapsulated probiotics was maintained at an average value of 3.48±0.32 μmol/hr/g microcapsule. **(Figure 5.1)**

5.4.2 Weight and diet profile

Bio F1B hamsters in all three experimental groups were weighed weekly. All groups showed a logarithmic increase weight profile; a faster initial rate of mass increase which slowly plateaus. Results show that the hamsters in the treatment group maintain a lower average growth curve than the HFD group, and a higher average growth curve than the NFD group. Endpoint weight measurements (week 12) show an average weight of 168.4 ± 3.5 g for the treatment group, 177.4 ± 3.7 g for the HFD group, and 155.45 ± 3.7 for the NFD group. Treatment group showed significantly lower final average weight as compared to the HFD group ($p \leq 0.05$). Results are shown in **Figure 5.2.** Dietary intake profile showed no significant difference between the three groups through the experimental period (**Figure 5.3)**

5.4.3 C-reactive proteins profile

C-reactive proteins (CRPs) were measured biweekly for all hamsters in the three experimental groups. Differences in CRP profiles were observed by week 10 between HFD, treatment and NFD groups. At week 12, serum CRP levels in the treatment group were measured at an average value of 0.71 ± 0.03 mg/L, which was found to be significantly below CRP levels in the HFD group of 0.94 ± 0.04 mg/L ($p \leq 0.0005$). Animals in NFD group remained low, at an average endpoint of 0.64 ± 0.02 mg/L at week 12. **(Figure 5.4)**

5.4.4 TNF-α and IL-6 profiles

Serum TNF-α and IL-6 cytokines were measured biweekly for all hamsters in the three experimental groups. TNF-α profiles in all groups showed no significant difference until week 10. At week 12, serum TNF-α levels in the treatment group were measured at an average value

of 11.58 ± 0.18 pg/ml. This was statistically lower than the endpoint TNF-α levels in the HFD group of 12.19 ± 0.18 pg/ml ($p \leq 0.05$) Profiles for IL-6 showed statistical significant differences between the three groups only at week 12. Endpoint (week 12) measurements for serum IL-6 levels were measured in the treatment group at 10.93 ± 0.08 pg/ml, in the HFD at 11.10 ± 0.04 pg/ml, and in the NFD group at 10.78 ± 0.07 pg/ml. Serum IL-6 levels in the treatment group was determined to be significantly lower than in the HFD group at the 12-week endpoint ($p \leq 0.05$) **(Figure 5.5)**

5.4.5 Fecal lipid profile

Fecal pellets were collected biweekly from hamster cages of all experimental groups, and analyzed for total lipid content. Results show that the fecal fat content of the NFD group increased over the 12-week period from 21.0 ± 0.6 mg/g at start of trial to 26.2 ± 1.56 mg/g at week 12. This increase is significantly less than the relative increase for both the treatment and HFD groups. Total fecal lipid contents of treatment group and HFD group showed statistically significant difference only by week 10. At week 12, the treatment group showed a fecal lipid content of 47.71 ± 1.19 mg/g feces, which was statistically higher ($p \leq 0.05$, n=6) than endpoint lipid content in the HFD group of 41.47 ± 1.65 mg/g feces. Results illustrated in **figure 5.6**.

5.4.6 Liver histology

Histological liver sections stained with H&E showed significantly ($p \leq 0.05$) reduced number of lipid deposits in treatment group as compared to HFD group. Fat deposition remained low in NFD group. Microscopy and fat deposition densities are shown in **Figure 5.7.**

5.4.7 Serum, liver and adipose tissue endpoint measurements

The liver and mesenteric adipose tissue was excised from all the animals following euthanasia at the experimental endpoint. Additionally, blood was collected from the heart via cardiac puncture. Serum and liver tissue FFA concentrations were determined from the collected blood and livers. Liver were weighed and their total lipid content was determined. Finally, relative cytokine production of TNF-α, IL-6 and IL-10 was determined in extracted mesenteric adipose tissue samples. The results for the endpoint measurement of these factors are shown in **Table 5.2** for all three experimental groups (n=12). Moderately significant differences ($p \leq 0.05$) were observed for body:liver weight ratio, serum and liver FFA and adipose IL-6 between the treatment group and the HFD group. Highly significant differences ($p \leq 0.0005$) were observed for liver total lipid content and adipose TNF-α between treatment group and HFD group. NFD group remained with the statistically lowest values for all endpoint measurements.

5.4.8 Serum lipids

Serum triglycerides, total cholesterol and HDL-c were measured at week 0 and week 12 within experimental period. LDL-c and atherogenic index were calculated, and all data was tabulated in **Table 5.3.** Serum triglycerides, total cholesterol and LDL-c showed significant ($p<0.0005$) decrease in treatment group by week 12 as compared to HFD group; respectively 27.9 ± 3.3%, 14.9 ± 1.9% and 27.8 ± 3.9%. HDL-c levels showed moderately significant ($p<0.05$) increase of 30.4 ± 6.2% in the treatment group as compared to the HFD group. Finally, the atherogenic index at week 12 of treatment group was significantly lower (0.62 ± 0.03) than HFD group (0.88 ± 0.03). ($p<0.0005$)

5.5 Discussion

The current study demonstrated the ability of APA-microencapsulated *L.reuteri* (NCIMB 30242) to lower factors of metabolic disease in Bio F1B Golden Syrian hamsters. Since both the viability and BSH-activity of the APA-coated probiotic were maintained at steady levels throughout the 12-week experimental period, the effective therapeutic dose of active BSH enzyme can be considered to have been constant for the duration of the trial. The delivery of an effective dose is further supported by the increased levels of total fecal lipids in the treatment group, as compared to the non-treated HFD group. Evidence therefore suggests that the BSH-active APA-*L.reuteri* treatment interferes with lipid absorption, thus increasing the density of excreted fecal lipids. However, excreted cholesterol due to the hydrolysis of bile salts likely also contributed to the observed increased levels of fecal lipids.

The cumulative decrease of the treatment on several metabolic and inflammatory factors demonstrated its therapeutic effect on disease. The treatment was shown to weight gain in this hamster model. While decrease in weight gain via probiotics has been shown in broilers [168], a decrease in Bio F1B via this APA-encapsulated strain has not been demonstrated. Low-grade inflammation was demonstrated to be reduced by this treatment, as shown by lower circulating levels of CRPs, TNF-α and IL-6. The effect of probiotic treatment on diabetes has also shown this beneficial reductive effect on systemic inflammation in mice [169]. However, the mechanism of action of this effect appeared to be prominently through the direct action of the gut microflora on the modulation of inflammatory factors. This study postulates that the mechanism of inflammation reduction is the result of the secondary effect of BSH-active probiotic on adipocyte expansion. This theory is supported by the lower levels of IL-6 and TNF-α (and IL-10, as a negative control) in the mesenteric adipose tissue. Locally elevated levels of TNF-α and IL-

6 in mesenteric adipose tissue have been shown to be strongly linked to elevated circulating cytokines, metabolic problems, and adipocyte dysfunction due to over-growth [2, 39, 42]. Therefore local reduction of these factor in adipose tissue is highly beneficial to counter the pathogenesis of metabolic diseases, as shown by the effect of endurance training on adipose tissue of obese mice [167]. Levels of FFAs in both serum and liver were shown to be decreased via treatment. Such a reduction via probiotic treatment has been demonstrated in rats fed high-energy diet [170]. These findings are in support that hamsters undergoing treatment have reduced levels of FFA leakage from adipocytes. This in turn signifies lower energy dependence of the system on fats and lower adipocyte growth, both of which are key markers of metabolic disease. Finally, the liver weight and total lipid content were both found to be significantly lower in treated hamsters versus untreated HFD animals. In addition, treatment group was marked by lower density of globular hepatic fat deposits than in HFD group at endpoint, as observed by liver histology. This evidence indicates the onset of fatty liver, a secondary effect in the development of metabolic disease [3]. Similar results have been previously observed for the onset of fatty liver disease in Bio F1B via high-fat diet [171].

Elevated serum lipids (triglycerides, total cholesterol, LDL-c) in HFD group at endpoint (week 12), as compared to treatment group, confirm the onset of metabolic problems. This is further supported by the increased atherogenic risk (increased AI) in the HFD group. Interestingly, increase HDL-c levels were observed in the treatment group as compared to HFD. This is likely a consequence of lower total cholesterol levels in the treatment group, which helps re-establish a normal equilibrium in HDL/LDL levels in the animal. BSH-active bacteria have indeed been previously shown for their cholesterol lowering capability [11, 158, 172].

Overall, treatment of APA-microencapsulated *L.reuteri* (NCIMB 30242) shows very positive effects on factors for metabolic disease; namely systemic inflammation and atherogenic risk. The treatment retains significant potential for clinical applications.

5.6 Conclusion

When studying the therapeutic effect of the microencapsulated doses of BSH-active *L.reuteri* (NCIMB 30242), it becomes evident that *overall* reduction in markers of metabolic disease is observed due to the intricate interconnection between them. The increased fecal lipids in the treated animals indicate reduced lipid absorption, which explains their reduced levels of serum triglycerides and body weight. In turn, this results in the reduced expansion of adipocytes and the reduced development of the inflammatory adipocyte/macrophage cycle; as elucidated by decreased levels FFAs, TNF-α, and IL-6 in serum, adipose tissue and liver. As a result of this, systemic metabolic problems such as fatty liver and atherogenesis are substantially alleviated; as shown by the reduced levels of hepatic lipids, LDL-c, and lower atherogenic index in the treated group. Overall, the broad-spectrum effects of this treatment may make it a good option in reducing metabolic disorders caused by hyper-alimentation.

Acknowledgements

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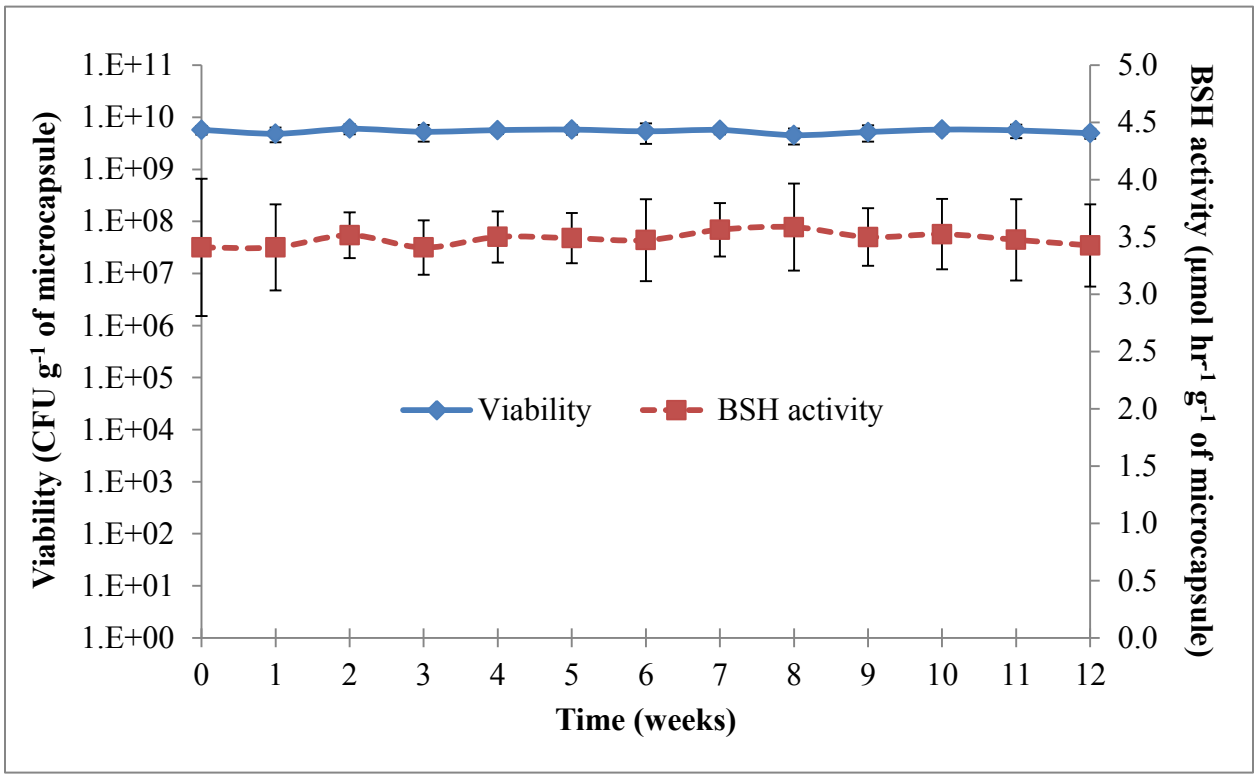

Figure 5.1: Maintenance of viability and BSH-activity for doses of APA-microencapsulated *L.reuteri* (NCIMB 30242) (error bars included ± SEM, n=3). Viability and BSH-activity of encapsulated probiotic doses were measured weekly by spectrophotometric assay and agar plate counts. Encapsulation was performed every 4 weeks. Doses remained stable at 5.42±1.39 x 10^9 CFU/g and 3.48±0.32 µmol/hr/g respectively, throughout the 12-week period.

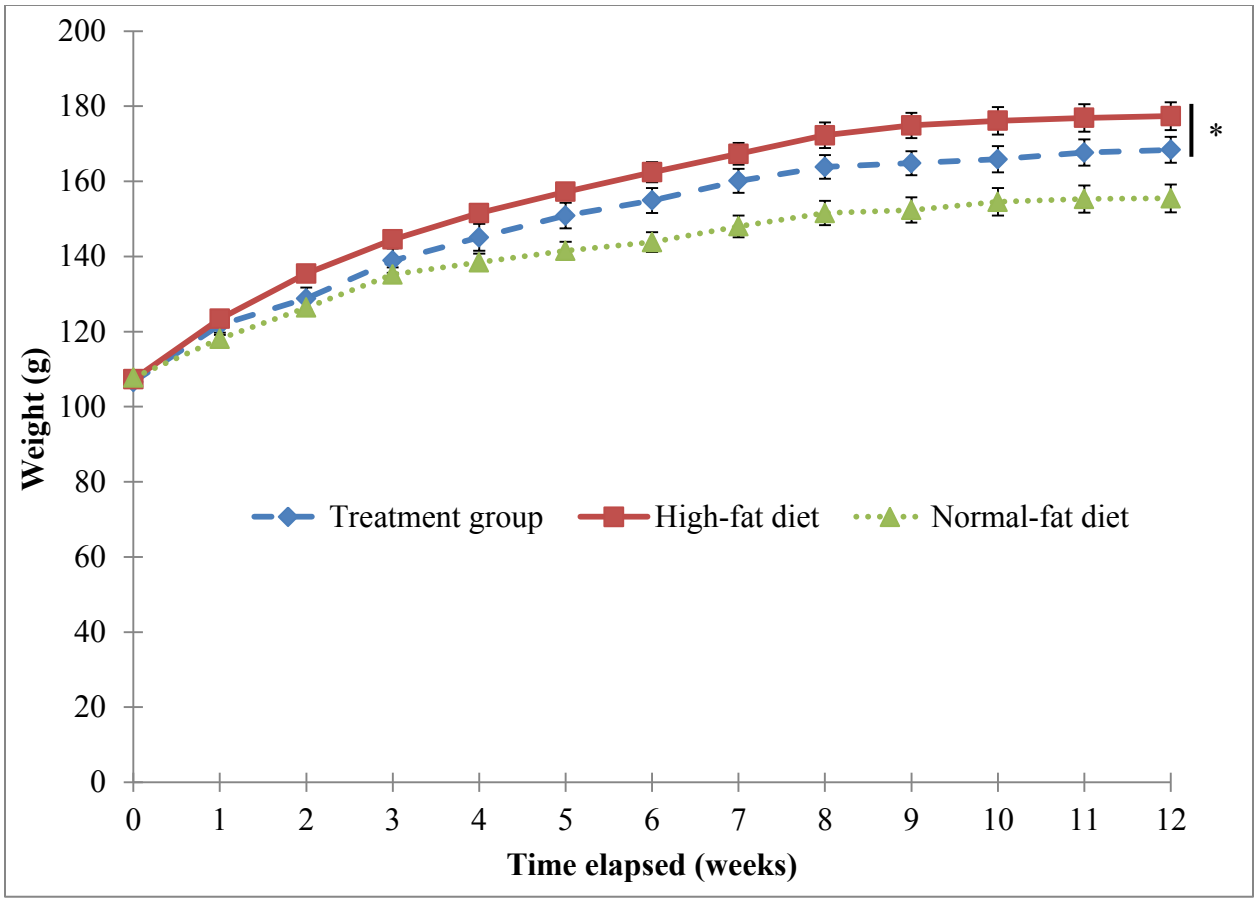

Figure 5.2. Weight profiles for all three experimental groups of Bio F1B hamsters
Treatment group: receiving high-fat diet and two daily doses of 1.5g APA-microencapsulated *L.reuteri* NCIMB 30242. High-fat diet group: receiving high-fat diet and no treatment. Normal-fat diet group: receiving normal-fat diet and no treatment. The treatment group growth curve remained lower than the HFD group, and higher than the NFD group, throughout the 12-week period. Endpoint weights (week 12) were found to be significantly lower for the treatment group (168.4 ± 3.5 g), as compared to the HFD group (177.4 ± 3.7 g). (*$p \geq 0.05$) (error bars included ± SEM, n=12).

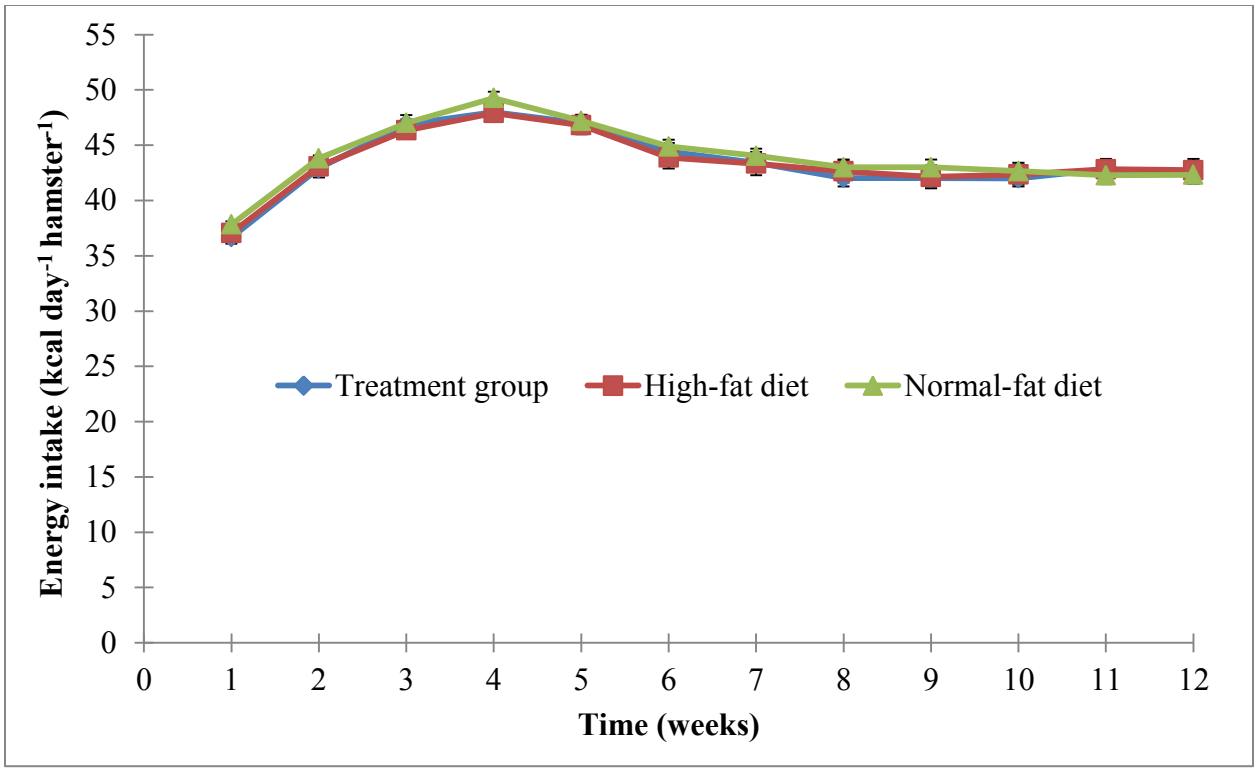

Figure 5.3: Food intake profiles for all three experimental groups.
Treatment group: receiving high-fat diet and two daily doses of 1.5g APA-microencapsulated *L.reuteri* NCIMB 30242. High-fat diet group: receiving high-fat diet and no treatment. Normal-fat diet group: receiving normal-fat diet and no treatment. Food was weighed weekly for all cages of each experimental group and standardized in terms of daily energy intake per hamster. Results show no significant difference between experimental groups of throughout the entire 12-week experimental period. (error bars included ± SEM, n=12)

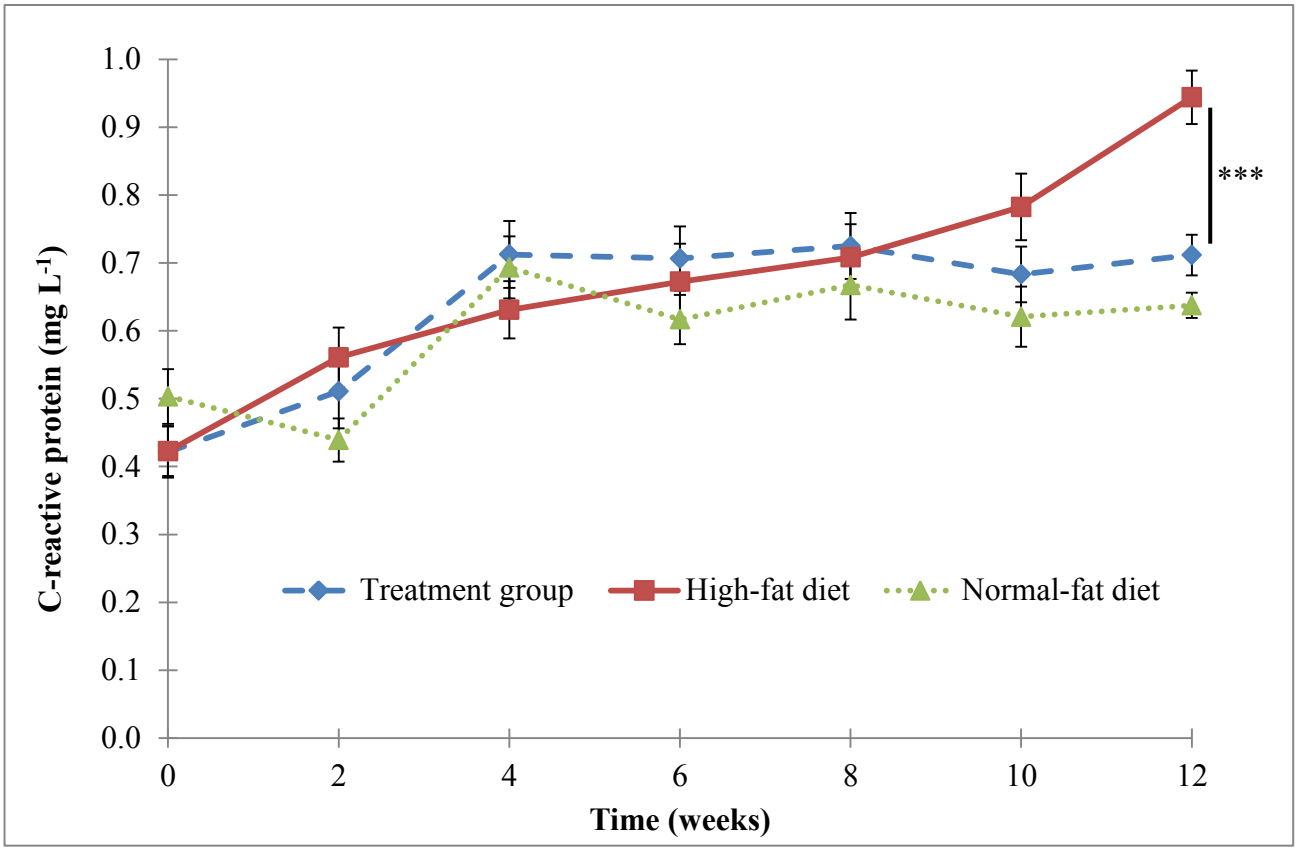

Figure 5.4: CRP profiles for all three experimental groups of Bio F1B hamsters.
Treatment group: receiving high-fat diet and two daily doses of 1.5g APA-microencapsulated *L.reuteri* NCIMB 30242. High-fat diet group: receiving high-fat diet and no treatment. Normal-fat diet group: receiving normal-fat diet and no treatment. Biweekly blood collections were used to assess levels. CRP profile showed increase in all three experimental groups over 12 weeks. Endpoint measurements at week 12 showed serum CRP levels of 0.71 ± 0.03, 0.94 ± 0.04 and 0.64 ± 0.02 mg/L for treatment group, HFD group, and NFD group respectively. Serum CRPs were significantly lower (***$p \geq 0.0005$) in treatment group as compared to the HFD group by week 12. (error bars included ± SEM, n=12).

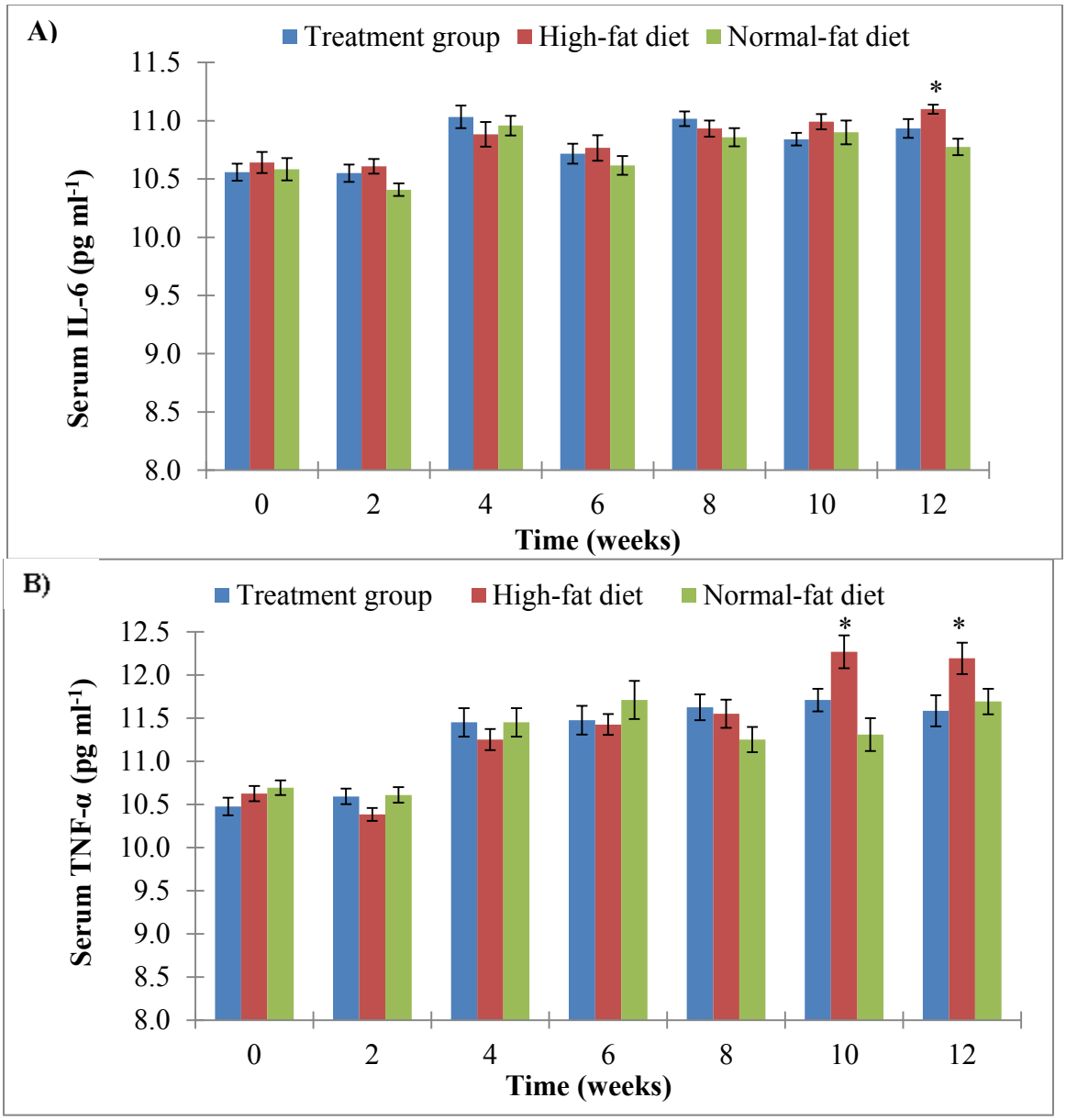

Figure 5.5: Inflammatory cytokines for the three groups of hamsters, with the treatment group administered two daily doses of 1.5 g APA-microencapsulated *L. reuteri* NCIMB 30242. Significant differences between the treatment and the HFD groups were observed at the 12-week endpoint for both serum (A) TNF-α and (B) IL-6 (*$p < 0.05$). Endpoint TNF-α and IL-6 levels in the treatment group were found to be 11.58 ± 0.18 and 10.93 ± 0.08 pg ml^{-1} respectively. Endpoint TNF-α and IL-6 levels in the HFD group were found to be 12.19 ± 0.18 and 11.10 ± 0.04 pg ml^{-1} respectively. Data is expressed as means ± SEM, n = 12.

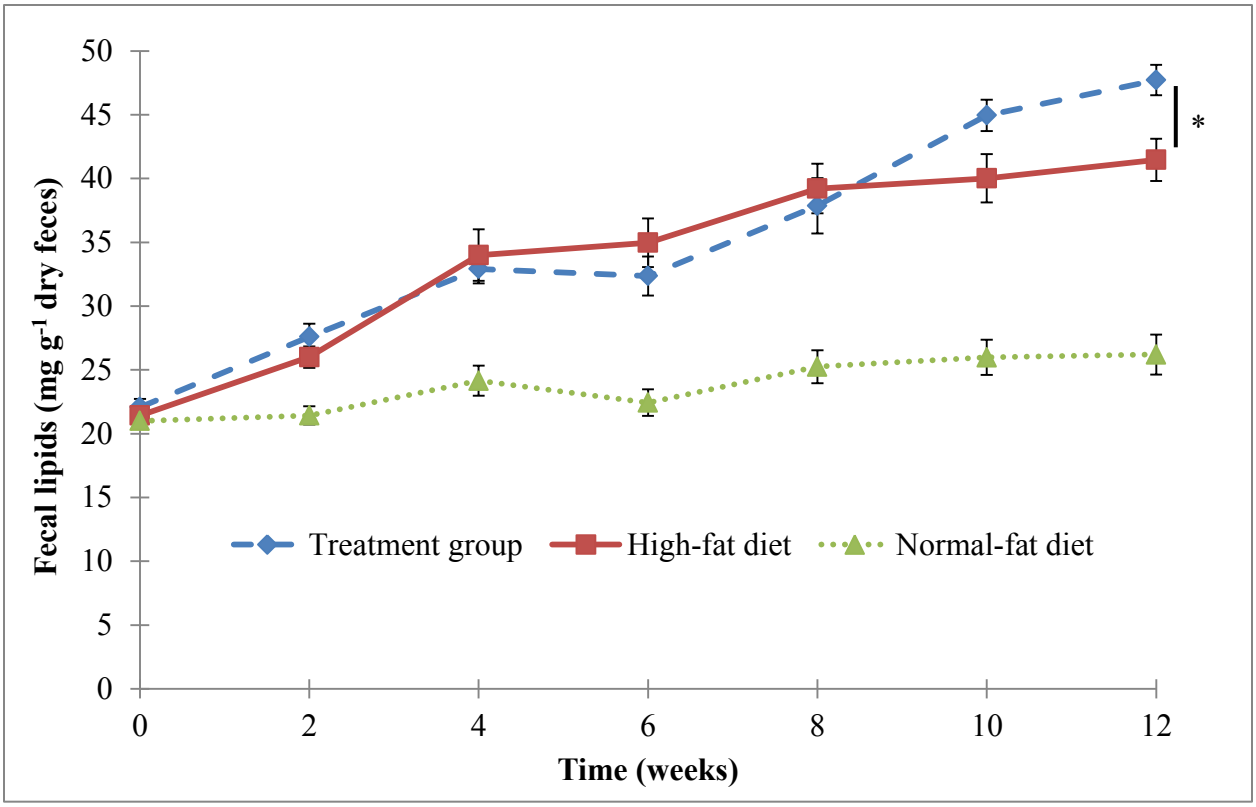

Figure 5.6: Fecal lipid profiles for all three experimental groups of Bio F1B hamsters. Treatment group: receiving high-fat diet and two daily doses of 1.5g APA-microencapsulated *L.reuteri* NCIMB 30242. High-fat diet group: receiving high-fat diet and no treatment. Normal-fat diet group receiving normal-fat diet and no treatment.Feces were collected from each cage biweekly and analyzed for lipid content. NFD group varied less over 12-week period (from 21.0 ± 0.6 mg/g to 26.2 ± 1.56 mg/g) as compared to treatment and HFD groups. Statistically significant difference ($p \leq 0.05$) was observed between HFD and treatment groups by week 10. Endpoint (week 12) measurements of fecal content in treatment group and HFD group remained statistically different at respective values of 47.71 ± 1.19 and 41.47 ± 1.65 mg/g feces. (*$p \leq 0.05$) (error bars included ± SEM, n=6).

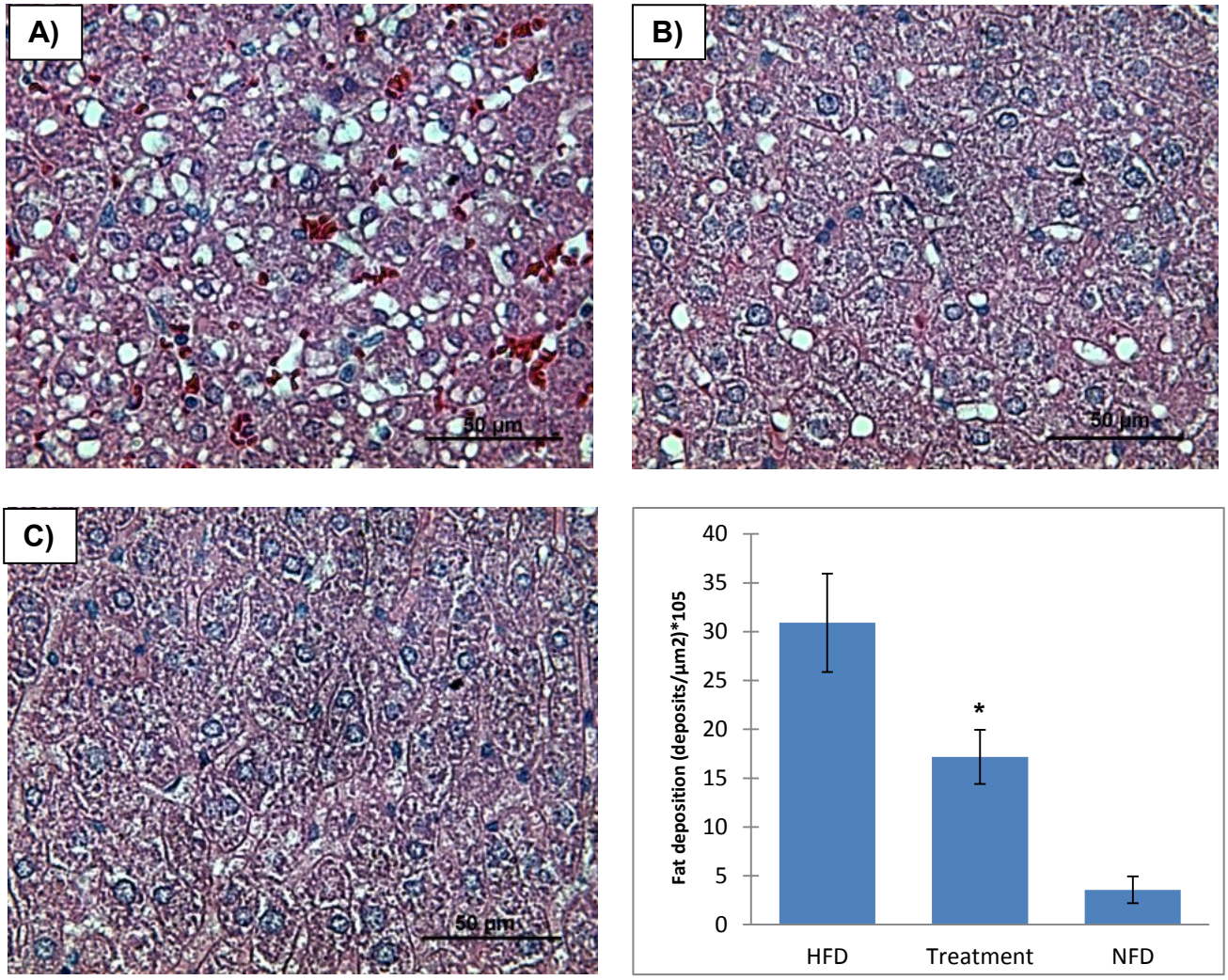

Figure 5.7: Hematoxylin and eosin (H&E) staining of liver tissue extracted from hamsters of all three experimental groups at endpoint (week 12).
Treatment group: receiving high-fat diet and two daily doses of 1.5g APA-microencapsulated *L.reuteri* NCIMB 30242. High-fat diet group: receiving high-fat diet and no treatment. Normal-fat diet group: receiving normal-fat diet and no treatment. Tissue slides samples from animals of all groups were prepared, stained with H&E and photographed at 400x magnification. Images are shown for all 3 groups above: A) HFD (high-fat diet) group. B) Treatment group. C) NFD (normal-fat diet) group. Hepatocytes are filled and interspersed with macrovesicular fat deposits (white globules) in treatment group in significantly lower number (*$p \leq 0.05$) as compared to both HFD group. Data shown as mean ± SEM. (n=5)

Treatment Endpoint (Week 12)

	Treatment group	High-fat diet group	Normal-fat diet group
Liver and blood			
Liver:Body weight ratio	**0.035 ± 0.001***	**0.038 ± 0.001**	**0.036 ± 0.001**
Liver total lipids (mg/ g liver)	**64.2 ± 1.3*****	**71.2 ± 0.7**	**53.3 ± 0.9**
Liver FFA (mg/g liver)	**0.79 ± 0.01***	**0.85 ± 0.02**	**0.69 ± 0.01**
Serum FFA (mmol/L)	**0.57 ± 0.01***	**0.61 ± 0.01**	**0.46 ± 0.01**
Adipose tissue cytokines (pg/ug protein)			
TNF-α	**0.105 ± 0.004 *** **	**0.126 ± 0.004**	**0.093 ± 0.003**
IL-6	**0.309 ± 0.022 ***	**0.371 ± 0.014**	**0.211 ± 0.007**
IL-10	**0.254 ± 0.009**	**0.241 ± 0.016**	**0.239 ± 0.011**

Table 5.1: Endpoint (week 12) measurements of serum, liver and adipose tissue factors in Bio F1B hamsters of all three experimental groups.
Treatment group: receiving high-fat diet and two daily doses of 1.5g APA-microencapsulated *L.reuteri* NCIMB 30242. High-fat diet group: receiving high-fat diet and no treatment. Normal-fat diet group: receiving normal-fat diet and no treatment. Serum FFAs were determined enzymatically from blood samples. Liver FFAs and adipose cytokines were determined enzymatically from their derived tissues. Liver lipids and liver:body weight ratio was determined gravimetrically. Moderately significant differences (*$p \leq 0.05$) were observed between treatment and HFD group for liver/body weight ratio, serum and liver FFA, and adipose IL-6. Highly significant differences (***$p \leq 0.0005$) were observed between treatment and HFD group for total liver lipid content and adipose TNF-α. NFD group retained the lowest endpoint values for all factors.

	Treatment group	**High-fat diet group**	**Normal-fat diet group**
Triglycerides (mg/dl)			
Week 0	**118.7 ± 9.4**	**118.9 ± 9.8**	**126.3 ± 9.8**
Week 12	**534.4 ± 24.5*****	**741.4 ± 28.5**	**174.7 ± 14.2**
Total cholesterol (mg/dl)			
Week 0	**188.7 ± 7.8**	**175.8 ± 8.3**	**175.5 ± 7.2**
Week 12	**375.7 ± 8.6*****	**441.4 ± 9.6**	**209.5 ± 3.6**
HDL-c (mg/dl)			
Week 0	**92.9 ± 7.7**	**81.3 ± 8.2**	**82.5 ± 6.5**
Week 12	**127.8 ± 6.1***	**97.9 ± 7.3**	**99.3 ± 8.1**
LDL-c(mg/dl)			
Week 0	**72.3 ± 4.4**	**70.8 ± 2.8**	**67.9 ± 5.9**
Week 12	**141.9 ± 7.8*****	**196.3 ± 7.8**	**75.5 ± 9.3**
Atherogenic index			
Week 0	**0.11 ± 0.06**	**0.17 ± 0.05**	**0.15 ± 0.07**
Week 12	**0.62 ± 0.03*****	**0.89 ± 0.03**	**0.25 ± 0.05**

Table 5.2: Week 0 and Week 12 serum lipids and atherogenic indexes for all three experimental groups. Treatment group: receiving high-fat diet and two daily doses of 1.5g APA-microencapsulated *L.reuteri* NCIMB 30242. High-fat diet group: receiving high-fat diet and no treatment. Normal-fat diet group: receiving normal-fat diet and no treatment.
Serum samples were collected from animals of all three groups (N=12) for quantification of triglycerides, total cholesterol and HDL-c. LDL-c and AI were subsequently calculated. Moderately significant difference (*$p \leq 0.05$) were observed between treatment and HFD group for HDL-c levels at week 12. Highly significant differences (***$p \leq 0.0005$) were observed between treatment and HFD group for triglycerides, total cholesterol, LDL-c, atherogenic index. NFD group retained the lowest endpoint values for all factors. (N=12)

CHAPTER VI: Summary of results

This thesis aimed to develop a BSH-activity quantification assay for the selection and stability study of a potential APA-microencapsulated probiotic. The effect of the microencapsulated BSH-active probiotic treatment on markers of metabolic disease was investigated in Bio F1B Golden Syrian hamsters. The results can be summarized as follows:

1. The first step was to develop an assay for the determination of BSH-activity in probiotics that is relatively quick and simple (for logistical purposes) while retaining sufficient accuracy and adaptability for APA-microencapsulated probiotics. The assay was developed from a modified version of the protocol from Stellwag et al. [153] and adapted for APA-microcapsules.
2. In order to test the assay, it was performed on five selected strain of *Lactobacillus* grown for 12h overnight in MRS broth. It was determined that *Lactobacillus reuteri* (NCIMB 30242) elicited the highest BSH-activity at 17.09 ± 4.37 µmol /hr/g. The *Lactobacillus reuteri* (NCIMB 30242) strain was selected as the best candidate for potential in vivo studies.
3. To determine the maximal BSH-activity of the selected strain, a time-course experiment was performed: both activity and viability were monitored for *Lactobacillus reuteri* (NCIMB 30242) at 4h intervals for 24h. Results show that maximal BSH-activity was obtained at at 16h in its growth at 42.82 ± 4.26 µmol of glycine/hr/g of bacterial pellet, with peak viability between 12-16h. The 16h time point of growth was thus selected at the incubation period for growth of the probiotic formulations

4. The *Lactobacillus reuteri* (NCIMB 30242) strain was encapsulated a 8% v/v in APA microcapsules at 16h within its growth curve. The BSH-activity assay was performed on these microcapsules (as well as a series of negative controls) to determine 1) if the adapted assay maintained accuracy for encapsulated cells and 2) if APA-microencapsulation process might affect the activity of the probiotic. The activity of APA-microencapsulated *Lactobacillus reuteri* (NCIMB 30242) was determined at 3.56 ± 0.24 in μmol/hr/g microcapsule , which once standardized by a multiplying factor of (x100/8) equals 44.50 ± 3.00 μmol of glycine/hr/g bacteria. This value is within range of the measured activity of the free bacteria. Negative controls showed no activity, supporting the use of this assay for determination of BSH-activity in APA-microencapsulated probiotics.
5. APA microcapsules containing *Lactobacillus reuteri* (NCIMB 30242) were tested for their therapeutic stability; mainly maintenance of viability, BSH-activity and morphology. As negative controls, free bacteria was used. Bacteria encapsulated within APA microcapsules maintained activity and viability for 4 weeks in 10% MRS at 4^{o}C, at average values of 43.37 ± 3.34 μmol/hr/ml and 5.2 ± 1.2 x 10^{9} CFU/ml respectively. The morphology of the APA microcapsules was shown to remain stable during the 4-week period. Free bacteria maintained viability and BSH-activity at comparable values for only 2 days and 2 weeks at 4^{o}C in saline and 10% MRS respectively.
6. With the BSH-active probiotic selected and its stability confirmed, the next step involved investigating its effect in vivo on factors of metabolic disease. Bio F1B hamsters were administered 3g daily of APA microcapsules containing *Lactobacillus*

reuteri (NCIMB 30242) for 12 weeks. A high-fat diet (HFD) group serve as a no-treatment control, and a normal-fat diet (HFD) group served as a negative-induction control of metabolic disease. The activity and viability of the doses were maintained at average values of 3.48±0.32 μmol/hr/g and 5.42±1.39 x 10^9 CFU/g respectively. The results from physical measurements, serum and tissue were as follows:

a. Weight profile: Treatment group showed lower average weight at experimental endpoint (168.4 ± 3.5 g) as compared to high-fat diet (HFD) group receiving no treatment (177.4 ± 3.7 g). ($p \leq 0.05$, n=12)
b. Diet profile: All three experimental groups showed no difference in dietary intake over the 12-week period.
c. CRPs: Treatment group showed lower average CRP levels at experimental endpoint (0.71 ± 0.03 mg/L) as compared to high-fat diet (HFD) group (0.94 ± 0.04 mg/L). ($p \leq 0.0005$, n=12)
d. Serum TNF-α: Treatment group showed lower average serum TNF-α levels at experimental endpoint (11.58 ± 0.18 pg/ml) as compared to high-fat diet (HFD) group (12.19 ± 0.18 pg/ml). ($p \leq 0.05$, n=12)
e. Serum IL-6: Treatment group showed lower average serum IL-6 levels at experimental endpoint (10.93 ± 0.08 pg/ml) as compared to high-fat diet (HFD) group (11.10 ± 0.04 pg/ml). ($p \leq 0.05$, n=12)
f. Fecal lipids: Treatment group showed higher fecal lipid levels at experimental endpoint (47.71 ± 1.19 mg/g) as compared to high-fat diet (HFD) group (41.47 ± 1.65 mg/g). ($p \leq 0.05$, n=6)

g. Endpoint serum and tissue measurements: As compared to HFD group, treatment group showed moderately significant ($p \leq 0.05$) lower levels of IL-6 in adipose tissue (0.309 ± 0.022 vs 0.371 ± 0.014 *pg/ug*), liver:body weight ratio (0.035 ± 0.001 vs 0.038 ± 0.001), hepatic FFAs (0.79 ± 0.01 vs 0.85 ± 0.02) and serum FFAs (0.57 ± 0.01 vs 0.61 ± 0.01). Highly significant ($p \leq 0.0005$) lower levels were observed for total liver lipids (64.2 ± 1.3 vs 71.2 ± 0.7) and TNF-α in adipose tissue (n=12)

h. Serum lipids: As compared to HFD group, treatment group showed very significant ($p \leq 0.0005$) lower levels of serum triglycerides (514.4 ± 24.5 vs 741.4 ±28.5 mg/dl), total cholesterol (375.7 ± 8.6 vs 441.4 ± 9.6 mg/dl), LDL-c (141.9 ± 7.8 vs 196.3 ± 7.8 mg/dl) and atherogenic index (0.62 ± 0.03 vs 0.89 ± 0.03) at week 12. In addition, at week 12, HDL-c levels showed moderately significant ($p \leq 0.05$) increase in treatment group as compared to HFD group; 127.8 ± 61 vs 97.9 ± 7.3 mg/dl. (n=12)

i. Histology: Treatment group liver samples showed reduced amount of fat deposits by the 12-week endpoint, as shown by H&E-stained histological examination ($1.6 \pm 0.1 \times 10^{-3}$ deposits/um^2 in treatment group as compared to $3.4 \pm 0.3 \times 10^{-3}$ in HFD group) (n=5)

CHAPTER VII: General discussion and conclusions

The treatment of disease via probiotics has been the subject of great research over the last few decades. Of the many varied enzymatic found amongst probiotics, BSH-activity offers great potential for therapeutic use. Characterizing and quantifying the level of BSH-activity in bacteria is often done via assays involving cholesterol precipitation [152] or HPLC [10]. However, glycine released from the hydrolysis of GDCA via the BSH enzyme offers an exploitable method for BSH-activity determination. While quantifying activity by measuring glycine release has been shown in previous studies [153, 154], these methods are limited to simple media. Given the increasing prominence of APA-microencapsulation as a means of probiotic delivery, a more versatile procedure is required that is adapted to various media. In this thesis, a spectrophotometric assay was developed to quantify BSH-activity in simple and complex media, as well as from APA-microencapsulated probiotics. A pool of probiotics was tested for activity, and the most active candidate was selected; *L.reuteri* NCIMB 30242. This activity was comparable to levels measured in BSH-active probiotics in other studies [9]. The in vitro results showed great potential for testing in an animal model. However, prior to proceeding to animal studies, studies on the stability of the APA-microencapsulation doses was performed to ensure sufficient maintenance of the active enzyme during treatment. Results showed improved maintenance of viability and BSH-activity in APA-microencapsulated *L.reuteri* NCIMB 30242 as compared to free bacteria. Microcapsule membrane morphology was maintained. These observations are consistent with results previously shown [8, 164] Finally, using Bio F1B hamsters as models for metabolic disease, oral treatment of APA-microencapsulated *L.reuteri* NCIMB 30242 was shown to decrease markers for metabolic disease over a 12-week experimental period. This is consistent with earlier findings in this field; treatment of

metabolic syndromes via BSH-active probiotics has had positive results in previous studies. Its potential treatment of hyperlipidemia and hypercholesterolemia has been demonstrated in a number of studies; reducing cholesterol levels as much as 22-33% [9, 11, 158, 172-174] and even preventing elevated cholesterol levels in mice fed with a high-fat diet [175]. Evidence that BSH-active probiotics beneficially impair lipid absorption has also been shown, reducing plasma triglyceride levels in animals. [159, 173] Finally, probiotic therapy has already shown potential in reducing inflammation for intestinal diseases such as Crohn's disease and ulcerative colitis [176-179].The effectiveness of probiotics treatment depends heavily upon the selection of a sufficiently active bacterial strain, as well as efficient delivery of the cells to their target site. Microencapsulation of probiotics within APA-microcapsules has been shown to improve their delivery by increasing bacterial survival [12]. This increases the therapeutic potential of the probiotic on metabolic problems. In this thesis, it was hypothesized that delivery of BSH-active bacteria in an appropriate animal model could reduce factors associated with metabolic disease. Bio F1B hamsters were chosen for the purpose of this study. The stability of the dose was critical to characterize prior to undergoing animal trials. Because metabolic diseases are systemic physiological problems with a number of interacting pathways, assessing the effectiveness of the treatment requires the measurements of several different factors. These include physical parameters (body and liver weight), inflammatory factors (TNF-α, IL-6, CRPs) and metabolic lipids (cholesterols, tissue and serum lipids).

In summary, after reviewing the results of this thesis, the following conclusions can be drawn. First, the developed spectrophotometric assay can quickly and easily determine BSH-activity in both free and APA-microencapsulated probiotics, in simple and complex media. Second,

Lactobacillus reuteri (NCIMB 30242) is a BSH-active probiotic strain appropriate for oral delivery within APA-microcapsules. Third, APA-microcapsules containing *L.reuteri* (NCIMB 30242) maintain stable viability, BSH-activity and microcapsule morphology for a minimum of 4 weeks when stored at 4^{o}C in 10% MRS. Finally, an animal trial on Bio F1B hamsters under high-fat diet demonstrated that oral treatment of APA-microencapsulated *L.reuteri* (NCIMB 30242) reduces physical, inflammatory and metabolic factors associated with metabolic disease. The primary mechanism of action of this treatment can be reasonably concluded from these results. In the case of dietary obesity and downstream metabolic problems, hyperalimentation of fats is the primary cause. Through adipocyte dysfunction, a heightened inflammatory state and hypercholesterolemia develops [2]. BSH-active bacteria can be administered orally to act as probiotics [9]. By orally delivering BSH-active bacteria, these probiotics can invade the intestinal tract of a hyperlipidemic and hypercholesterolemic host. Inside, the probiotics can hydrolyze bile salts. These deconjugated bile salts are incapable of emulsifying lipids, thus reducing their absorption into the system and alleviating hyperlipidemia and all downstream problems [9]. In addition, deconjugated bile salts are significantly less well reabsorbed into the system than conjugated bile salts, and are excreted into the feces. The liver responds by synthesizing new bile salts from cholesterol, thus reducing hypercholesterolemia [11, 173]. Via these two mechanisms, BSH-active probiotics can reduce factors which lead to the inflammatory state and high cardiovascular risk associated with metabolic syndrome.

While the positive results observed in this thesis leave room for optimism, limitations and future work remains to be addressed. These are further specified in the subsequent chapter.

CHAPTER VIII: Limitations and future recommendations

Despite the demonstrated effect of the probiotic treatment on factors of metabolic disease, several limitations exist. Firstly, the spectrophotometric assay, while significantly quicker and cheaper than most alternatives, sacrifices precision. Further quantitative analysis on the BSH-activity of a given probiotic should utilize more accurate assays, e.g HPLC [10]. Secondly, further screening of bacteria should be performed to find more superior candidates for oral treatment. These probiotic candidates should maintain high viability and activity over time within microcapsules. By utilizing the assay developed in this thesis, such a study could be performed. BSH-activity is an activatable system; therefore attempts at increasing probiotic expression of the BSH enzyme (using varying growth conditions) should be pursued.

Next, dosage storage and optimization are a serious limitation. For the purposes of an animal study, a 4-week period of maintenance is sufficiently long. However, for industrial applications; long-distance shipping and long-term storage will become necessary. Lyophilization or freeze-drying are potential techniques which should be explored for BSH-active probiotics to improve shelf life and product quality [180]. While, the maximum dose allotted by the Animal Ethics Committee was administered in the animal trial presented in this thesis, dose optimization studies should be performed in the future to determine the minimum required dose for an effect. This is a currently serious limitation, compounded by possible variability in the host's response to probiotic entry in the GI tract, which forces studies to utilize the maximum permitted dose in the trial.

While the Bio F1B hamster served as a useful animal model, one prominent limitation of the current study is predicting the effect in humans or other pertinent animal models. Trials should be repeated with this treatment on other animal models prone to the symptoms of

metabolic problems under a high-fat diet; e.g atherosclerosis, high basal inflammation, fatty liver disease. This would support the use of the treatment in alleviating metabolic diseases. Another limitation is the lack of information pertaining to long-term toxicity of the treatment. Studies must be performed in vivo to assess this risk. Ultimately, clinical trials should be undertaken on humans, utilizing a non-toxic, highly active, dose-optimized microencapsulated bacterial strain to test its effect.

In summary, while limitations and unanswered questions do exist, there remains significant potential of this treatment. This potential justifies future work and improvements.

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